ABSTRACT

C57BL/6d ApcMin/+ (ApcMin/+ mice) spontaneously develop preneoplastic adenomas into the intestinal mucosa. We studied the relationship between the intestinal immune responses and adenoma formation in ApcMin/+ mice and compared ApcMin/+ mice with their wild-type siblings. Three time points (5, 8, and 15 weeks of age) and three high-fat dietary treatments (a non-fiber control with beef or inulin amendment) were included. The numbers of CD8+ T lymphocytes and tissue macrophages (Mac-1+ cells) per villus in ileal mucosa were determined by immunohistology, and the concentrations of secretory IgA, residual prostaglandin E2 (PGE2), tumor necrosis factor α, and interleukin (IL)-12 in ileal contents were analyzed by ELISA. The crypt-villus ratio of the ileal mucosa was determined histologically. An immunostimulation, characterized by an increase in several parameters (PGE2, IgA, Mac-1, and CD8), was observed in both genotypes between weeks 5 and 8. Most of the adenomas in ApcMin/+ mice also appeared during the same period of sexual maturation. Females had smaller adenomas than males, and the beef group had fewer and smaller adenomas than the control group at 15 weeks. Females had less IgA and fewer Mac-1+ and CD8+ cells in ileal tissue than males at 15 weeks and more luminal IL-12 than males at 8 weeks. Puberty may have affected both tumorigenesis and intestinal immune responses in the ApcMin/+ mouse. The beef group showed less luminal IgA and tumor necrosis factor α but more IL-12 than the control group. The concentration of PGE2 correlated positively with the number and size of adenomas and was higher in the ApcMin/+ mice than in wild-type mice at 15 weeks. IgA and Mac-1 were positively correlated with the size of adenomas at 15 weeks. The positive correlations between tumor size and IgA and between tumor number and size and PGE2 suggest that a balance toward the Th2 type immune response may affect the pace of tumorigenesis in this model. The general similarity of the intestinal immune responses in both genotypes and the lack of intestinal inflammation in the ApcMin/+ mouse suggest that the mutation in the adenomatous polyposis coli gene does not lead to major alterations in intestinal immune function and that the intestinal immunology does not explain tumorigenesis in the ApcMin/+ mouse model.

INTRODUCTION

Immune responses can be roughly divided into cytotoxic (Th1) and humoral (Th2) types, and the balance between these response types has an impact on the development of chronic diseases. The cytotoxic immune responses eliminate pathologically altered cells, e.g., such as neoplastic cells (1, 2), or cells that have been infected with microbes or viruses. The recruitment of macrophages and CD8+ cells has been linked to an improved clearance of transformed cells and improved prognosis for colon cancer (3, 4). TNF-α plays an important role in the activation of cytotoxicity-inducing cells as well as in the apoptosis of cancer cells (5–7). However, long-term intestinal inflammation increases the risk for colorectal cancer, e.g., in patients of inflammatory bowel disease (8).

The constant flow of food-derived antigens and the presence of microbes in the intestinal tract demand special down-regulation of the immune system to prevent unnecessary inflammation. In addition to other inflammation-controlling mediators (9), PGE2 may direct the intestinal immune system toward Th2-type responses by enhancing switching to IgA production (10, 11). The humoral immune responses can be correlated with poor prognosis of cancer (12), and patients with advanced colorectal cancer often show an induction of Th2 cells and a suppression of Th1 cells (13).

The ApcMin/+ mouse, a model for colorectal cancer, is used to study the effects of genetics, diet, or chemical compounds on the incidence and development of intestinal precancerous lesions, the adenomas. The germ-line mutations in the APC gene lead to FAP, but inactivation of APC is also found in 80% of sporadic colorectal cancers (14). In addition to the mutation in APC, tumor progression is dependent on mutations in other genes such as c-Myc, K-ras, p53, and members of the transforming growth factor β signaling pathway (14). Evidence for the effect of dietary factors on both intestinal immunology and small intestinal adenoma formation in the ApcMin/+ mouse has been found. Resveratrol, a compound in wine and grapes, reduced adenoma formation and regulated the expression of several genes involved in the recruitment and activation of immune cells (15). Curcumin, a plant-derived phenolic compound with immunomodulatory properties (16), decreased tumorigenesis (17) and modulated intestinal T- and B-lymphocyte populations (18). Anti-inflammatory drugs such as aspirin (19) and sulindac (20) have also been found to reduce the tumor formation in the ApcMin/+ mouse. Sulindac suppressed the production of intestinal tissue prostaglandin PGE2 (20) by the enzyme COX-2, an enzyme that is often overexpressed in tumor tissue (21) and in intestinal macrophages (22). COX-2 inhibitors can reduce intestinal tumorigenesis (23) and are therefore used to treat FAP patients (24) and patients with colorectal cancer (25).

