Lymphoid Hyperplasia, Autoimmunity, and Compromised Intestinal Intraepithelial Lymphocyte Development in Colitis-Free Gnotobiotic IL-2-Deficient Mice

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Lymphoid Hyperplasia, Autoimmunity, and Compromised Intestinal Intraepithelial Lymphocyte Development in Colitis-Free Gnotobiotic IL-2-Deficient Mice

Nikhat V. Contractor,¹ Hamid Bassiri,²* Tannishtha Reya,³* Audrey Y. Park,* Daniel C. Baumgart,*† Mariusz A. Wasik,‡ Stephen G. Emerson,§ and Simon R. Carding 4

IL-2-deficient (IL-2−/−) mice develop disorders of the hemopoietic and immune systems characterized by anemia, lymphocytic hyperplasia, and colitis. The mechanisms responsible for these abnormalities remain unclear. To investigate the underlying basis of autoimmunity, the particular role of commensal gut flora in the initiation of colitis, and the role of IL-2 in the development of intestinal intraepithelial lymphocytes (iIEL), we evaluated IL-2−/− mice reared and maintained under gnotobiotic (germfree) conditions. By 8 wk of age, 80% (20 of 25) of germfree IL-2−/− mice show signs of disease, including anemia, disturbances in bone marrow hemopoietic cells, lymphocytic hyperplasia, and generalized autoimmunity, similar to those seen in specific pathogen-free (SPF) IL-2−/− mice. In striking contrast to SPF IL-2−/− mice, germfree IL-2−/− mice do not develop colitis. However, the numbers of γδ T and TCRβ⁺CD8α⁺ iIELs are reduced, and in lethally irradiated SPF IL-2−/−/+ mice, reconstituted with IL-2−/− bone marrow TCRγδ⁺ iIELs fail to develop, consistent with an important role of IL-2/IL-2R signaling in the development of γδ iIELs. Consequently, our findings demonstrate that the colitis seen in SPF IL-2−/− mice depends upon the presence of intestinal bacterial flora and that environmental Ags are not responsible for the anemia and extraintestinal lymphoid hyperplasia that occur in IL-2−/− mice. Thus, germfree IL-2−/− mice represent a unique system in which the role of IL-2 deficiency in hemopoietic and immune system disorders can be investigated in dissociation from complications that may arise due to colitis. The Journal of Immunology, 1998, 160: 385–394.

The hypothesis that disease induction is caused by a breakdown in the regulation of peripheral T cells is supported by several observations. First, IL-2 has been demonstrated to mediate the termination of clonal expansion by inducing apoptosis (6). Second, superantigen-stimulated T cells from IL-2−/− (7) and IL-2R α-chain-deficient (8) mice are more resistant to Fas-induced apoptosis than those from wild-type mice. Finally, the onset of the disease in IL-2−/− mice reared and maintained in a specific pathogen-free (SPF) environment is delayed (1). Together, these observations suggest that disease is caused by dysregulated peripheral T cell responses to environmental Ags.

Despite the initial analysis of IL-2−/− mice indicating normal T cell development (9), the recent finding that IL-2−/− athymic mice fail to develop autoimmune or lymphoproliferative disorders demonstrates that disease initiation depends upon the intrathymic differentiation of IL-2−/− T cells (10). Attempts to identify abnormalities in intrathymic T cell development and negative selection in IL-2−/− mice, however, have produced contradictory results. The marked skewing toward the CD4⁺8⁺ phenotype in the thymus of nonmanipulated IL-2−/− mice (11) and the decreased cortical apoptosis and loss of immature CD4⁺8⁺ (double-positive) and accumulation of CD4⁺ and CD8⁺ (single-positive) thymocytes in IL-2−/− mice following antigenic stimulation (12) are consistent with abnormal intrathymic development. However, the normal selection and deletion of thymocyte subsets by endogenous superantigens or following chronic administration of Ag to MHC class I-restricted TCR-transgenic mice in the absence of IL-2 (13) argue for T cell selection mechanisms being intact in these mice.

