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Defective Lymphoid Development in Mice Lacking Expression of the Common Cytokine Receptor γ Chain

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Summary

The common γ chain (γ_c) of the IL-2, IL-4, IL-7, IL-9, and IL-15 receptors is defective in humans with XSCID. Mice lacking γ_c expression had hypoplastic thymuses; the thymocytes responded to γ_c-independent mitogens, but not γ_c-dependent stimuli. Splenic T cells were diminished at 3 weeks of age, but $CD4^+$ T cells markedly increased by 4 weeks. B cells were greatly diminished, in contrast with the situation in XSCID. NK cells, γδ intestinal intraepithelial lymphocytes, dendritic epidermal T cells, peripheral lymph nodes, and gut-associated lymphoid tissue were absent. These findings underscore the importance of γ_c in lymphoid development. Moreover, differences in humans and mice lacking γ_c expression indicate species-specific differences in the roles of γ_c-dependent cytokines or in the existence of redundant pathways. These mice provide an important model for studying the pathophysiology of and gene therapy for human XSCID.

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Introduction

X-linked severe combined immunodeficiency (XSCID) in humans is characterized by profoundly diminished cell-mediated and humoral immunity, with death typically occurring in the first year of life in the absence of successful bone marrow transplantation (Conley, 1992; Leonard et al., 1994). T cells and natural killer (NK) cells are absent or profoundly reduced in numbers and B cells, while present in normal or increased numbers, are nonfunctional, ostensibly due to a combination of lack of T cell help as well as an intrinsic B cell defect. XSCID results from mutations in the gene encoding the common γ chain, γ_c (Noguchi et al., 1993c), which is an essential signaling component of the receptors for interleukin-2 (IL-2) (Takeshita et al., 1992), IL-4 (Kondo et al., 1993; Russell et al., 1993), IL-7 (Noguchi et al., 1993b; Kondo et al., 1994), IL-9 (Russell et al., 1994), and IL-15 (Giri et al., 1994). The clinical manifestations in XSCID, therefore, result from the concomitant inactivation of at least five different cytokine systems. γ_c associates with and mediates the activation of the Janus family tyrosine kinase, Jak3 (Boussiotis et al., 1994; Russell et al., 1994; Miyazaki et al., 1994). Thus, at least some of the signaling and developmental defects found in XSCID are likely to result from a failure to activate Jak3.

To advance our understanding of the developmental role of γ_c as well as to create a murine model for XSCID gene therapy, we have generated mice in which the γ_c gene was disrupted by homologous recombination. Whereas heterozygous ($\gamma_c^{+/-}$) female mice exhibited normal immune function, males lacking γ_c expression ($\gamma_c^{-/Y}$) had severe cellular abnormalities characterized by an absence of NK cells and greatly diminished B and T cells, including a complete absence of $Thy1^+$ γδ dendritic epidermal T cells (DETC) and intraepithelial lymphocytes (IEL). However, it was also striking that T cells were present and could proliferate in response to mitogenic stimuli. The implications of these findings are discussed.

Results

Characterization of the Murine γ_c Gene, Preparation of a Targeting Construct, and Creation of γ_c-Deficient Mice

Like human γ_c, the murine γ_c gene is located on the X chromosome (Cao et al., 1993). The organization of the gene (Figure 1A), including its restriction map, sequence (5267 bp spanning all exons and introns, deposited in GenBank), and exon–intron boundaries were determined. Both human (Noguchi et al., 1993a) and murine (Figure 1A) γ_c genes have eight exons and similar exon–intron boundaries. This information was utilized to prepare a sequence replacement targeting construct, denoted pPNTγ_c1.3/2.0 (Figure 1A), designed to delete by homologous recombination part of exon 3 and all of exons 4–8, thereby resulting in the loss of much of the γ_c extracellular domain as well as the entire transmembrane and cyto-

