IL-2-deficient mice raised under germfree conditions develop delayed mild focal intestinal inflammation

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pifies immune stimuli and influences B cell differentiation. IL-2-deficient mice spontaneously develop intestinal inflammation if raised under specific pathogen-free (SPF) condi-
tions. We quantitatively determined the aggressiveness and kinetics of gastrointestinal and hepatic inflammation in the presence or absence of viable bacteria in IL-2-deficient mice. Breeding colonies were maintained under SPF and germfree (GF) conditions. Intestinal tissues, serum, and mesenteric lymph nodes were obtained from mice at different ages for blind histological scoring, immunoglobulin measurements, mucosal T cell infiltration, and cytokine secretion. GF IL-2−/− mice developed mild, focal, and nonlethal intestinal inflammation with delayed onset, whereas the more aggres-
sive inflammation in SPF IL-2−/− mice led to their death between 28 and 32 wk. Periportal hepatic inflammation was equal in the presence or absence of bacterial colonization. Intestinal immunoglobulin secretion decreased significantly by 13 wk of age in IL-2−/− mice in both GF and SPF envi-
ments. In contrast to other genetically engineered rodents, IL-2−/− mice develop mild focal gastrointestinal and active portal tract inflammation in the absence of viable bacteria.

interleukin-2-deficient mice; luminal flora; colitis; animal models

RECOMBINANT DNA TECHNOLOGY allows genes of interest to be either introduced or deleted in rodents by targeted manipulation. To determine the functions of cytokines in vivo, mice with selective deletion of various cytokine genes have been generated. The development of sponta-
aneous colitis in a number of these “knockout” mice has led to new insights into the pathogenesis of chronic intestinal inflammation (38).

Since its introduction several years ago, the interleukin-2 knockout mouse (IL-2−/−) has been regarded as a model for chronic, immune-mediated colitis in hosts with dysregulated T cell function (1, 35, 36). IL-2 is an important regulatory cytokine, produced by a subset of activated CD4+ T cells that amplifies immune stimuli by promoting T lymphocyte growth and expansion, possibly contributing to B cell differentiation and activa-
ting macrophages, LAK cells (lymphocyte-activated killer cells), and NK cells (natural killer cells) (28, 46). However, conflicting data exist regarding the role of IL-2 in vivo. In 3- to 6-wk-old IL-2-deficient mice, immune responses are still relatively normal, and no intestinal or hepatic inflammation is evident (20, 41); however, as they age, IL-2-deficient mice raised in a conventional environment progressively develop aggressive colitis, first detectable between 6 and 15 wk of age (36), that is mediated by T cells, especially CD4+ T cells, and not B cells (25, 43). These T cells are thought to be thymus dependent and invade the colon and the bone marrow to cause colitis, anemia, and loss of B cells, respectively (18). Recent studies suggested that the extraintestinal manifestations are independent of the microbial environment (8).

In the original description of colitis in IL-2-deficient mice, Sadlack et al. (36) demonstrated attenuated colitis in knockout mice raised in a specific pathogen-
free (SPF) environment and no clinical or histological evidence of colitis in a limited number of germfree (GF, sterile) IL-2−/− mice. The absence of histological evidence of colitis in young GF IL-2−/− mice was confirmed recently by Contractor et al. (8), who also reported that anemia, hepatic inflammation, and gener-
ized lymphoid hyperplasia in these mice was not dependent on bacterial colonization. These observations suggest that luminal microbial agents provide the persistent antigenic stimulus for this T cell-mediated colitis. A number of other rodent models support this hypothesis (37, 38). One of the best characterized models in this respect is the HLA-B27/human β2-microglobulin transgenic rat. Normal resident luminal bacteria play an important role in the pathogenesis of inflammation in this model since these rats do not develop colitis, gastritis, or arthritis if raised in a sterile environment (30, 47). Furthermore, Bacteroides species preferentially induced colitis in reconstitution studies (30, 32), consistent with the ability of metroni-
dazole and broad-spectrum antibiotics to attenuate disease (31). Similarly, IL-10-deficient mice, depending...
on their genetic background, and T cell receptor (TCR) knockout mice develop colitis, anemia, and growth retardation in the SPF environment and stay healthy in the GF state (12, 19, 42). Luminal bacteria and bacterial products also contribute to the inflammatory response in the indomethacin-induced enterocolitis rat model. GF rats have attenuated acute small intestinal ulceration, no chronic enteritis, and no cecal ulcers (10, 34, 39), while monoaassociation with Escherichia coli accentuated small intestinal ulcers (34). Furthermore, chronic indomethacin-induced inflammation is attenuated by treatment with tetracycline and metronidazole (3, 51).