An obligatory role for IL-2 in the normal development of at least some T cell populations is supported by the recent finding that the composition of the intestinal intraepithelial lymphocytes (iIEL) compartment is greatly altered in IL-2R β-chain-deficient mice.
injected with 1

To investigate further the mechanism of disease in IL-2−/− mice, gnotobiotic (germfree) IL-2−/− mice were rederived and evaluated for signs of hemopoietic and immune disorders. The composition of the iIEL repertoire was also evaluated in these animals. The results show that germfree IL-2−/− mice develop hemopoietic disorders similar to those in SPF IL-2−/− mice, consistent with the hypothesis that defective central and/or peripheral tolerance to self Ags results in autoimmunity. The absence of colitis and abnormalities in the development of TCRβgd mice may arise as a consequence of an abnormal mucosal immune response to enteric Ags.

Materials and Methods

Mice

Gnotobiotic (germfree) IL-2−/− mice (Taconic Farms, Germantown, NY), backcrossed eight generations onto the C57BL6 background, were housed in isolator cages within a flexible film isolator in the gnotobiotic facility of the Biology Department, University of Pennsylvania. The germfree status of IL-2−/−, IL-2+/−, and IL-2+/+ animals was verified by bacteriologic, histologic, and serologic analysis of tissues and fluids at autopsy. IL-2−/− mice obtained from heterozygous matings were identified from samples of tail DNA using a previously described PCR-based method (9). Heterozygote and/or wild-type littermates were used as controls. B6.SJL-P7tpc/PepR2/B220/H-2a (B6Cd45.1) mice were a gift from Dr. Edward Scott (University of Pennsylvania, Philadelphia, PA).

Histopathology

Tissues removed at autopsy were fixed in formalin and embedded in paraffin. Sections were stained according to standard protocols with hematoxylin-eosin or stains for the detection of microorganisms that included Gram, acid-fast, Warthin-Starry silver, and Germori melanamine-silver nitrate stains.

Cell preparation

Blood was obtained by tail bledding or cardiac puncture and was collected in polypropylene tubes containing heparin. Blood counts were obtained using a Baker 1000 automated cell counter (Coulter, Hialeah, FL) and leukocyte differential counts were performed manually on Wright-stained blood smears. Mononuclear cells from thymus and spleen were prepared by disrupting intact tissues into ice-cold PBS. Bone marrow cells were obtained from femurs and tibias by flushing the bones with PBS and scraping the bone fragments. To prepare iIEL, the small intestine was opened longitudinally in HBSS, Peyer’s patches were removed, and the intestine was cut into small fragments that were incubated in HBSS containing 0.1 M EDTA and 5 mM DTT in an Endemeyer flask in a shaking water bath at 37°C for 30 min. Filtered cells were enriched for lymphocytes by Percoll gradient centrifugation, removing cells between the 65 and 45% interface. The purity of the iIEL was assessed by the degree of contamination by nonlymphoid cells. The tissue was then disrupted by passing it through a steel cannula, injected with 1 × 106 unmanipulated bone marrow cells from B6Cd45.1 syngeneic donors or IL-2−/− (Cd45.2) congenic donors. Control mice were lethally irradiated and injected with an equal volume of PBS. Control mice died 10 to 14 days postirradiation. Experimental mice were euthanized at 90 days postirradiation, and iIELs were isolated as described.

Abs and flow cytometry

The following mouse and rat mAbs were used to stain and analyze thymocytes isolated from germfree IL-2−/− and IL-2+/+ mice: CD90/Thy 1.2 (30H.12; Life Technologies, Gaithersburg, MD), CD1 (29B; Life Technologies), CD4 (CT-CD4; Caltag Laboratories, San Francisco, CA), CD8α (53-6.7; Life Technologies), CD8β (CT-CD8b; Caltag Laboratories), CD11b/Mac-1 (M1/70.15; Caltag Laboratories), CD24/heat stable Ag (M1/69; PharMingen, San Diego, CA), CD25/IL-2Rα (PC61.5.3, American Type Culture Collection, Rockville, MD), CD45RB/B220 (RA3-6B2; Life Technologies), CD69 (HI.2F3; PharMingen), TCRβ (H57-597; PharMingen), TCRγδ (GL3; PharMingen), and Gr-1 (RB6-8C5; PharMingen). Donor-derived hemopoietic cells in bone marrow chimeras were detected using mouse-anti-CD45.2 (104; PharMingen). Streptavidin-fluorescein isothiocyanate, streptavidin-PE-Cy5.5 (670 Life (Technologies), and/or streptavidin-allophycocyanin (Caltag Laboratories) were also used to detect the reactivity of biotinylated primary Abs. Ab staining and three- or four-color flow cytometric analyses were conducted using a FACScan and CellQuest software (Becton Dickinson, San Jose, CA) as previously described (17).

