Brief Definitive Report

T Cells, but Not B cells, Are Required for Bowel Inflammation in Interleukin 2-deficient Mice

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Summary

Interleukin-2 (IL-2)-deficient (IL-2−/−) mice develop anemia and colonic inflammatory bowel disease. To elucidate the mechanism of this disease, we have bred IL-2−/− mice to two strains of immunodeficient mice, RAG-2-deficient (RAG-2−/−, lacking B and T cells) and JH-deficient mice (JH−/−, lacking B cells). IL-2−/−, RAG-2−/− double-mutant mice are disease free, while IL-2−/−, JH−/− double-mutant mice succumb to bowel disease at the same rate as IL-2−/− littermates. IL-2−/−, JH−/− mice do not, however, succumb to anemia. Thus, spontaneous intestinal inflammation in IL-2−/− mice requires mature T cells, not B cells, while anemia is dependent on B cells.

Materials and Methods

Breeding and Housing of IL-2−/−, JH−/−, and RAG-2−/− Mice. All mice were housed and bred within one track in the specific pathogen-free barrier facility of the Animal Research Facility at Children's Hospital. Animals were monitored weekly for the development of loose bowel movements, wasting, or anemia. Clinically ill animals were monitored twice per week and killed when severely ill.

Colonic Histopathology. Colons from freshly killed animals were flushed with PBS to remove stool contents. Cross-sections were frozen directly in OCT compound (Miles Laboratories, Inc., Naperville, IL) using a cryostat (Bright Instruments, Huntingdon, UK). Frozen sections were cut on the cryostat and stained with hematoxylin and eosin.

Assessment of Anemia. Cardiac puncture was performed immedi-
ately after cervical dislocation and blood was drawn into heparinized microhematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA). Capillary tubes were then centrifuged for 5 min, and hematocrits were directly measured in the tubes.

**Analysis of Lamina Propria Lymphocytes (LPLs).** LPLs were harvested from individual colons of diseased mice, or from two to three nondiseased controls using an adapted version of a previously described protocol (9). Briefly, colons were rinsed with HBSS (GIBCO BRL, Gaithersburg, MD), incubated with serial washes of HBSS with 1 mM EDTA, washed, equilibrated with RPMI with 10% FCS and β-mercaptoethanol, and incubated with RPMI with collagenase and DNAse (0.1 mg/ml; Sigma immunochromicals, St. Louis, MO) in a shaking incubator at 37°C. Liberated LPLs were purified via Percoll gradient (Sigma), washed, resuspended in PBS with 5% FCS, and stained with PE- or FITC-conjugated mAbs specific for CD3, CD4, CD8, CD69, B220, IgM, and M290 antigens (Pharmingen, San Diego, CA; 10) for 20 min at 4°C. After washing, lymphocytes were analyzed on a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA).

**Results**

Bowel Inflammation in IL-2−/−, but not in JH−/− or RAG-2−/− Mice. Because the manifestation of disease pheno-
types in transgenic animals can vary between animal facilities (1, 11), we initially established the course of disease of IL-2-/-/ mice in our facility by breeding and observing heterozygote and homozygote IL-2-/- mice. Mice were maintained together in one track (such that their cages would be changed together). IL-2-/- mice generated from breedings of IL-2+/- parents regularly developed anemia, splenomegaly, and bowel disease, and became moribund or died between 18 and 24 wk of age. Some mice (3 out of 10 IL-2-/- mice) developed profound anemia and died between 11 and 16 wk of age. These mice had normal colons at necropsy. IL-2+/+ mice were maintained in the same cages as their IL-2-/- littermates, and all IL-2+/- mice lived >40 wk without visible or histopathological signs of illness. A total of 27 mice (10 IL-2-/- and 17 IL-2+/- mice) were studied before cross-breeding of these mice with JH-/- and RAG-2-/- mice. All IL-2-/- mice and no IL-2+/- mice became ill. Thus, IL-2-/- mice regularly develop bowel disease in our facility.