The ability of sterile bacterial components to induce intestinal inflammation is documented by the observations that luminal peptidoglycan-polysaccharide polymers (PG-PS), the primary structural cell wall component of nearly all bacterial species, potentiates small intestinal ulcers in indomethacin-treated rats (10) and acetic acid-induced colitis (40) and that intramural injections of purified PG-PS (26) or muramyl dipeptide in incomplete Freund's adjuvant (21) induce chronic, immune-mediated granulomatous enterocolitis in susceptible hosts. In vitro studies with colonic epithelial cell lines further document the ability of bacteria and bacterial cell wall components to stimulate inflammatory responses. Jung and co-workers (16) demonstrated that bacterial invasion of epithelial cells stimulates a characteristic array of proinflammatory cytokines, and we have shown that a colonic epithelial cell line can be activated with PG-PS and lipopolysaccharide (LPS) (15). This cumulative evidence strongly suggests that products of the normal enteric gut flora can induce colitis in the genetically susceptible host and are essential for perpetuation of chronic disease. However, the recent observation that GF mice fed dextran-sodium-sulfate (DSS) develop aggressive colitis with increased mortality indicates that acute colonic injury can develop in the absence of viable luminal bacteria (2).

The aim of this study was to investigate in a quantitative fashion the relative time of onset, rate of progression, and degree of aggressiveness of colitis, gastritis, and hepatitis in IL-2-deficient mice at various ages in the presence or absence of luminal bacteria. Previous studies of colitis in GF IL-2-/- mice have investigated only early time points and have not quantified mucosal inflammatory responses (8, 36). In contrast to previous reports in this and other rodent model systems, we found that mild colitis and active gastritis and portal inflammation occur in IL-2-deficient mice in the absence of viable luminal bacteria.

MATERIALS AND METHODS

Mice. GF C57BL/6 x 129/Ola outbred IL-2-deficient mice were derived at the University of Wisconsin by cesarian section as previously described (30, 50). SPF and GF breeding colonies (heterozygous x heterozygous) were established at the Laboratory Animal Facilities of the University of North Carolina at Chapel Hill and the Gnotobiotic Facilities of the Center of Gastrointestinal Biology and Disease at the North Carolina State University, College of Veterinary Medicine at Raleigh, respectively. Mice were genotyped by amplification of the IL-2 gene by PCR with IL-2-specific primers (Nucleic Acid Core Facility, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC) in tail digests as described elsewhere (41). The GF colonies were housed in Trexler flexible film isolators with autoclaved food and water ad libitum (50). Sterility was tested on a monthly basis by fecal Gram stain and cultures of the feces and bedding. In addition, cecal contents were documented to be sterile by aerobic and anaerobic culture and Gram stain at the time of necropsy of selected mice. PCR analysis of feces from SPF IL-2-deficient mice excluded Helicobacter infection (4). Animals were used between 4 and 46 wk of age.

Clinical assessment. Clinical symptoms of intestinal inflammation, including diarrhea, progressive wasting, and rectal prolapse, were assessed twice weekly. Before necropsy, mice were weighed and then killed by CO2 asphyxiation. Cardiac blood was taken for serum immunoglobulin measurements. Rectum, colon, cecum, small intestine, stomach, and liver were processed for histology, immunohistochemistry, immunoglobulin, and cytokine measurements. In addition, mesenteric lymph nodes were obtained for flow cytometric analysis.

Histological assessment of intestinal inflammation. Intestinal tissues were fixed for 24 h in 10% buffered Formalin. Tissues were embedded in paraffin and stained with hematoxylin and eosin. A gastrointestinal histological inflammatory score ranging from 0 to 4 (Table 1), adapted from a scoring system previously validated in HLA-B27 transgenic rats (30), was applied in a blinded fashion to quantitate intestinal inflammation. An overall large intestinal score was obtained by adding the individual scores from the rectum, transverse colon, and cecal tip and dividing by three.