Statistical analysis

Data are presented as the mean ± SD. Statistical significance was assessed by Student’s t test.

Results

Onset of disease in germfree IL-2−/− mice

To test the hypothesis that disorders of the hemopoietic and immune systems and colitis that develops in IL-2−/− mice are initiated by an uncontrolled peripheral activation of T cells by environmental Ags (1, 10), IL-2−/− mice were rederived and maintained in a germfree environment. Histopathologic and microbial analyses of germfree IL-2−/− mice confirmed the absence of contagious pathogens (e.g., Gram-positive and Gram-negative (including Helicobacter sp) bacteria, fungi, and parasites). In addition, serologic tests for Abs against the most common murine pathogens (mouse hepatitis virus, polyoma virus, minute virus of mice, mouse adenosivirus, mouse CMV, reovirus type 3, mouse pneumonia virus, Theliers’ virus, Sendai virus, and lymphocytic choriomeningitis virus) were negative.

Up to approximately 8 wk of age, IL-2−/− mice exhibited no clinical signs of disease, although most were distinguishable from heterozygote or wild-type littermates by their smaller size and weight. Between 8 and 12 wk of age, 80% (20 of 25) of IL-2−/− mice developed anemia (Table I), the onset and severity of which varied with age among individual homozygous mice within the same and different litters. All the germfree heterozygous and wild-type mice have, to date, been free of any clinical or histopathologic signs of disease. In addition, a subset (5 of 25) of germfree IL-2−/− mice remained clinically healthy beyond 6 mo of age. The reason for this heterogeneity in disease progression is currently not known, but the involvement of any murine commensal or pathogenic microorganisms has been excluded. Visual inspection at autopsy of 8- to 12-wk-old IL-2−/− mice revealed a moderate to severe splenomegaly and lymphadenopathy, similar to those seen in SPF IL-2−/− mice. In contrast, the gastrointestinal tract, with the exception of an enlarged cecum ordinarily seen in germfree mice, was normal by visual inspection and histopathology in all germfree IL-2−/− mice analyzed. Thus, germfree IL-2−/− mice do not develop colitis. However, they still exhibit signs of immune system and hemopoietic disorders. To investigate this in further detail, we evaluated nonlymphoid, lymphoid, and hemopoietic organs for signs of disease.

Lymphoid hyperplasia

The most consistent histopathologic finding of diseased germfree IL-2−/− mice was a moderate to severe lymphocytic hyperplasia (Fig. 1) affecting hemopoietic, lymphoid, and other organs of all (n = 20) animals analyzed. Among nonlymphoid tissues, lymphocytic infiltrates were seen in the lungs, liver, pancreas, and kidneys.
of myeloperoxidase, and presence of tartrate-resistant acid phosphatase, as defined by their large blast-like appearance, lack of nuclear condensation, and presence of tartrate-sensitive acid phosphatase. Histochemical analysis of cytocentrifuge preparations of the islets of Langerhans were unaffected by the inflammatory process. Intrahepatic lymphocytes in germfree IL-2−/− mice were unaffected by the inflammatory process. Intrahepatic lymphocytes in germfree IL-2−/− mice were unaffected by the inflammatory process.

Changes in splenic lymphocyte phenotype

By 6 wk of age the number of mononuclear cells recovered from the spleens of IL-2−/− mice increased by 1.5- to 2-fold compared with that in IL-2+/+ mice. This change was attributable to increased numbers of both CD4+ and CD8+ T cells (Fig. 3) and of mature myeloid (Mac-1high) cells similar to those seen in SPF IL-2−/− mice (Fig. 2 and 3). The CD4+ T cells exhibited an activated phenotype, as shown by expression of CD69 and CD25 (Fig. 3 and Table II). The T cell counts in germfree IL-2−/− mice (Fig. 2), a phenotype normally expressed by activated T cells that accumulate and undergo apoptosis in the liver (18).