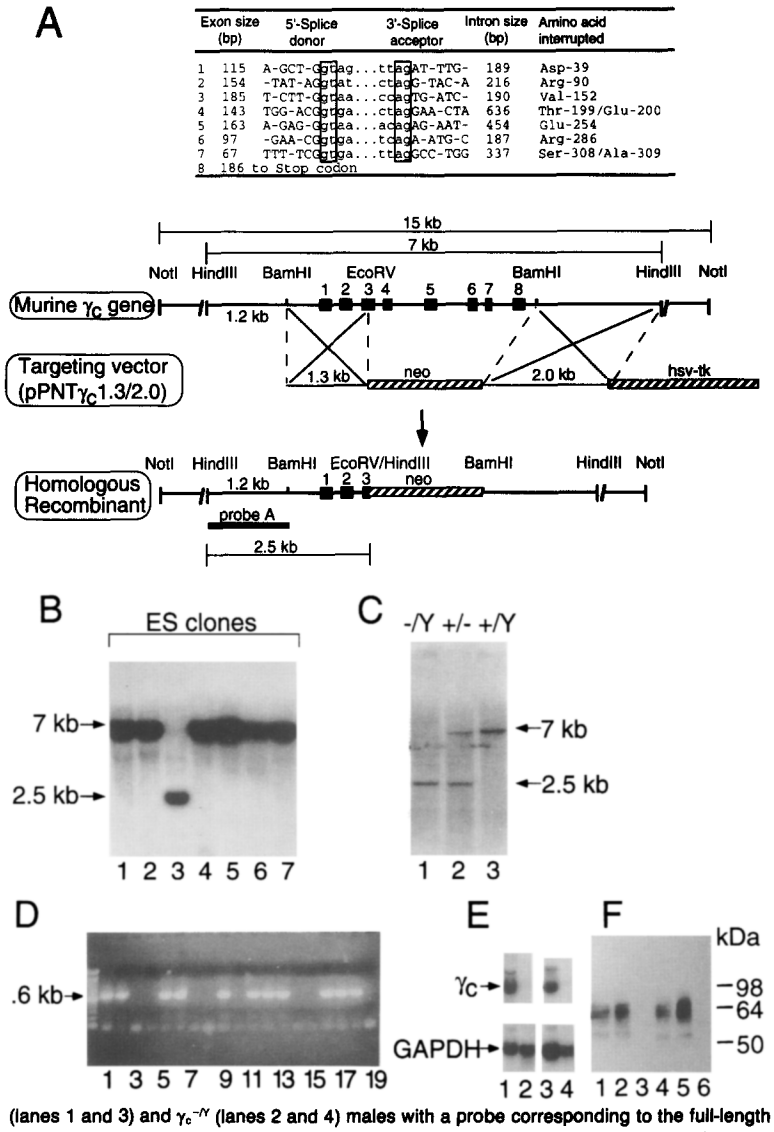


Figure 1. Inactivation of the γ_c Gene by Homologous Recombination

(A) At the top, the exon–intron organization of the murine γ_c gene is shown, with exon and intron sequences in upper and lower case letters, respectively. Exon and intron sizes are given, as well as the amino acids interrupted by each intron. The consensus splice donor and acceptor sequences are boxed. Below are schematics of the normal murine γ_c gene (with the positions of the eight exons indicated by closed boxes, partial restriction map, and the 15 kb NotI and 7 kb HindIII genomic fragments), the targeting vector, pPNTγ_c1.3/2.0 (showing the positions of the *neo* and *hsv–tk* genes and the regions of identity with the γ_c gene), and the predicted homologous recombinant (including the position of the 2.5 kb HindIII fragment that is detected upon hybridization with probe A).

(B) Identification of an ES cell clone in which the γ_c gene has been targeted by homologous recombination. DNA was digested to completion with HindIII and then Southern blotted using probe A (see [A]). Shown are six ES clones with the normal 7 kb germline configuration (lanes 1, 2, and 4–7), and one in which homologous recombination occurred to yield a 2.5 kb band (lane 3).

(C) Southern blotting of HindIII-digested DNA from a $\gamma_c^{-/Y}$ male (lane 1), heterozygous female (lane 2), and wild-type male (lane 3) using probe A. As expected, a 7 kb band was seen in lanes 2 and 3, and a 2.5 kb band was seen in lanes 1 and 2.

(D) Nested PCR was used to identify rapidly the homologously recombined γ_c–*neo* gene construct that was present in $\gamma_c^{-/Y}$ males (lanes 1, 2, 6, 13, and 16–18) and heterozygous ($^{+/-}$) females (lanes 5, 9, 11, 12) but not in wild-type males (lanes 7, 14, 15) or females (lanes 3, 4, 8, 10, 19). The 19 mice are from three litters derived from the mating of heterozygous females to wild-type males.