Immunohistochemical staining of colonic tissues. Frozen sections (5 μm) of colons of GF IL-2-/- and wild-type (WT) mice 14 wk of age were acetone fixed (10 min) and then washed in PBS. To block endogenous peroxidase the sections were then incubated with 3% H2O2 (10 min) and washed in PBS. To block endogenous peroxidase the sections were then incubated with 3% H2O2 (10 min). After washing in PBS, nonspecific binding sites were blocked with 10% normal goat serum (Vector; 20 min). Next, sections were incubated with the primary antibody rat anti-mouse CD4 or rat anti-mouse CD8 (both rat IgG2a from Pharmingen) in a concentration of 2 μg/ml (30 min). Negative controls were incubated only in PBS. After a wash in PBS the secondary antibody (Vector biotinylated) rabbit anti-rat biotinylated was added at a concentration of 5 μg/ml (20 min). All incubations were done at room temperature. After washing in PBS, sections were incubated with horseradish peroxidase (HRP)-conjugated avidin-biotin complexes (Vectastain ABC method, Vector Laboratories) followed by 3-ethylcarbazole (AEC) substrate from Vector) staining. Counterstaining was done with hematoxylin.

Intestinal fragment cultures. Sections of the transverse colon of individual mice were prepared as previously described (7). Briefly, colon sections were washed with 1× PBS

Table 1. Histological grading of gastrointestinal inflammation

<table>
<thead>
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<th>Score</th>
<th>Criteria</th>
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<tr>
<td>0</td>
<td>No inflammation</td>
</tr>
<tr>
<td>1</td>
<td>Slightly infiltrating cells in lamina propia</td>
</tr>
<tr>
<td>2</td>
<td>Infiltration with mononuclear cells leading to separation of crypts, mild mucosal hyperplasia</td>
</tr>
<tr>
<td>3</td>
<td>Massive infiltration with inflammatory cells leading to disturbed mucosal architecture, loss of goblet cells, marked mucosal hyperplasia</td>
</tr>
<tr>
<td>4</td>
<td>Crypt abscesses, ulceration</td>
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(pH 7.5) to remove fecal contents, gently shaken at room temperature for 30 min in PBS, and cut into small fragments. Tissue fragments (100 mg) were incubated in 1.0 ml of RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 100 U/ml penicillin, 100 mM streptomycin, 0.25 mM L-glutamine, 1 mM sodium pyruvate, 5% heat-inactivated FCS (Irvine Scientific, Santa Ana, CA), 2 mM L-glutamine, 1 mM sodium pyruvate, 5 mM 2-mercaptoethanol, and 50 µg/ml gentamicin (Sigma, St. Louis, MO) at 37°C for 24 h. Supernatants were collected and stored at −20°C until further processing.

Isotype-specific ELISA to measure immunoglobulin. Detection of immunoglobulin production in colon culture supernatants and sera was performed as described in detail previously (48). The following affinity-purified antibodies were used for capture: goat anti-mouse IgA and goat anti-mouse IgM (Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD), goat anti-mouse IgG1 and goat anti-mouse IgG2a (Southern Biotechnology, Birmingham, AL). HRP-labeled goat anti-mouse IgA and goat anti-mouse IgG2a (Southern Biotechnology) were used for capture: goat anti-mouse IgA and goat anti-mouse IgG2a (Southern Biotechnology) were used to detect the different isotypes. The concentration of antibody was calculated by comparison with a standard curve of purified mouse IgA, IgM, IgG1, and IgG2a (PharMingen, San Diego, CA).

Cytokine measurements by ELISA. IL-12 secretion in colon culture supernatants was measured by ELISA using commercially available anti-IL-12 antibodies C15.6 and biotinylated C17.8 (PharMingen) for capture and detection, respectively (48). The following affinity-purified antibodies were commercially available anti-IL-12 antibodies C15.6 and biotinylated C17.8 (PharMingen) for capture and detection, respectively, as previously described (49). Hybridomas (GK1.5 and 53–6.72) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Supernatants were prepared in this laboratory. Cells were then washed twice and incubated with 25 µg/ml FITC-labeled goat anti-rat IgG (PharMingen) adsorbed with mouse serum (KPL) for 30 min at 4°C and then washed twice and analyzed. For detection of B cells, cells were incubated for 30 min at 4°C with either FITC-labeled goat anti-mouse IgA-IgG-IgM, 12.5 µg/ml (KPL), and washed twice. All analyses were done in a FACScan (Becton Dickinson, San Jose, CA).