Intrahepatic lymphocytes in germfree IL-2−/− mice

Kramer and colleagues (10) have previously described the accumulation of CD4−CD8− T cells that express intermediate levels of the TCRαβ in the livers of SPF IL-2−/− mice. Compared with the livers of 5-wk-old IL-2+/+ littermates, approximately 10-fold more mononuclear cells were recovered from the livers of germfree IL-2−/− mice (Fig. 2). Flow cytometric analysis of these cellular infiltrates showed that the increase in cellularity in IL-2−/− mice was attributable to increased numbers of CD4−CD8− and, to a lesser extent, CD4+CD8− T cells (Fig. 2). Whereas the number of CD4−CD8− T cells increased by approximately 13-fold on the average, there was a >30-fold increase in the number of CD4+CD8− T cells. Although the IL-2−/− liver contained proportionately fewer B220+ (TCRβ−) B cells than those in littermate control mice (Fig. 2), when the difference in cellularity of IL-2−/− and IL-2+/+ liver mononuclear cells was taken into consideration the numbers of B cells present in germfree IL-2−/− and IL-2+/+ mice were similar. Also of note was the >30-fold increase in the number of B220+ TCRαβlow/int cells in the livers of germfree IL-2−/− mice (Fig. 2), a phenotype normally expressed by activated T cells that accumulate and undergo apoptosis in the liver (18).

Hemopoietic disorder

Visual inspection of germfree IL-2−/− mice revealed bristle bones that upon a more comprehensive analysis identified striking changes in bone marrow composition that were evident as early as 4 wk of age (Fig. 4 and Table II). These changes were primarily characterized by a progressive loss of mature myeloid cells that corresponded to a 20-fold reduction in mature, Mac-1high and Gr-1high, polymorphonuclear cells (Table II). The majority of resident cells expressed low levels of Mac-1/Gr-1 (Fig. 4), characteristic of immature myeloid cells (19, 20). B cell development was similarly affected in germfree IL-2−/− mice, as evidenced by the >30-fold reduction in immature, B220low, and the 10-fold reduction in more mature, B220high, B cells in the marrow (Fig. 4). Accompanying the breakdown in B cell development was the infiltration of large numbers of T lymphocytes that were primarily CD4+ T cells, of which a large proportion was CD69+CD25+ (Table II). The T cell infiltration occurred at approximately 6 wk of age, and since the changes in myeloid cells were seen before this, it is likely that the

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>IL-2 Genotype</th>
<th>RBC (10^6/μl)</th>
<th>WBC (10^3/μl)</th>
<th>Neut</th>
<th>Lymph</th>
<th>Mono</th>
<th>Eosin</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-5</td>
<td>+/-</td>
<td>7.9 ± 0.9</td>
<td>15.0 ± 0.5</td>
<td>33 ± 1</td>
<td>7.6 ± 1.5</td>
<td>0.6 ± 0.2</td>
<td>6.2 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>+/−</td>
<td>7.2 ± 1.2</td>
<td>14.8 ± 0.3</td>
<td>32 ± 2</td>
<td>6.8 ± 2.0</td>
<td>0.7 ± 0.2</td>
<td>6.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>7.4 ± 0.8</td>
<td>13.1 ± 1.1</td>
<td>28 ± 5</td>
<td>8.0 ± 2.6</td>
<td>0.3 ± 0.2a</td>
<td>7.0 ± 1.4</td>
</tr>
<tr>
<td>8</td>
<td>+/-</td>
<td>8.6 ± 0.2</td>
<td>14.2 ± 0.7</td>
<td>31 ± 1</td>
<td>11.0 ± 1.9</td>
<td>1.7 ± 0.3</td>
<td>9.7 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>+/−</td>
<td>8.9 ± 0.9</td>
<td>15.6 ± 1.0</td>
<td>31 ± 2</td>
<td>10.1 ± 2.1</td>
<td>1.6 ± 0.3</td>
<td>8.6 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>−/−</td>
<td>3.8 ± 0.6</td>
<td>10.0 ± 0.5</td>
<td>15 ± 6</td>
<td>9.8 ± 2.5</td>
<td>0.6 ± 0.6a</td>
<td>6.3 ± 1.2*</td>
</tr>
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<td>12</td>
<td>+/-</td>
<td>9.9 ± 1.9</td>
<td>14.9 ± 1.8</td>
<td>33 ± 4</td>
<td>13.1 ± 2.1</td>
<td>1.8 ± 0.3</td>
<td>11.0 ± 3.0</td>
</tr>
<tr>
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<td>+/−</td>
<td>7.6 ± 2.5</td>
<td>3.7 ± 2.3</td>
<td>12 ± 3</td>
<td>14.0 ± 2.2</td>
<td>0.2 ± 2.0a</td>
<td>5.9 ± 2.0a</td>
</tr>
</tbody>
</table>