In the present study, the intestinal immune responses of ApcMin/+ mice and their wild-type siblings were compared by measuring both cellular and humoral arms of the intestinal defenses. We also studied the relationship of intestinal immune responses and tumorigenesis in the ApcMin/+ mouse model. Dietary beef and inulin treatments were compared with the non-fiber control diet because both inulin and beef have previously shown a tendency to increase tumorigenesis in this model (26). The abundance of ileal tissue macrophages (Mac-1+ cells) and CD8+ T lymphocytes and the levels of secretory IgA, PGE2, IL-12, and TNF-α in ileal contents were compared in ApcMin/+ and wild-type C57BL/6j mice. A series of time points (5, 8, and 15 weeks) was included to study the association between local immune responses and the development of adenomas over time.

MATERIALS AND METHODS

Animals. C57BL/6j ApcMin/+ males and wild-type females, originating from an inbred strain of Jackson Laboratory (Bar Harbor, ME), were used in a breeding program to produce the experimental animals at the Experimental Animal Unit of the University of Helsinki (Helsinki, Finland). The mice were genotyped by a PCR assay (27), and both genotypes were used in the experiment. At the age of 5 weeks, the animals were grouped according to the
feeding treatments of 3 or 10 weeks in duration or to the starting point sampling. The number of animals used was 12–14 Apc<sup>Min<sup>+/−</sup> mice and 8–10 wild-type mice per dietary treatment and time point. An equal number of male and female mice were included in each treatment. The mice were housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Feed and water were available to the animals ad libitum. The mice were weighed once a week during the experiment. The Laboratory Animal Ethics Committee of the University of Helsinki approved the study protocol.

Diet. Before the change to the experimental diets at 5 weeks, the mice were fed a pelleted, standard rodent chow (Teklad Global 14% Protein Rodent Maintenance Diet; Harlan Teklad, Madison, WI). A modified semisynthetic AIN93-G diet (28) was prepared for the feeding treatments (Table 1). The diet contains 40% fat, and the fatty acid profile is similar to that in a Western-type diet. In the non-fiber control diet, the sources of carbohydrates and protein were dextrose and casein, respectively. The beef diet contained freeze-dried, ground, low-fat beef as the protein source, instead of casein. The inulin diet was prepared by adding 10% (w/w) inulin (Raftiline; Orafti, Tienen, Belgium) to the control diet. The other ingredients of the beef and inulin diets were adjusted to keep the proportion of energy from carbohydrate, protein, and fat similar to that in the control diet. Before use, the diets were stored at −20°C. The control and beef diets were used for the 3- and 10-week feeding periods, and the inulin diet was only used for the 3-week feeding period. In addition, five female Apc<sup>Min<sup>+/−</sup> mice were fed the standard rodent chow diet until the age of 8 weeks.

Adenoma Enumeration and Classification. The mice were killed by CO2 asphyxiation, and the small intestine was excised and divided into five segments of equal length. The intestinal sections were opened longitudinally, rinsed with 0.9% NaCl solution, and spread onto microscope slides. The adenomas were counted, and the diameter of each adenoma was measured to the nearest 0.1 mm under an inverse light microscope with a screen at a magnification of ×2.5. The location and diameter of each adenoma was recorded with a millimeter scale placed on the screen. The adenomas were classified as small (0.3 mm < diameter < 1 mm), medium-sized (1 mm < diameter ≤ 1.5 mm), or large (diameter > 1.5 mm). The relative proportions of the size classes in the entire small intestine were counted and given as a percentage of the total number of adenomas. The adenoma burden was counted by summing the areas (mm<sup>2</sup>) of all adenomas of an individual mouse. The adenomas are approximately round, and their diameter was measured; therefore, the area of each adenoma was counted by the following equation: area = πr<sup>2</sup> (r = 0.5 × diameter).