Statistical analysis. Values are expressed as means ± SE. For each measurement, one-way ANOVA was carried out to compare groups. P < 0.05 was regarded as significant.

RESULTS

Clinical parameters of mice with disrupted IL-2 gene in variable microbial environments. Offspring of both colonies (GF and SPF) appeared normal after birth, but in the perinatal period IL-2−/− mice in both environments were already generally smaller than WT animals, and were more difficult to maintain. At 8 wk of age there was a mortality rate of 50% affecting only the SPF colony, following severe growth retardation and progressive wasting. Sadlack et al. (36) provided evidence that this early death is the result of severe autoimmune anemia. By 16–18 wk of age, all of the surviving IL-2−/− mice in the SPF environment (n = 17) developed diarrhea and gained less weight than the WT controls (P < 0.0001, Fig. 1). Rectal prolapse was seen rarely (8% in SPF IL-2−/−). IL-2−/− mice in the SPF environment died usually between 28 and 32 wk of age (n = 9). In contrast, IL-2−/− mice raised in the GF environment remained clinically healthy during the observation period of up to 46 wk (n = 12) with no mortality, diarrhea, or rectal prolapse, and gained weight normally in the first 8 wk of life but then...
gradually lost weight and were generally smaller than age-matched WTs (P < 0.05, Fig. 1). However, GF IL-2 2/2 mice weighed more than SPF IL-2 2/2 mice in the first 13 wk (P < 0.0001, Fig. 1).

Gross and histological evaluation of gastrointestinal tract. SPF IL-2-deficient animals (n = 6) showed mild splenomegaly by 4–8 wk of age. Grossly, the intestines of SPF IL-2 2/2 mice appeared normal at these time points, although blinded histological examination at 8 wk revealed mild large intestinal inflammation (Fig. 2), predominantly affecting the rectum (rectal histological score: SPF IL-2 2/2 1.0 ± 0.2, SPF WT 0.1 ± 0.1; P < 0.0001). By 13 wk of age, intestinal inflammation in SPF IL-2 2/2 mice rapidly progressed and became characterized by mucosal hyperplasia and massive infiltration of the lamina propria with mononuclear cells, which in some cases extended into the submucosa. By 28 wk of age, the entire gastrointestinal tract, including liver, stomach, small intestine (jejunum and ileum), and colon was involved in SPF IL-2 2/2 mice (Fig. 3). At this time point, advanced disease was grossly manifested by a rigid, thickened colon, mesenteric lymphadenopathy, and marked splenomegaly. These later stages of the disease were characterized histologically by a further loss of goblet cells, frequent crypt abscesses, and widely separated crypts (Fig. 4B). Ulcerations of the mucosa were rarely seen. The involvement of the stomach was similar, with prominent lymphocytic infiltration and hyperplasia of the mucosa. The glandular region (Fig. 4E) was more affected than the squamous cell portion of the stomach, and inflammation extended into the duodenum.