* Cells consisted of atypical blast-like cells that were tartrate sensitive, acid phosphatase-positive, and myeloperoxidase-negative.
disruption in bone marrow hemopoiesis occurs before or coincident with the lymphoproliferative disorder. The progressive neutropenia seen in the blood of germfree IL-2\(^{-/-}\) mice (Table I) is also consistent with bone marrow failure. The loss of mature neutrophils was accompanied by a progressive anemia and the presence of large numbers of atypical blast-like lymphocytes that were tartrate resistant, acid phosphatase positive, and myeloperoxidase negative.

**Absence of colitis in germfree IL-2\(^{-/-}\) mice**

The incidence of colitis in our SPF IL-2-deficient mouse colony was comparable to that described in other colonies (2). Of the animals that survived early disease (50%), characterized by splenomegaly, lymphadenopathy, and anemia, all subsequently developed colitis, with disease symptoms present between 6 and 10 wk of age. In contrast to the colitis seen in SPF IL-2\(^{-/-}\) mice, none \((n = 25)\) of the germfree IL-2\(^{-/-}\) mice developed any intestinal pathology. The small and large intestines of germfree IL-2\(^{-/-}\) animals displayed normal architecture, with no detectable lymphocyte infiltrates, epithelial cell damage, crypt hyperplasia, or signs of regeneration (Fig. 1, e and f).

**Changes in the distribution of iIEL populations**

To investigate whether the absence of colitis and gut flora was accompanied by changes in the intestinal T cell repertoire, we examined iIELs from both IL-2\(^{-/-}\) and IL-2\(^{+/+}\) mice. The iIEL repertoire of the small intestine in IL-2\(^{-/-}\) germfree mice was distinct from that in littermate control mice. Although the total numbers of iIEL recovered from IL-2\(^{-/-}\) and IL-2\(^{+/+}\) mice were equivalent, those from IL-2\(^{-/-}\) mice contained fewer CD8\(^{+}\)-expressing \(\gamma\delta^+\) and \(\alpha\beta^+\) T cells (Fig. 5B). Whereas \(\gamma\delta^+\) cells represented the major iIEL population (63%) in wild-type germfree mice, they comprised only 34% of iIEL in their IL-2\(^{-/-}\) counterparts (Fig. 5A). Similarly, the number of TCR\(\alpha\beta^+\)CD8\(\alpha\alpha^+\) iIELs was reduced in germfree IL-2\(^{-/-}\) mice, but due to an increase in CD8\(\alpha\beta^+\) iIEL, the overall number of TCR\(\alpha\beta^+\) iIEL was higher in
germfree IL-2−/− (~1.5 × 10⁶) than that in IL-2+/+ (~1.0 × 10⁶) mice (Fig. 5B). By contrast, the TCRαβ⁺ CD4⁺ and TCRβ⁺ CD4⁺ subsets of iIELs did not appear to be significantly affected by the absence of IL-2. Thus, the absence of IL-2 results in a decrease in the numbers of CD8αα⁺, particularly γδ⁺ T cells, in the iIEL compartment.