ELISA Methods for IgA, PGE<sub>2</sub>, IL-12, and TNF-α. The contents of the three most distal segments of the small intestine, approximately representing distal jejunum and ileum, were collected and stored at −70°C. Before the analyses, the digesta samples were treated with 1.0% BSA-50 mM Tris buffer (pH 7.5) for 60 min at room temperature to separate food matrix and cellular material. The samples were then centrifuged at 50,000×g for 15 min. The supernatants were stored at −70°C and used later for measurements of IgA (Bethyl Laboratories Inc., Montgomery, TX), PGE<sub>2</sub> (Cayman, Ann Arbor, MI), IL-12 (R&D Systems, Minneapolis, MN), and TNF-α (R&D Systems) with specific ELISA kits according to the manufacturers’ instructions. The concentrations were recorded per digesta fresh weight.

Tissue Sampling and Immunohistochemical Methods. A tissue sample was cut from the middle of the second most distal small intestinal section of each animal. The tissue sample was thus obtained from the middle of the area from which the digesta sample was collected. The sample was placed in Tissue-Tek OCT compound (Sakura Finetek, Zoeterwoude, the Netherlands), frozen in liquid nitrogen, and stored at −70°C. The tissue samples were cut with a cryostat microtome at 7 μm, and the slices were placed on SuperFrost microscope slides (Menzel-Gläser, Braunschweig, Germany), air-dried, and stored at −70°C.

The slices were fixed with acetone, rehydrated in TBS, and incubated with the biotinylated antinmouse CD8a monocolonal antibody or the biotinylated antimonoclonal Mac-1 monoclonal antibody, both from Cedarlane (Hornby, Ontario, Canada). The slices were washed in TBS and incubated with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). The slides were again washed in TBS, and the staining was developed with 3,3’-diaminobenzidine peroxidase substrate kits of Vector Laboratories or Zymed Laboratories (South San Francisco, CA) for CD8 and Mac-1 analysis, respectively. All of the kits were used according to the manufacturers’ instructions. The slides were counterstained with hematoxylin and mounted with Aquamount Improved (BDH Laboratory Supplies, Dorset, United Kingdom). Negative control samples were produced by incubating the samples in TBS plus 1% BSA without either of the biotinylated primary antibodies. The same TBS-BSA solution was also used for optimal dilution of the primary antibodies.

The stained samples were studied under a light microscope at ×100 magnification. A minimum of 4 villi/sample was counted for positively stained cells, with an average number of 15 and 24 for the CD8 and Mac-1 analysis, respectively.

CVR. The CVR was measured from micrograph prints taken from the histological slides. A representative area was chosen from the prints, and the depth of the crypts and the length of the villi were measured with a ruler to the nearest millimeter (see Fig. 2D). The CVR ratio was counted by dividing the depth of the crypts by the length of the villi.

Statistics. A MLR technique was used to analyze the data. Within each age group, a MLR model with principal terms (diet, sex, and genotype) and first-degree interaction terms was tested to discover the most significant sources of variations with a stepwise exclusion of nonsignificant terms. Furthermore, mean values from each age group were compared using a t test. Correlations between immunoparameters were calculated using all data within each age group, whereas correlations of immunoparameters with the adenoma parameters were calculated from the Apc<sup>Min<sup>+/−</sup> mouse data only, excluding the wildtype mice.

ANOVA (Systat 7.0; SPSS Inc.) was also used to analyze differences between treatments. The Ps obtained by ANOVA are indicated as such in the text. Otherwise, Ps are from the MLR analysis.