Even though GF IL-2-deficient animals showed no clinical symptoms or mortality up to 46 wk of age, they developed splenomegaly, lymphadenopathy, and histological features of mild gastrointestinal inflammation (Figs. 2 and 4C). Sterility of the colony was confirmed by monthly culture and Gram staining of feces and...
Fig. 4. Histological evidence of colitis in GF and SPF mice. A: stained normal colonic section from healthy, 28-wk-old IL-2 WT mouse, raised under SPF conditions [hematoxylin and eosin (H&E), ×100]. B: colon section from 26-wk-old SPF IL-2-deficient mouse (×100). Characteristics of inflammatory process are marked mucosal hyperplasia due to infiltrating mononuclear cells, loss of goblet cells, and disrupted mucosal architecture. C: section of colon of diseased GF IL-2-deficient mouse, 36 wk of age (×100). Inflammation is focal (arrowheads), manifested by mild infiltration of mononuclear cells and the loss of goblet cells. Adjacent mucosa is entirely normal. In contrast to SPF animals, there was no hyperplasia of mucosa. D: low-power view of colon of 28-wk-old GF IL-2-deficient mouse demonstrating focal normal areas with abundant goblet cells (open arrow) with more generalized mild inflammation with mild infiltration of a small number of mononuclear cells and loss of goblet cells, but no crypt hyperplasia. Focal areas of moderate mononuclear cellular infiltration are present (solid arrow) (×40). E: section of glandular part of stomach from 26-wk-old SPF IL-2-deficient mouse (×100). Inflammatory pattern is similar to that of colon with abundant lymphocytic infiltration and hyperplasia of mucosa. F: moderate mononuclear cell infiltration of antrum of 28-wk-old GF IL-2-deficient mouse (×100).
culture and Gram staining of cecal contents from selected mice at necropsy. The disease in GF animals was delayed (onset after 8 wk) but exhibited a 100% penetrance by 13 wk of age. Colonic inflammation progressively increased between 8 and 28 wk, but then reached a plateau. At each time point after 4 wk histological colonic inflammation in GF IL-2−/− mice was significantly less than in SPF IL-2−/− mice (P < 0.05; Fig. 2) and became significantly greater than in WT controls by 13 wk of age (P < 0.05; Fig. 2). Inflammation in the large intestine in GF IL-2−/− mice was quite focal, and mucosal hyperplasia was much less prominent than in the SPF environment (compare Fig. 4B with Fig. 4, C and D). Crypt abscesses were occasionally present but no ulcerations or submucosal inflammation was noted even at late time points in the colons of GF IL-2−/− mice. GF WTs showed no inflammation at any time point. Immunohistochemical staining indicated infiltration of CD4+ T lymphocytes in the colonic mucosa of GF IL-2−/− mice (Fig. 5A); CD4+ T cells were rare in the mucosa of GF WT mice (Fig. 5B). CD8+ T lymphocytes were less evident in the mucosa of GF IL-2−/− mice (data not shown). By 28–46 wk of age GF IL-2−/− mice exhibited increased histological inflammation in all gastrointestinal regions (Fig. 3), including focally aggressive mononuclear cell infiltration into basal regions of the antral mucosa (Fig. 4F).

Colonic IL-12 production. Mucosal secretion of IL-12 (p40 subunit) was measured as an independent objective marker of immune-mediated colitis. The p40 subunit of IL-12 is upregulated with inflammation in a number of experimental mouse models of chronic colitis (9, 33, 42). IL-2−/− mice of both environments expressed increased colonic levels of IL-12 only with advanced disease. Colonic IL-12 secretion corresponded to the aggressiveness of colitis by histological criteria. Although colons of GF IL-2−/− mice 38–46 wk of age secreted approximately twofold more IL-12 than those of GF WT animals (443 ± 72 vs. 229 ± 68 pg/100 mg tissue weight, P < 0.05), SPF IL-2−/− mice at 28 wk of age secreted as much as 20-fold more colonic IL-12 than SPF WT mice (4,211 ± 769 vs. 199 ± 32 pg/100 mg tissue, P < 0.01). INF-γ and TNF-α could not be detected in cultured colonic supernatants.

Immunoglobulin production. GF and SPF IL-2-deficient mice exhibited similar patterns of serum and colonic mucosal immunoglobulin isotype production. After an initial rise at 8 wk of age, which was less pronounced in GF IL-2−/− animals, colonic IgG2a production decreased dramatically with age, with ~100-fold lower values at 26 wk of age (Fig. 6), compared with 8 wk of age. Similar patterns were seen with serum and colonic IgM, IgA, and IgG1 (data not shown). In SPF IL-2−/− mice, all of the analyzed isotypes (IgA, IgM, IgG1, IgG2a) were already increased at the time of very mild histological inflammation (at 8 wk of age) and before clinical signs of colitis became evident. At this time point, SPF IL-2−/− mice showed 13-fold more secreted colonic IgG2a compared with WT of the same age and twofold more IgG2a relative to GF IL-2−/− mice (Fig. 6). However, by 13 wk of age, colonic production of immunoglobulins (Fig. 6) of IL-2−/− mice progressively decreased and was very low at 28 wk of age in SPF and at 38 wk of age in GF IL-2−/− animals compared with WT mice.