Table II. Cellular composition of hematopoietic organs in germfree IL2−/− mice

<table>
<thead>
<tr>
<th>Age (weeks)</th>
<th>IL-2 Genotype</th>
<th>n</th>
<th>Splenomegalyb</th>
<th>Colitisc</th>
<th>Total (×10⁶)</th>
<th>B220⁺</th>
<th>CD3⁺</th>
<th>Immatured</th>
<th>Matured</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>+/+</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>51 ± 7</td>
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<td>3 ± 1</td>
<td>31 ± 6</td>
<td>67 ± 8</td>
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<tr>
<td></td>
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<td>4</td>
<td>-</td>
<td>-</td>
<td>33 ± 8</td>
<td>33 ± 6</td>
<td>2 ± 2</td>
<td>56 ± 5</td>
<td>40 ± 7</td>
</tr>
<tr>
<td>6</td>
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<td>4</td>
<td>+/+</td>
<td>-</td>
<td>60 ± 10</td>
<td>33 ± 4</td>
<td>8 ± 2</td>
<td>66 ± 4</td>
<td>28 ± 8</td>
</tr>
<tr>
<td></td>
<td>+/−</td>
<td>4</td>
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<td>24 ± 6</td>
<td>21 ± 9</td>
<td>10 ± 3</td>
<td>84 ± 10</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>8</td>
<td>+/+</td>
<td>3</td>
<td>+/+</td>
<td>-</td>
<td>59 ± 5</td>
<td>29 ± 7</td>
<td>7 ± 1</td>
<td>66 ± 10</td>
<td>31 ± 9</td>
</tr>
<tr>
<td></td>
<td>+/−</td>
<td>5</td>
<td>++</td>
<td>-</td>
<td>19 ± 7</td>
<td>16 ± 4</td>
<td>18 ± 4</td>
<td>83 ± 12</td>
<td>18 ± 6</td>
</tr>
</tbody>
</table>

a Phenotype and composition of bone marrow and spleen cells determined by flow cytometry. Data represent the mean ± SD.
b Size of IL2−/− spleen relative to that of IL2+/+ mice; −, no change; +, 1 to 2 times larger; ++, >2 times larger.
c Based upon histologic analysis.
d Gr-1high and/or Mac-1high cells.
e Gr-1low cells.
f 60–75% TCRαβ⁺CD4⁺CD69⁺ and/or CD25⁺.
Failure of γδ iIELs to develop in bone marrow radiation chimeras

To determine whether the decreased numbers of γδ⁺ iIELs in germfree IL-2⁻/⁻ mice was due to a defect in the ability of these cells to differentiate, lethally irradiated CD45.1⁺ (BL/6 congenic) mice were reconstituted with IL-2⁻/⁻ (CD45.2⁺) bone marrow. The majority of mice (four of five) reconstituted with IL-2⁻/⁻ bone marrow survived through 90 days postreconstitution. At necropsy, tissues were grossly normal, and there were no signs of...
colitis in these animals maintained under SPF conditions (for the duration of the experiment). Analysis of the iIELs in mice reconstituted with syngeneic (host) bone marrow showed that these cells were able to fully reconstitute the iIEL compartment (Fig. 6A), such that \( \alpha \beta^+ \) and \( \gamma \delta^+ \) iIELs were present in the same proportions as those seen in unmanipulated wild-type mice (Fig. 5). On the other hand, while IL-2\(^{-/-}\) bone marrow was able to reconstitute the majority (71%) of the iIEL compartment (Fig. 6B), they were unable to fully reconstitute TCR\(\gamma \delta^+\) cells. The iIELs present consisted almost exclusively of \( \alpha \beta^+ \) (91%), with very few \( \gamma \delta^+ \) iIELs (10% or less). Additionally, the endogenously derived (host) iIELs in IL-2\(^{-/-}\) bone marrow-reconstituted mice were not comprised of \( \alpha \beta^+ \) and \( \gamma \delta^+ \) T cells in the same proportions as those in the mice reconstituted with syngeneic cells (Fig. 6B). Since these cells most likely arose from endogenous progenitors that survived radiation, one possible explanation for this disparity is that \( \gamma \delta^+ \) iIEL progenitors are more radiosensitive than \( \alpha \beta^+ \) iIEL progenitors. The presence of TCR\(\gamma \delta^+\) iIELs in animals reconstituted with syngeneic bone marrow (Fig. 6A) argues against the possibility that the absence of these cells in mice reconstituted with IL-2\(^{-/-}\) bone marrow is due to differences in the kinetics of \( \gamma \delta \) vs \( \alpha \beta \) iIEL generation and reconstitution. Instead, our results of iIEL analysis