During the same period in which we observed the clinical course of IL-2-/- mice in our colony, we observed and killed multiple JH-/- and RAG-2-/- mice maintained in the same track as IL-2-/- mice. These mice have been described before, but colonic histopathology has not been performed (7, 8). Our studies revealed that JH-/- mice have grossly normal colons. Although immunohistochemical stains of colonic sections from JH-/- mice confirm the absence of κ-positive staining lymphocytes, consistent with the inability of JH-/- mice to rearrange Ig genes, the histological appearance of the colonic epithelium was otherwise normal and no epithelial damage, crypt hyperplasia, or signs of regeneration were noted (Fig. 1 C). Moreover, no wasting, weight loss, diarrhea, premature death, or other signs of clinical bowel disease were evident in JH-/- mice maintained for 40 wk. Thus, unlike cytokine- and T cell-deficient mice, B cell-deficient mice do not spontaneously develop bowel inflammation.

RAG-2-/- mice were also placed in the same track with IL-2-/- and JH-/- mice, and they were observed and histologically studied in the same fashion. Among 18 RAG-2-/- mice observed for 40 wk, only 1 mouse became ill. This mouse developed a wasting illness; autopsy revealed pulmonary lesions consistent with pneumonia caused by the protozoan Pneumocystis carinii, an opportunistic infection that infrequently affects RAG-2-/- mice. No significant bowel inflammation or epithelial damage was seen on histopathology. Furthermore, necropsy of six additional 40-wk-old mice failed to reveal evidence of bowel disease. Colonic sections from RAG-2-/- mice revealed no gross evidence of colonic inflammation mediated by polymorphonuclear cells, monocytes, or other nonlymphoid immune cells. In addition, epithelial cells were maintained in a normal architecture (Fig. 1 E). Thus, RAG-2-/- mice, like JH-/- mice, remained healthy during the 40-wk observation period. Colonic histology of RAG-2-/- and JH-/- mice, like IL-2-/- mice, was grossly normal (Fig. 1 A).

**Figure 2.** Survival of single- and double-mutant IL-2-/-, JH-/-, and RAG-2-/- mice. Surviving single- and double-mutant mice expressed as a percentage of the initial number of animals in each genotype group are plotted. All animals were observed until moribund or until 24 wk old, at which time necropsies were performed with colonic histopathology and hematocrit measurement. Wild type (combined survival of IL-2+/-, JH-/- and IL-2-/-, RAG-2-/- mice (- -); IL-2-/-, JH-/- mice (- -); RAG-2-/- mice (- -); IL-2-/- mice (- -); IL-2-/-, JH-/- mice (- -); and IL-2-/-, RAG-2-/- mice (- -).

**Absence of Bowel Inflammation in IL-2-/-, RAG-2-/- Mice.** We then bred IL-2+/- mice into both JH-/- and RAG-2-/- backgrounds. All parental strains were from a mixed C57Bl/6/SV129 background. All offspring were genotyped by PCR and/or Southern analysis of genomic DNA isolated from tail tips at 3 wk of age. Genotypes were confirmed by repeat analysis of genomic DNA at necropsy. Double-heterozygote mice from F1 breedings, i.e., IL-2-/-, JH-/-, and IL-2-/-, RAG-2-/- mice, remained healthy and were interbred for two generations. Fourth, fifth, and sixth generation IL-2-/-, JH-/- and IL-2-/-, RAG-2-/- mice were observed along with their littermate controls until they developed gross disease, became moribund, or until 24 wk of age if they remained healthy.

IL-2-/-, JH-/- and IL-2-/-, RAG-2-/- animals exhibited a similar wasting disease to IL-2-/- mice by 24 wk, including weight loss, anemia, a hunched stance, abdominal

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Figure 1. Histology of single- and double-mutant IL-2-/-, JH-/-, and RAG-2-/- mice. Colonic sections from various mice were stained with hematoxylin and eosin. Paired littermates are shown (A and B, C and D, and E and F). All sections are ×1,300. (A) IL-2+/-, JH-/- (wild type); (B) IL-2-/-, JH-/- (IL-2-/-); (C) IL-2+/-, JH-/- (JH-/-); (D) IL-2-/-, JH-/- (IL-2-/-, JH-/-); (E) IL-2+/-, RAG-2-/- (RAG-2-/-); (F) IL-2-/-, RAG-2-/- (IL-2-/-, RAG-2-/-).
retraction, loose stools (but rarely rectal bleeding), and occasional rectal prolapse. At autopsy, distal segments (1–5 cm) of IL-2−/− descending colons were uniformly thickened and enlarged. Mesenteric lymph nodes were increased 3–10-fold in size as compared to control littermates. Colonic histopathology revealed gross infiltration of colonic lamina propria with lymphocytes, lesser numbers of polymorphonuclear cells, crypt abscesses, and crypt hyperplasia and branching, when compared to normal colons (Fig. 1, A and B). Loss of mucin from crypt goblet cells and polymorphonuclear cell infiltration of crypts was documented by scanning electron microscopy (data not shown).