Flow cytometric analysis of mesenteric lymph node cells. Macroscopically there was an increase in size and numbers of the mesenteric lymph nodes in SPF and GF IL-2−/− mice with colitis compared with their healthy IL-2−/− littermates. We therefore examined the cellular composition of these lymphoid organs. The loss of
circulating and mucosal immunoglobulin production was accompanied by a loss of mature surface Ig^+^ B cells from mesenteric lymph nodes in older IL-2-deficient mice, regardless of luminal bacterial colonization (Fig. 7A). Initially, at 4 wk of age, all mice had comparable proportions of mesenteric lymph node cells expressing surface immunoglobulin, with a slightly higher percentage in SPF IL-2^−/−^ compared to IL-2 WT 41.5 ± 7.0% vs. IL-2 WT 35.0 ± 2.2%, not significant (NS) and GF: IL-2^−/−^ 33.5 ± 3% vs. IL-2 WT 35.2 ± 2.1%, NS). However, during later stages of the disease, the differences became very apparent, such that by 28 wk of age, very few B cells were detected in SPF and GF IL-2^−/−^ mice (SPF: IL-2^−/−^ 2.7 ± 0.3% vs. IL-2 WT 26.6 ± 3.4%, P < 0.0001 and GF: IL-2^−/−^ 1.6 ± 0.4% vs. IL-2 WT 30.6 ± 5.2%, P < 0.001). At the same time point, we observed a reciprocal increase of CD4^+^ and CD8^+^ T cells in IL-2^−/−^ mice with no significant differences between SPF and GF mice (Fig. 7B and C). Proportions of CD4^+^ and CD8^+^ T cells remained increased in GF IL-2^−/−^ mice at 42 wk of age (Fig. 7B and C).

Inflammation of liver. The liver of SPF IL-2-deficient mice showed early signs of multifocal periportal infiltration, composed of macrophages and lymphocytes as early as 4–8 wk of age (Fig. 8). By 13 wk of age there were diffuse periportal infiltrates with abundant extramedullary hematopoiesis. The inflammation by 28 wk of age was characterized by severe periportal infiltration of macrophages and lymphocytes with penetration of the limiting plate, bile duct proliferation, and single cell necrosis of hepatocytes adjacent to lesions (Fig. 9B). In contrast to the less severe gastrointestinal inflammation in GF IL-2^−/−^ mice, portal inflammation was similar at all time points in SPF and GF IL-2^−/−^ mice (Figs. 8 and 9B, B and C). Interestingly, serum transaminases were not elevated even with advanced histological inflammation (results not shown). WT mice showed minimal hepatic inflammation (Fig. 9A).
DISCUSSION

In this study we were able to confirm previous observations (8, 36) that IL-2−/− mice raised under SPF conditions developed histological inflammation; however, in contrast to these previous reports of absent colitis in GF IL-2−/− mice, we demonstrated mild, delayed, and focal gastrointestinal inflammation in GF IL-2-deficient mice, which was first detected at 13 wk of age. Colitis in GF IL-2−/− mice was mild, focal, delayed in onset, and reached a plateau at 28 wk. Mononuclear infiltrates in the lamina propria consisted predominantly of CD4+ T lymphocytes with both CD4+ and CD8+ T cells increased in the mesenteric lymph nodes of GF IL-2−/− mice. There were no clinical symptoms or mortality in GF IL-2−/− mice up to 46 wk of age, and histological inflammation and colonic IL-12 production were significantly less than in the SPF environment. The intestinal inflammation in SPF IL-2−/− mice was accompanied by a progressive loss of body weight and dramatically increased colonic IL-12 production. In addition, we observed clinical and gross evidence of progressive and lethal intestinal inflammation in our SPF IL-2−/− colony, in contrast to the absence of all but mild histological evidence of colitis in the original report (36). The mild, focal nature of the colitis in GF IL-2−/− mice older than 13 wk of age, our inclusion of older mice, and the different genetic background of our animals may explain the absence of observed colonic inflammation in other studies (8, 36). For example, the study by Contractor et al. (8) concen-

Fig. 8. Blinded liver histological scores at different ages. SPF (●, n = 26) and GF (○, n = 12) IL-2-deficient mice were compared with GF (△, n = 14) WTs. *P < 0.05 and **P < 0.0001 vs. GF WT.