RESULTS

Number of CD8+ Cells in Ileal Mucosa. The number of CD8+ cells per ileal villus did not differ between genotypes or dietary treatments. The 5-week-old mice had fewer CD8+ cells than the 8-week-old (P < 0.01) or 15-week-old mice (P < 0.05), whereas no further increment between 8 and 15 weeks was found (Fig. 1A). Males had more CD8+ cells per villus than females (P < 0.05) at 15 weeks. The number of CD8+ cells correlated positively with the number of Mac-1+ cells at 8 and 15 weeks and luminal IgA concentration at 8 weeks (Table 2). Most of the CD8+ cells were located intrapithelylial, but some were also observed in the lamina propria area (Fig. 2A).

Number of Mac-1+ Cells in Ileal Mucosa. Except for the 5-week time point at which the Apc<sup>Min<sup>+/−</sup> mice exhibited fewer Mac-1+ cells than the wild-type mice (P < 0.01), the numbers of mucosal Mac-1+ cells were similar in both genotypes and in all dietary treatments. The number of Mac-1+ cells/villus increased from 5 weeks to a higher level at 8 weeks and decreased again by the age of 15 weeks (Fig. 1B). The number of Mac-1+ cells was significantly increased at 8 weeks when compared with 5- and 15-week-old mice (Fig. 1B, P < 0.01).
Males had more Mac-1+ cells/villus than females at 15 weeks ($P < 0.05$). At 15 weeks, the number of Mac-1+ cells correlated positively with the number of CD8+ cells, the diameter of adenomas, and the relative proportion of large adenomas, whereas it correlated negatively with the relative proportion of small adenomas (Table 2). The Mac-1+ cells were located in the lamina propria area of the villi (Fig. 2B).

**Luminal IgA Concentrations.** The genotypes did not differ at any time point in the concentration of luminal IgA, which increased between 5 and 15 weeks in all mice ($P < 0.01$ from 5 to 8 weeks and from 8 to 15 weeks; Fig. 1C). At 8 weeks, no dietary or gender effects were observed, but at 15 weeks, mice on beef diet exhibited less luminal IgA than mice on control diet ($P < 0.001$; Fig. 3), and females exhibited less luminal IgA than males ($P < 0.05$; Fig. 3). The concentration of IgA correlated positively with the number of CD8+ cells at 8 weeks. In addition, a positive correlation between the diameter of adenomas and the relative proportion of medium-sized adenomas and a negative correlation between the diameter of adenomas with the relative proportion of small adenomas were found at 15 weeks (Table 2).

**Luminal PGE$_2$ Concentrations.** The concentration of luminal PGE$_2$ was higher at 8 weeks than at 5 weeks or at 15 weeks ($P < 0.001$; Fig. 1D), and no differences between dietary treatments or genders were found. The Apc$^{Min/+}$ mice had higher levels of PGE$_2$ than the wild-type mice at the age of 15 weeks ($P < 0.01$). At 15 weeks, the concentration of PGE$_2$ correlated positively with the total number of adenomas, adenoma burden, and the relative proportion of medium-sized and large adenomas. PGE$_2$ correlated negatively with the relative proportion of small adenomas (Table 2).

**Luminal IL-12 Concentrations.** Concentration of luminal IL-12 was measured from most 8- and 15-week-old mice ($N = 6–13$ mice/treatment). Lack of sample material prevented us from analyzing IL-12 from some animals. The variation between individuals was quite high, and in many samples a value near the detection limit of the assay was observed. The average luminal IL-12 concentration was 47.6 pg/g digesta fresh weight (SE, 4.8; $N = 99$), with no difference between the genotypes. At 8 weeks, IL-12 concentration was higher in females (mean, 61.0 pg/g digesta; SE, 11.7) than in males (mean, 30.2 pg/g digesta; SE, 3.1; $P < 0.05$, ANOVA). The IL-12 concentration was especially high in beef-fed females (mean, 77.5 pg/g digesta; SE, 33.5), with a statistical difference from other diet-gender groups at 8 weeks ($P < 0.05$). Otherwise, the ideal IL-12 concentration did not differ between the age groups, dietary treatments, or genders.
Luminal TNF-α Concentrations. The concentration of luminal TNF-α was measured from most 8- and 15-week-old mice (N = 6–13 mice/treatment). Lack of sample material prevented the analysis of TNF-α from some animals. The mean TNF-α concentration was 379.0 pg/g digesta fresh weight (SE, 23.3; N = 90), with no difference between the genotypes. At 8 weeks, when genotypes were combined, the luminal TNF-α concentration was lower in the beef diet (mean, 264.4 pg/g digesta; SE, 52.2) than in the control diet (mean, 462.9 pg/g digesta; SE, 56.2) or inulin diet (mean, 399.4 pg/g digesta; SE, 60.9; P < 0.01). There were no other differences in the TNF-α concentrations between age groups, dietary treatments, or sexes.