While IL-2−/−, RAG-2+/− mice reproducibly developed anemia and colonic inflammation within 24 wk of age, most IL-2−/−, RAG-2−/− double-mutant littermates remained disease free while sharing cages with IL-2−/−, RAG-2+/− littermates (Fig. 2). One IL-2−/−, RAG-2−/− mouse became ill during the 24-wk observation period; necropsy of this mouse had hepatic and pulmonary lesions and no colonic inflammation. To determine whether IL-2−/−, RAG-2−/− mice developed disease in a delayed fashion, several IL-2−/−, RAG-2−/− mice were maintained for 12 mo, after which they were killed, and again no colonic lesions were noted. Thus, the elimination of mature lymphocytes prevented bowel inflammation and anemia in IL-2−/−, RAG-2−/− mice, and demonstrated that either mature B or T cells are required for these manifestations of disease.

Bowel Disease in IL-2−/−, JH−/− Mice. To determine whether B cells or autoantibodies are necessary for the development of inflammatory bowel disease or anemia in IL-2−/− mice, we monitored IL-2−/−, JH−/− mice along with IL-2−/+, JH−/− and IL-2−/−, JH+/− littermates. Except for the absence of early (<16 wk old) deaths in IL-2−/−, JH−/− mice (see below), IL-2−/− mice developed a wasting disease of comparable severity at a similar onset regardless of the genotype at the JH locus (Fig. 2). Histopathological analyses revealed the same lymphocyte predominant infiltrates in IL-2−/−, JH−/− mice as seen in IL-2−/−, JH+/− mice (Fig. 1, C and D). Flow cytometric analyses of purified colonic lamina propria lymphocytes confirmed that inflammatory infiltrates from both IL-2−/−, JH−/− and IL-2−/−, JH+/− mice are comprised predominantly of activated CD3+, CD4+, CD69+, and M290− T cells, and virtually no B cells (Fig. 3). Since most murine intraepithelial lymphocytes bear the αM290β7 integrin, this data suggests that intraepithelial lymphocytes are not a major population infiltrating the colons of IL-2−/− mice (10). Crypt branching, loss of mucin, and crypt abscesses in both these mice indicate that bowel inflammation leading to epithelial dam-

Figure 3. FACS® analysis of LPLs from IL-2−/− and IL-2−/−, JH+/− mice. LPLs from freshly killed normal (left panels), IL-2−/− (middle panels) and IL-2−/−, JH+/− (right panels) mouse colons were stained with mAbs directed against the following antigens: PE-B220/FITC-IgM; PE-CD4, FITC-CD8; PE-CD69; FITC-CD3; and PE-M290/FITC-CD3.
age, rather than simple lymphocyte infiltration, has occurred (Fig. 1, C and D). The similarity of disease onset and severity in IL-2−/−, JH−/− and IL-2−/−, JH+/− mice indicates that mature B cells and anticolon autoantibodies are not necessary for the bowel inflammation that occurs in IL-2−/− mice.

Amelioration of Anemia in IL-2−/−, JH−/− Mice. Some IL-2−/− mice developed severe anemia leading to death within 17 wk of age, before the development of bowel disease, while older mice developed severe anemia concomitantly with the development of severe bowel disease. No deaths occurred in IL-2−/−, JH+/− mice before the age of 17 wk. The mean hematocrit from IL-2−/−, JH+/− mice (19 ± 3.6%) was markedly lower than from terminally ill IL-2−/−, JH−/− littermates (36 ± 2.9%) (P <0.001, Fig. 4). Heterozygote JH+/− mutant mice possessed a mean of 48 ± 3.0%, which was not significantly different from the mean from JH−/− mutant mice, 44 ± 3.4% (P >0.05, Fig. 4). The amelioration of anemia in IL-2−/−, JH−/− mice, as compared to IL-2−/−, JH+/− mice, indicates that B cells contribute significantly to the development of anemia seen in IL-2−/− mice. This anemia is associated with splenic hemosiderin deposits and an abundance of early erythroblast forms in peripheral blood smears and splenic sections (data not shown). The moderate anemia seen in IL-2−/−, JH+/− mice, as compared to normal mice, may result from malnutrition associated with bowel disease.