Fig. 9. Histological evidence of portal tract inflammation in GF and SPF. A: hematoxylin and eosin (H&E)-stained liver section of 28-wk-old SPF IL-2 WT (×400). B: portal tract inflammation in 28-wk-old SPF IL-2-deficient animal with characteristic periportal infiltrates and bile duct proliferation (×400). C: H&E stained liver section of 28-wk-old GF IL-2-deficient mouse showing similar infiltration (×400).
trated on histological evidence of colonic inflammation at early time points (4–8 wk of age), whereas our GF IL-2 −/− mice did not develop detectable colitis by histological criteria until 13 wk of age and increased colonic IL-12 secretion was not evident until 36–38 wk of age. In addition, our SPF and GF colonies of IL-2-deficient mice were C57BL/6 × 129/Ola outbred populations derived from the same progenitors and were not selectively backcrossed, whereas the previous studies were performed on backcrossed C57BL/6IL-2 −/− mice (8, 36). In other models, C57BL/6 mice are low responders. For example, Berg et al. (5) showed that IL-10-deficient mice on an inbred 129/SvEv background developed more aggressive colitis than those on an inbred C57BL/6 background and that outbred 129/C57BL/6 mice had an intermediate phenotype (5). The importance of the genetic background is further illustrated by observations that IL-2 knockout mice are more sensitive to both SPF bacterial and GF environments than IL-10 knockout mice and HLA-B27 transgenic rats. In previous studies, we demonstrated that no mortality or jejunocolitis inflammation occurred in IL-10 −/− mice or B27 transgenic rats housed in the same SPF environment used in the current study and that neither of these two rodent models developed inflammation if housed in identical conditions as the GF IL-2 −/− mice (30, 42). Additional differences in the environmental conditions could account for disparate results of the present and previous studies, since subtle alterations in luminal bacteria (30), food (29), and temperature (45) can influence experimental inflammation. We cannot exclude spontaneous mutations, which enhance susceptibility to colitis, but we were careful to exclude the presence of all detectable microbial pathogens, including Helicobacter species, in our SPF colony.

The IL-2 knockout mouse model of chronic intestinal inflammation described here is unique because it spontaneously develops mild colonic, small bowel, and gastric lesions when raised under sterile conditions. In other well-established genetically engineered models of colitis, the involvement of the luminal bacterial flora on gastrointestinal inflammation is more pronounced. IL-10 and TCR-α/β knockout mice, as well as HLA-B27 transgenic rats, do not develop colitis or gastritis if raised under GF conditions (12, 30, 39, 42, 47), and IL-10 −/− mice develop even greater inflammation under conventional conditions where environmental pathogens are common, compared with the SPF state (19). Colitis in GF mice fed DSS established a precedent for mucosal inflammation in the absence of luminal bacteria (2), but in this acute situation luminal DSS could be considered the injurious agent.

Although our GF IL-2 −/− mice were documented to have no viable bacteria, they were still exposed to dead bacteria and bacterial constituents in the autoclaved food and bedding. We have previously postulated that nonviable bacterial products can provide the constant antigenic stimulus that drives chronic, relapsing colitis in genetically susceptible hosts (30, 37) and demonstrated that sterile bacterial components can induce or potentiate enterocolitis and extraintestinal disease. For example, luminal sterile PG-PS can potentiate toxin-induced intestinal inflammation in the indomethacin and acetic acid models (10, 40). Similarly, chronic granulomatous enterocolitis, hepatitis, and arthritis can be induced in susceptible Lewis rats by the intramuscular injection of purified bacterial PG-PS polymers (26), and heat-killed E. coli causes hepatic inflammation in rabbits (17). Furthermore, mice with experimental colitis display humoral and cellular immune responses to luminal bacterial antigens, establishing immunologic mechanisms by which nonviable bacterial antigens could cause inflammation (6, 13). It is possible that nonviable bacterial cell wall components and protein antigens present in the autoclaved food or bedding of GF animals provide sufficient stimulation to generate the mild, focal gastrointestinal inflammation seen in our GF IL-2 −/− mice, but that the less susceptible IL-10 −/− mice and HLA-B27 transgenic rats do not respond to identical stimuli. The autoclaved food in our GF facility contains dead bacteria by Gram stain and readily detectable LPS. In keeping with our suggestion of heightened susceptibility to luminal bacterial stimuli, SPF IL-2 −/− mice have more aggressive gastrointestinal disease with frequent crypt abscesses, higher mortality by 28–32 wk of age, and increased extraintestinal disease in the portal tracts and bone marrow than do SPF IL-10 −/− mice (19, 42). Alternatively, dysregulated T cells may react to self antigens for which bacterial constituents provide an adjuvant effect. Although we stress the unique presence of spontaneous gastrointestinal inflammation in sterile IL-2 −/− mice, we also need to emphasize the delayed onset of this mild, focal, nonproliferative, and nonprogressive colitis and the lack of a lethal inflammatory response, which is much more aggressive in the presence of viable luminal bacteria.