CVR in Ileum. The general inflammatory status of the gut mucosa was determined by CVR measurements. Intestinal inflammation was not observed in the samples of the present study (Fig. 2D). The mean CVR was 0.49 (SE, 0.007; N = 124), with no difference between the genotypes, age groups, dietary treatments, or genders.

Infiltration of Immune Cells into Adenomas. The number of CD8+ and Mac-1+ cells within the adenoma tissue was not the main focus of this study. However, some adenomas appeared in the samples taken from the ApcMin+/+ mice. The number of CD8+ cells per adenoma ranged from a few to none (Fig. 2E). In contrast, the Mac-1+ cells were observed in all adenomas, and they were often relatively abundant (Fig. 2F). The normal-appearing mucosa adjacent to adenomas did not appear to harbor abnormally high or low numbers of CD8+ or Mac-1+ cells, compared with other mucosal areas (Fig. 2E).

Development of Adenomas. Age was the most significant factor explaining the adenoma parameters. At the age of 5 weeks, most ApcMin+/+ mice exhibited only a few small or medium-sized adenomas (Table 3). The number of adenomas increased significantly between weeks 5 and 8 (P < 0.0001) but stayed at the same level thereafter (Table 3). The mean diameter of adenomas and the adenoma burden increased significantly between weeks 5 and 8 and between weeks 8 and 15 (P < 0.0001 in each case; Table 3). The percentage proportion of small adenomas decreased throughout the experimental period, whereas the proportions of medium-sized and large adenomas increased (P = 0.01–0.0001; Table 4).

At the age of 5 weeks, the adenoma parameters did not differ between the two genders. At 8 and 15 weeks, females had proportionally more small adenomas and fewer medium-sized adenomas than males (P < 0.05; Table 4). The inulin group had a higher average adenoma diameter (P < 0.001), proportionally more large and medium-sized adenomas (P < 0.05), and a lower percentage of small adenomas (P < 0.001) than the control and beef groups at 8 weeks (Tables 3 and 4). At 15 weeks, the beef-fed females had fewer adenomas and a lower adenoma burden than the other diet-gender groups (Table 3). ApcMin+/+ mice that were fed the rodent chow diet until week 8 also developed a comparable number of adenomas (mean, 33.0; SE, 6.9; N = 5) with the other dietary groups at 8 weeks (P = 0.49, ANOVA).

Mouse Weight Development. As expected, female mice were smaller and gained less weight than males. At the 5 and 8 week time

| Table 3 The number, diameter, and burden (total adenoma area per individual) of adenomas in the small intestine of ApcMin+/+ mice |
|-----------------|-----------------|-----------------|-----------------|
| Age (wks)       | Diet            | Gender | Adenoma<sup>a</sup> | Diameter<sup>b</sup> | Burden<sup>c</sup> |
|                 |                 |        | No. | SE | mm | SE | mm² | SE |
| 5               | Peller<sup>c</sup> | M     | 14.7 | 3.40 | 0.6 | 0.05 | 6.1 | 1.93 |
|                 | M               | F     | 12.8 | 2.23 | 0.6 | 0.02 | 3.6 | 0.81 |
| 8               | Control         | M     | 60.0 | 5.29 | 1.0 | 0.03 | 43.3 | 3.94 |
|                 | F               | F     | 56.7 | 12.87 | 0.8 | 0.04 | 29.4 | 6.56 |
|                 | M               | F     | 39.6 | 8.57 | 0.9 | 0.03 | 27.9 | 7.29 |
|                 | M               | F     | 35.7 | 10.86 | 0.8 | 0.03 | 19.6 | 6.25 |
|                 | Control         | M     | 31.7 | 2.23 | 0.9<sup>d</sup> | 0.05 | 26.1 | 3.23 |
|                 | F               | F     | 47.7 | 10.78 | 1.0<sup>e</sup> | 0.05 | 37.0 | 5.97 |
| 15              | Control         | M     | 61.5 | 5.89 | 1.2 | 0.02 | 83.5 | 6.56 |
|                 | Control         | F     | 55.8 | 8.46 | 1.1<sup>f</sup> | 0.07 | 72.3 | 15.27 |
|                 | M               | F     | 49.8 | 12.33 | 1.2 | 0.09 | 76.2 | 23.16 |
|                 | Beef            | F     | 28.7 | 3.77 | 1.0<sup>g</sup> | 0.05 | 30.9 | 4.90 |