Discussion

Bowel inflammation has been noted in several strains of transgenic mice bearing immune deficiencies resulting from gene-targeted mutations. This condition could directly result from one or several types of infections caused by the abundant microbiological flora of the colon, or it could represent an autoimmune response to intestinal antigens. By establishing that both JH−/− and RAG-2−/− mice grow and live normally without developing bowel inflammation in our colony, we have established that (a) immune deficiency per se does not lead to bowel inflammation from putative intestinal pathogens in our colony; and (b) neither of these immune-deficient conditions leads to spontaneous autoimmune bowel inflammation. Furthermore, the absence of gross bowel disease in both strains of animals makes them appropriate for directly testing the roles of B and T lymphocytes in murine models of inflammatory bowel disease.

Previous work demonstrated that intestinal luminal contents influence the onset and severity of bowel inflammation in IL-2−/− mice (3). The absence of bowel inflammation and general health of IL-2−/−, RAG-2−/− mice formally demonstrates that infectious agents alone do not cause bowel disease in IL-2−/− mice. Rather, antigen-specific immune cells are required for this process. This process can be distinguished from conditions such as the bowel inflammation induced by oral administration of dextran sulfate sodium, since dextran sulfate sodium induces colitis in both SCID (12) and RAG-2−/− mice (Ma, A., unpublished data). It should also be distinguished from the cecal bowel inflammation seen in IL-2 γ chain-deficient mice, where lymphopenia is associated with the presence of Helicobacter helicis organisms (13).

The presence of bowel inflammation in several strains of transgenic mice with compromised T cell compartments suggested that the loss of a particular subset(s) of T cells might lead to dysregulated B cell activity and autoimmune bowel disease (6). Our finding that IL-2−/−, JH−/− double-mutant mice develop disease with comparable severity and onset as IL-2−/−, JH+/− mice demonstrates that neither B cells nor autoantibodies are necessary for this disease. While this does not completely rule out a role for B cells in the colonic immune response, the similarity of IL-2−/−, JH−/− and IL-2−/−, JH+/− mice suggests B cells play at most a minor role. Formal testing of the role of particular T cell subsets in mediating IL-2−/− bowel disease by breeding of IL-2−/− mice with TCRα−/−, TCRβ−/−, or MHC II−/− mice may be complicated by the spontaneous appearance of bowel inflammation in the latter strains of mice (3). Direct comparisons of these mouse models of bowel inflammation await simultaneous studies of the various strains in the same inbred background, in the same facility, and ideally as interbred littermates.

The amelioration of anemic symptoms in IL-2−/−, JH−/− vs IL-2−/−, JH+/− mice suggests that B cells are important for mediating an autoimmune hemolytic anemia in IL-2−/− mice. The absence of MHC class I molecules on mature
erythrocytes makes them unlikely targets for T cell-mediated destruction. The finding that IL-2−/−, JH−/− mice die at approximately the same age as IL-2−/−, JH+/− mice despite retaining significantly higher hematocrits indicates that bowel inflammation (vs anemia) is likely to be the major cause of death in mice of this age. The separation of bowel inflammation from anemia in IL-2−/−, JH−/− mice demonstrates the use of B cell-deficient mice in distinguishing the immune mechanisms involved in autoimmune processes. JH−/− mice have previously been used to demonstrate the critical role of B cells in mediating nephritis and vasculitis in lpr/lpr mice (14). Bowel inflammation in IL-2−/− mice is thus pathogenetically distinct from the autoimmune processes seen in lpr/lpr mice. Finally, IL-2−/−, JH−/− mice are now a useful reagent for studying the specific mechanism(s) by which IL-2−/− T cells cause bowel inflammation.

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