**FIGURE 4.** Cellular composition of bone marrow from 8-wk-old germfree IL-2\(^{-/-}\) mice. Representative FACS profiles of bone marrow mononuclear cells from IL-2\(^{-/-}\) mice and wild-type (IL-2\(^{+/+}\)) littermates using lineage-specific Abs to identify myeloid (Gr-1), monocytic/macrophage (Mac-1), and B (B220) cells. The values shown for Gr-1 profiles represent the frequency of mature, Gr-1\(^{\text{high}}\), granulocytes. Those shown for Mac-1 and B220 represent the relative proportions of immature (Mac-1\(^{\text{low}}\); B220\(^{\text{low}}\)) and mature (Mac-1\(^{\text{high}}\); B220\(^{\text{high}}\)) cells present.
in germfree IL-2\(^{-/-}\) mice and bone marrow chimeras are consistent with a requirement for IL-2 in the generation of \(\gamma^\delta^+\) iIELs.

**Discussion**

Splenomegaly, lymphadenopathy, anemia, and inflammatory bowel disease are all characteristic disorders displayed by IL-2\(^{-/-}\) mice. However, the precise nature of the role of IL-2 and the nature of the factors that initiate these disorders remain unclear. It has been proposed that the disease may be a result of aberrant responses to environmental Ags. Conversely, the disease in IL-2\(^{-/-}\) mice may result from a breakdown in central and/or peripheral tolerance. To distinguish between these possibilities, we have rederived germfree IL-2\(^{-/-}\) mice and evaluated them over a period of 10 mo for signs of disease.

Our observations that germfree IL-2\(^{-/-}\) mice develop hemopoietic and lymphoid disorders similar to those seen in SPF IL-2\(^{-/-}\) mice, with the exception of colitis, demonstrate that environmental Ags are not responsible for the extraintestinal lymphoproliferative disorder and autoimmunity. The ability to distinguish between different mechanisms of disease in IL-2\(^{-/-}\) mice is analogous to the distinction between colitis and extraintestinal disease in HLA-B27 transgenic rats (21, 22). In contrast to the inflammatory disorders that affect the intestine, skin, and joints of SPF transgenic rats, the intestine and joints remain histologically normal in germfree animals (22).

The possibility that food Ags could initiate disease in IL-2\(^{-/-}\) mice seems unlikely, since the intestines of germfree animals remain histologically normal, and abnormal mucosal immune responses to food Ags would be expected to result in lymphocyte infiltration and tissue damage, as seen, for example, in celiac’s disease (23, 24). Instead, our findings are consistent with defects in intrathymic T cell development and a breakdown in central and/or peripheral tolerance as the causes of the hemopoietic disorders in IL-2\(^{-/-}\) mice. The accumulation of B220\(^+\) TCR\(^{\gamma^\delta^+}\) T cells that normally undergo apoptosis (18) in the livers of germfree IL-2\(^{-/-}\) mice and the defective elimination of superantigen-stimulated peripheral T cells in IL-2\(^{-/-}\) (7) and IL-2Ra\(^{-/-}\) mice (8) strongly suggest that a breakdown in peripheral tolerance is at least partially responsible for the disorders seen in IL-2\(^{-/-}\) mice. Additionally, our findings that IL-2 is produced by dying thymocytes and that activation-induced cell death in the thymi of IL-2\(^{-/-}\) mice is defective (H. Bassiri, P. J. Egan, E. Samoilova, Y. Chen, and S. R. Carding, unpublished observations) suggest that a breakdown in thymic tolerance may also be ultimately responsible for the splenomegaly, lymphadenopathy, and anemia seen in IL-2\(^{-/-}\) mice.
Comparison of the iIEL repertoire in wild-type mice maintained in an SPF or germfree environment clearly demonstrates the influence of the gut flora on shaping the iIEL repertoire. The presence of TCR$\alpha$CD8$\alpha$ iIELs appears to be largely dependent upon bacterial colonization, whereas the CD8$\alpha$a subset, which is thought to arise in the gut mucosa (25), is generated independently of microbial Ags. Among these cells, the generation or maintenance of TCR$\gamma$D iIELs appears to be dependent upon IL-2. The inability of IL-2$^{-/-}$ bone marrow cells to generate $\gamma$D iIELs in lethally irradiated hosts is consistent with an intrinsic defect within hemopoietic or lymphoid progenitor cells. The absence of TCR$\gamma$D iIELs in SPF IL-2$^{-/-}$ bone marrow chimeras demonstrates that the reduced number of these cells in germfree IL-2$^{-/-}$ mice is not due to autoimmunity, the absence of gut flora, or differences in food Ags or intestinal microenvironments that may exist between SPF and germfree mice. The increased numbers of CD$\alpha$B+ iIELs in germfree IL-2$^{-/-}$ mice may simply reflect their infiltration into the epithelia and/or their expansion to fill a niche created by the failed generation of TCR$\gamma$D+ iIELs.