<sup>a</sup> The 5-week age group differs statistically from the 8- and 15-week groups (P < 0.0001).

<sup>b</sup> Statistical difference between all three age groups (P < 0.0001).

<sup>c</sup> Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Teklad, Madison, WI.

<sup>d</sup> Higher in inulin than in control and beef diets, both genders combined (P < 0.001).

<sup>e</sup> Lower in females than in males, both diet groups combined at 15 weeks (P < 0.01).

<sup>f</sup> Lower in beef-fed females than in other diet-gender groups (P < 0.01).
Intestinal immune responses of the mutation in the APC gene does not lead into major defects in the similar immunological maturation in the intestine, suggesting that the peripheral tissues, and testosterone and estrogen have different effects at puberty, the T cells differentiate in thymus and migrate into pubertal rise in sex steroid hormones. Puberty begins around the fifth week of life in most inbred mouse strains such as C57BL/6J (30, 31). At puberty, the CVR analysis, which did not show differences between genotypes and the adjacent mucosa. The absence of inflammation was supported by the cytokine IL-12, than in other diets (34). Estrogen may directly protect the females against tumorigenesis because ovariectomy has been shown to increase intestinal adenoma number by 77% in ApcMin/+ females (40). However, it is also possible that some of the tumor growth-modulating effects of these hormones are mediated via their effects on the immune system. Females had a higher ileal concentration of the Th1- and males. Androgens have been described to influence the immune system because after the pubertal period, sex differences were observed in both adenoma parameters and immunoparameters. In the present study, as observed previously in both ApcMin/+ mouse (38) and rat (39), females seemed to have resisted intestinal tumorigenesis better than males. Estrogen may directly protect the females against tumorigenesis because ovariectomy has been shown to increase intestinal adenoma number by 77% in ApcMin/+ females (40). However, it is also possible that some of the tumor growth-modulating effects of these hormones are mediated via their effects on the immune system. Females had a higher ileal concentration of the Th1-type cytokine IL-12 than males at 8 weeks and had fewer Mac-1+ cells and less PGE2 and IgA than males at 15 weeks. This implies that the Th2-type immune response was more pronounced in males than in females.

### Table 4

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### DISCUSSION

#### The Effects of Age, Genotype, and Gender

In the present study, the age of the mice was the most important factor affecting the immunological and adenoma parameters. Most of the effects of the other main factors (genotype, diet, and gender), as well as the correlations between the studied parameters, were observed at the 15 week time point. In general, the wild-type and ApcMin/+ mice showed similar immunological maturation in the intestine, suggesting that the mutation in the APC gene does not lead into major defects in the intestinal immune responses of the ApcMin/+ mice. The lack of genotype differences at week 8, the time when most of the adenomas appeared, suggests that the adenomas do not induce inflammation in the adjacent mucosa. The absence of inflammation was supported by the CVR analysis, which did not show differences between genotypes at any time point. During inflammation, the crypt area thickens due to infiltration of immune cells, and concomitantly, the villi shorten (29). Based on the results of the present study, it is not possible to explain the tumorigenesis in the ApcMin/+ mouse model by immunological responses.