Histological inflammation was present in the gastric antrum, small intestine, colon, and hepatic portal tracts of GF IL-2 −/− mice. However, not all organs responded equally to the bacterial environment. Although the gastrointestinal inflammation was delayed and attenuated in GF animals, the periportal hepatic inflammation with necrosis of adjacent hepatocytes in GF IL-2 −/− mice was of similar intensity and started at the same time as in SPF animals; however, liver enzymes remained relatively normal throughout. Similarly, Contractor et al. (8) documented increased numbers of intrahepatic lymphocytes in IL-2 −/− mice at 5 wk of age, when mice did not have colitis, but did not compare hepatic inflammation in GF vs. SPF IL-2 −/− mice. Therefore, portal tract inflammation in this model does not appear to be secondary to the colonic inflammation and is independent of viable bacteria, in contrast to studies by Lichtman et al. (23, 24) in the rat jejunal bacterial overgrowth model. In addition, the degree of splenomegaly was similar in GF and SPF IL-2 −/− mice. A discrepancy between gastrointestinal and certain systemic manifestations was also noted in GF HLA-B27/β2-microglobulin transgenic rats, where colitis, gastritis, and arthritis were absent, but dermatitis
and testicular inflammation were not influenced by the absence of viable bacteria (30, 47).

The progressive and dramatic selective loss of B cells and immunoglobulins in IL-2-deficient mice was not influenced by the bacterial environment, inasmuch as both GF and SPF animal colonies reacted similarly. Similar to the report by Sadlack et al. (36), we demonstrated normal serum and secreted mucosal immunoglobulin levels at 4 wk in IL-2−/− mice, with increased levels at 8 wk of age, followed by a rapid and progressive decrease in immunoglobulin levels and B lymphocytes in the mesenteric lymph nodes in older IL-2−/− mice with colitis. Low levels of immunoglobulins were observed in all subclasses examined (IgA, IgM, IgG1, IgG2a). At the same time, we showed increasing proportions of T lymphocytes in the mesenteric lymph nodes, which could either reflect preservation of T cells in the face of the diminishing B cell population or an actual increase in the number of T cells. The loss of B cells and immunoglobulin secretion during progressive colitis provides further evidence that B cells are not involved in the pathogenesis of the intestinal inflammation. Ma et al. (25) demonstrated that IL-2−/−×RAG-2−/− double-mutant mice (T and B cell deficient) were disease free, whereas IL-2−/−×JH−/− double-mutant mice (selective B cell deficient) developed equivalent disease as IL-2−/− (25).

Colonic secretion of IL-12 was significantly increased in SPF IL-2−/− mice, and to a lesser extent in older GF IL-2-deficient mice. We measured IL-12 p40 because this subunit is upregulated in antigen-presenting cells by microbial stimuli (33) and because IL-12 is a key immunoregulatory molecule in experimental colitis (44) and Crohn’s disease (14). Most rodent models of chronic enterocolitis, with the notable exception of TCRRα−/− mice, display a Th1 profile of cytokines (38), despite the fact that human ulcerative colitis patients have more of a Th2 profile of lymphokines (14). Increased colonic IFN-γ expression has been reported in SPF IL-2−/− mice with colitis (1). IL-12 p40 is upregulated in colonic tissue of SPF IL-10−/− mice (9, 42) and mice with chronic trinitrobenzene sulfonic acid-induced colitis (27). In each of these models, blockade of endogenous IL-12 with neutralizing antibody dramatically reversed experimental colitis (9, 27).

In conclusion, we demonstrate that, in contrast with other genetically engineered models, older GF IL-2-deficient mice develop mild, focal, subclinical gastrointestinal inflammation in a sterile environment. Although mild colitis, small intestinal inflammation, mucosal infiltration of CD4+ T cells, upregulation of colonic IL-12, and antral gastritis are present in the absence of viable luminal bacteria, SPF mice have a much more rapidly progressive and aggressive clinical course, confirming the importance of resident luminal bacteria in the pathogenesis of chronic intestinal inflammation in genetically susceptible hosts. The mild colitis in GF IL-2−/− mice is accompanied by moderate portal tract inflammation and a progressive loss of mucosal B lymphocytes and immunoglobulin secretion, which is not dependent on viable luminal bacterial stimulation. The hypothesis that nonviable luminal bacterial fragments play a role in the gastrointestinal inflammation seen in GF IL-2−/− mice could be further investigated by eliminating all bacterial antigens from the food and bedding of GF animals.

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