Although we have not been able to identify the defective progenitor cell(s) in IL-2$^{-/-}$ bone marrow, the presence of donor-derived TCR$\alpha$B+ iIELs (Fig. 6) and $\alpha$B T cells and B cells in the blood$^6$ of IL-2$^{-/-}$ bone marrow chimeras suggests that the defective cell resides in a $\gamma$D progenitor population rather than in a common lymphoid progenitor or hemopoietic stem cell population. Furthermore, the normal number of $\gamma$D T cells in extraintestinal tissues of SPF (26) and germfree (T. Reya, H. Bassiri, and S. R. Carding, unpublished observations) IL-2$^{-/-}$ mice suggests that the defect is restricted to a $\gamma$D iIEL progenitor population. The reduced number of TCR$\gamma$D+ iIELs in IL-2R$\beta$-/- mice (14) is consistent with our findings in IL-2$^{-/-}$ mice and suggests that IL-2/IL-2R signaling may directly influence $\gamma$D progenitor cell development. Expression of IL-2Rs by a subset of T lymphoid progenitors within cryptopatches (27) of the gut is consistent with this interpretation.

The source and utilization of IL-2 within the intestinal mucosa could be autocrine or paracrine in nature (28, 29), with IL-2 being produced in response to endogenous or food Ags. We cannot, however, exclude the possibility that IL-2 may influence TCR$\gamma$D$^+$

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iIELs in other ways. For example, IL-2 may serve as a survival factor or a competence factor, enabling γδ iIEL progenitors to acquire responsiveness to other growth and differentiation factors. These possibilities are not mutually exclusive, since IL-2 may have multiple effects during γδ iIEL development, with its effect being determined by the developmental stage of the IL-2R-bearing cell. Finally, since the block in γδ iIEL generation is incomplete in germfree IL-2<sup>−/−</sup> mice or IL-2<sup>−/−</sup>/bone marrow chimera, it is possible that there are compensatory roles for other factors (30, 31) and/or IL-2-independent pathways of γδ iIEL development. Regardless of the role of these other factors, our findings clearly demonstrate the heterogeneity in the requirement for IL-2 for the development of iIELs and the existence of IL-2-dependent (TCRγδ<sup>+</sup>) and independent pathways of iIEL development.

The absence of colitis in germfree IL-2<sup>−/−</sup> mice is in agreement with the previous observations of Sadlack and co-workers (1) and strongly suggests that enterocolitis is a direct result of an abnormal immune response in the mucosa to the intestinal bacterial flora (32–34). It is possible that TCRγδ iIEL populations, which are reduced in the absence of IL-2, possess immunoregulatory functions that contribute to the regulation of mucosal immune responses and to the maintenance of tolerance to endogenous Ags (35–37).

In summary, the results of our analysis of germfree IL-2<sup>−/−</sup> mice show that the entero and extraintestinal lymphocytic infiltrate seen in SPF IL-2<sup>−/−</sup> mice are most likely the result of uncontrolled responses to endogenous Ags and not to environmental ones. In contrast, the colitis that develops in SPF IL-2<sup>−/−</sup> mice occurs in response to intestinal bacterial flora. The germfree IL-2<sup>−/−</sup> mice represent, therefore, a system in which the role of IL-2 in development and regulation of the hemopoietic and immune systems can be assessed in detail in dissociation from the complications of colitis.

References