The number of CD8+ and Mac-1+ cells and the luminal concentrations of IgA and PGE2 increased significantly from 5 to 8 weeks of age in both genotypes, suggesting an intestinal immunostimulation during that time period. The immunostimulation may be linked to the pubertal rise in sex steroid hormones. Puberty begins around the fifth week of life in most inbred mouse strains such as C57BL/6J (30, 31). At puberty, the T cells differentiate in thymus and migrate into peripheral tissues, and testosterone and estrogen have different effects on this process (32). In the present study, at 15 weeks, the females had fewer CD8+ and Mac-1+ cells in the mucosa, less luminal IgA and TNF-α, and more luminal IL-12, in comparison with males. It is likely that the sex steroids have a role in these differences between females and males. Androgens have been described to influence the immune functions specifically through their action on stromal cells (33). Thymus, like the intestine, harbors stromal cells that continuously express PGE2 (34).

In ApcMin/+ mice, most of the adenomas appeared between weeks 5 and 8, and no significant increase in the number of adenomas was observed thereafter. At week 5, the mice were transferred from the standard rodent chow to semisynthetic experimental diets. However, the dietary change is unlikely to be the primary factor for the tumorigenesis because the ApcMin/+ mice that were fed the rodent chow diet until week 8 developed a similar number of adenomas as animals in the three experimental diets. It is noteworthy that the majority of adenomas appeared concomitantly with the suggested pubertal immunostimulation. The relevance of the pubertal events in the development of adenomas is supported by the clinical knowledge that the polyps of FAP patients are seldom seen before 10 years of age (35), and the colonoscopy screenings of FAP patients usually start at puberty (36). The ApcMin/+ mice and human FAP patients carry a similar mutation in the APC gene (37). Gender-related hormones may have a role in both tumorigenesis and the shaping of the intestinal immune system because after the pubertal period, sex differences were observed in both adenoma parameters and immunoparameters. In the present study, as observed previously in both ApcMin/+ mouse (38) and rat (39), females seemed to have resisted intestinal tumorigenesis better than males. Estrogen may directly protect the females against tumorigenesis because ovariectomy has been shown to increase intestinal adenoma number by 77% in ApcMin/+ females (40). However, it is also possible that some of the tumor growth-modulating effects of these hormones are mediated via their effects on the immune system. Females had a higher ileal concentration of the Th1-type cytokine IL-12 than males at 8 weeks and had fewer Mac-1+ cells and less PGE2 and IgA than males at 15 weeks. This implies that the Th2-type immune response was more pronounced in males than in females.

#### Intestinal Immune Responses and Tumorigenesis in the ApcMin/+ Mouse

The relationship between adenoma formation and intestinal immune responses in ApcMin/+ mice was also studied. The positive correlations between adenoma size and number and PGE2 and that between adenoma size and IgA suggest that these Th2-type immune responses are associated with increased tumorigenesis in the ApcMin/+ mouse model. The positive correlation between tissue macrophages (Mac-1+ cells) and tumor size indicates that these COX-2-producing cells may be involved in tumorigenesis as well.

Fig. 4. The average weekly body weight development in male ApcMin/+ or wild-type mice in the 15-week beef and control diet groups. There were 4 wild-type mice and 6–8 male ApcMin/+ mouse per time point and dietary treatment.
Both COXs (COX-1 and COX-2) convert arachidonic acid to PGE₂ in the intestine (41). PGE₂ is a strong immunomodulator, which suppresses inflammation in the intestine and increases the Th2-type immune response (11, 42, 43). According to Hull et al. (22), COX-2 is up-regulated in intestinal macrophages of ApcΔMin/+ mice. In the present study, the concentration of PGE₂ was higher in mature ApcΔMin/+ than wild-type mice. The possible tumor-promoting effect of PGE₂ is supported by the findings that fish oil, or more precisely n-3 polysaturated fatty acids, reduced both the concentration of PGE₂ in intestinal contents (44) and the neoplastic growth (e.g., Refs. 45 and 46). Moreover, a novel finding by Pai et al. (47) indicates that PGE₂ directly increases the growth of the intestinal epithelium and intestinal tumor cells by the transactivation of epidermal growth factor receptor and the induction of the extracellular signal-regulated kinase 2-mitogenic signaling pathway.

In the present trial, dietary inulin increased the size of adenomas at 8 weeks (the 15 week time point was not studied). It is possible that inulin, a dietary fiber with prebiotic properties (48) in mice, affects tumorigenesis in the ApcΔMin/+ mouse model partly by modulating the intestinal immune response profile toward the Th2 direction as described with many prebiotics and probiotics (49). In contrast to the present findings, inulin suppressed the formation of colonic aberrant crypts in chemical carcinogenesis rat models (e.g., Refs. 50 and 51). According to Buddington et al. (52) dietary inulin reduced the mortality of mice in several disease models, including dimethylhydrazine-induced intestinal carcinogenesis. It is possible that the benefits and controversies of some dietary ingredients vary depending on the model system used, and these differences should be taken into account when drawing conclusions on the safety of dietary ingredients in human nutrition.

In the present study, dietary beef decreased the number and size of adenomas at the 15 week time point. The milder disease development in the beef group as compared with the control group was also implicated in the later cessation of growth and the less severe loss of body mass in the beef-fed males than in the control-fed males. Dietary beef suppressed the luminal concentration of IgA at 15 weeks in both sexes and increased the concentration of IL-12 in 8-week-old females. These results suggest that dietary beef decreased the Th2-type immune responses and increased the Th1-type immune responses. At 8 weeks, the concentration of TNF-α was lower in the beef group than in the other diet groups, indicating less recruitment and activation of macrophages in the tissue by the smaller number of developing adenomas. Dietary beef and female sex seemed to synergistically protect the ApcΔMin/+ mice against intestinal tumorigenesis. Mutanen et al. (26) observed that the level of cytosolic β-catenin in the intestinal mucosa of ApcΔMin/+ mice was significantly lower in beef diet than in inulin diet group, with an intermediate value for the mice in the non-fiber control diet group. With regard to its oncogenic nature, the decrease in the cytosolic β-catenin may decrease the formation of adenomas in this model. The mechanism behind the tumor-suppressing effect of beef in the present study remains unknown. One has to keep in mind that the quality of beef can vary a lot and that the beef used was freeze-dried rather than cooked, so the heat-induced toxic substances usually associated with meat consumption do not apply in this case. Angiogenic processes may be involved because the beef group appeared to have less blood in the intestinal contents than the control group at 15 weeks (general observations during the collection of digesta samples). The Th2-type immune response may be involved in the development of tumor vasculature because COX-2, the enzyme producing PGE₂, has been suggested to affect tumor angiogenesis in another APC-mutated mouse model of intestinal tumorigenesis, the ApcΔ747 knockout mouse (53). Besides immunological effects, PGE₂ also has other physiological effects that may play a role in tumor angiogenesis, and its potential role in tumor angiogenesis in the ApcΔMin/+ mouse model deserves further study.

Activation of recruited T lymphocytes and macrophages via pro-inflammatory cytokines appears necessary for an efficient clearance of neoplastic cells (5–7). The immunosurveillance against neoplastic cells has not been studied in the ApcΔMin/+ mouse model. In the present study, the adenoma tissue present in the immunohistological analyses contained few CD8+ cells, indicating that their recruitment for adenoma clearance was inefficient. In contrast, the Mac-1+ cells were always present in adenoma tissue. A complex relationship between tumors and tumor-associated macrophages has been suggested by Sica et al. (54), according to whom tumors may attract macrophages by chemokines, and the tumor microenvironment may regulate the localization and function of the macrophages. The role of macrophages in the ApcΔMin/+ mouse model should be further studied, and the identification of PGE₂-secreting cells in the tissue should be attempted.

The present study showed that the intestinal immune responses are mostly similar in ApcΔMin/+ and wild-type mice, suggesting that the tumorigenesis in the ApcΔMin/+ mouse model cannot be explained by intestinal immunology. For the first time, a general enhancement phase of intestinal immune responses was described in association with age, peaking by the time of puberty. The immunological enhancement was accompanied by a major increase in the number and growth of adenomas in ApcΔMin/+ mice. The luminal concentrations of IgA and PGE₂ and the number of macrophages in the ileal tissue were positively correlated with increased tumorigenesis in the ApcΔMin/+ mouse, indicating that the balance between the cytotoxic and humoral immune responses may affect the pace of the spontaneous intestinal tumorigenesis in this murine model of colorectal cancer.

REFERENCES

Intestinal Immune Responses in Wild-Type and Apc\textsuperscript{Min/+} Mouse, a Model for Colon Cancer

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