(E) Northern blotting of spleen (lanes 1 and 2) and thymus (lanes 3 and 4) from wild-type (lanes 1 and 3) and $\gamma_c^{-/Y}$ (lanes 2 and 4) males with a probe corresponding to the full-length murine γ_c cDNA (Cao et al., 1993) or GAPDH. Lanes 1 and 2, top subpanel, were exposed for 3 days whereas the other three subpanels were exposed for 8 hr. Although a γ_c–*neo* fusion transcript might have been expected (see [A]), this mRNA is apparently unstable.

(F) Western blotting with R878 antiserum to γ_c and lysates from spleens (lanes 1–3) and thymuses (4–6) from a wild-type male (lanes 1, 4), heterozygous female (lanes 2, 5), and $\gamma_c^{-/Y}$ male (lanes 3, 6).

plasmic domains. This is a more extensive truncation of γ_c than is found in many cases of XSCID in humans (Noguchi et al., 1993c; Puck et al., 1993; DiSanto et al., 1994a; Ishii et al., 1994), and would be expected to result in a construct incapable of cell surface expression and lacking regions of γ_c required for signaling.

J1 strain ES cells were transfected with pPNTγ_c1.3/2.0 and homologous recombination was detected by Southern blotting (a 2.5 kb band instead of a 7 kb band indicates recombination, Figure 1B) using the 5′ flanking probe (see Figure 1A, probe A), and confirmed by hybridization with a 3′ flanking probe (data not shown). Hybridization with a murine γ_c cDNA probe corresponding to exons 4–8 confirmed that this region of the gene had been deleted (data not shown). Following injection of C57BL/6 blastocysts with the embryonic stem (ES) cells containing a disrupted γ_c gene, chimeric progeny mice were mated to C57BL/6 mice. $\gamma_c^{-/Y}$ males and heterozygous females derived from these and subsequent matings were identified by Southern blotting and polymerase chain reaction (PCR) (Figures 1C and 1D). Mating of wild-type males to heterozygous females yielded wild-type males and females, heterozygous females, and $\gamma_c^{-/Y}$ males with the expected Mendelian frequency. Northern and Western blotting confirmed that $\gamma_c^{-/Y}$ males did not express γ_c mRNA (Figure 1E, lanes 2, 4) or protein (Figure 1F, lanes 3, 6) in spleen or thymus, but heterozygous females had normal expression of γ_c (Figure 1F, lanes 2 and 5).

Microscopic and Flow Cytometric Analyses of Immunological Organs in γ_c-Deficient Mice

Heterozygous females and $\gamma_c^{-/Y}$ males, ages 3–9 weeks, were similar in size and appearance to age-matched wild-type control males and females. No gross lesions were

detected in any nonlymphoid organs. As expected based on analogy to human XSCID carrier females, heterozygous female mice had normal lymphoid development and were indistinguishable from normal controls. In contrast, $\gamma_c^{-/Y}$ males had marked abnormalities, by gross (Figures 2A and 2B) as well as by microscopic (Figure 2; Figure 3) and flow cytometric (see Figure 4) analyses. They had very small thymuses (Figure 2A), and the cortex and medulla of the thymus of a representative 3-week-old $\gamma_c^{-/Y}$ male were severely hypoplastic. The cortex was reduced in size and the corticomedullary junction was indistinct (Figures 2D and 2E, compare with normal thymus in Figure 2C), but the basic thymic architecture was retained. Hassall's corpuscles were readily identified (Figure 2E, arrowhead). In comparison, SCID/NCr mouse thymuses lack obvious small lymphocytes and corticomedullary demarcations (Figure 2F). Peripheral lymph nodes were not identifiable at necropsy. The mesenteric lymph node was small, with a few lymphoid follicles containing small lymphocytes but no germinal centers; paracortical lymphocytes were not obvious (Figures 2H and 2I, compare with normal lymph node in Figure 2G). There was a mild diffuse proliferation of spindle cells resembling dendritic cells (Figure 2I). Gut-associated lymphoid tissue was absent in the small and large intestine (data not shown).

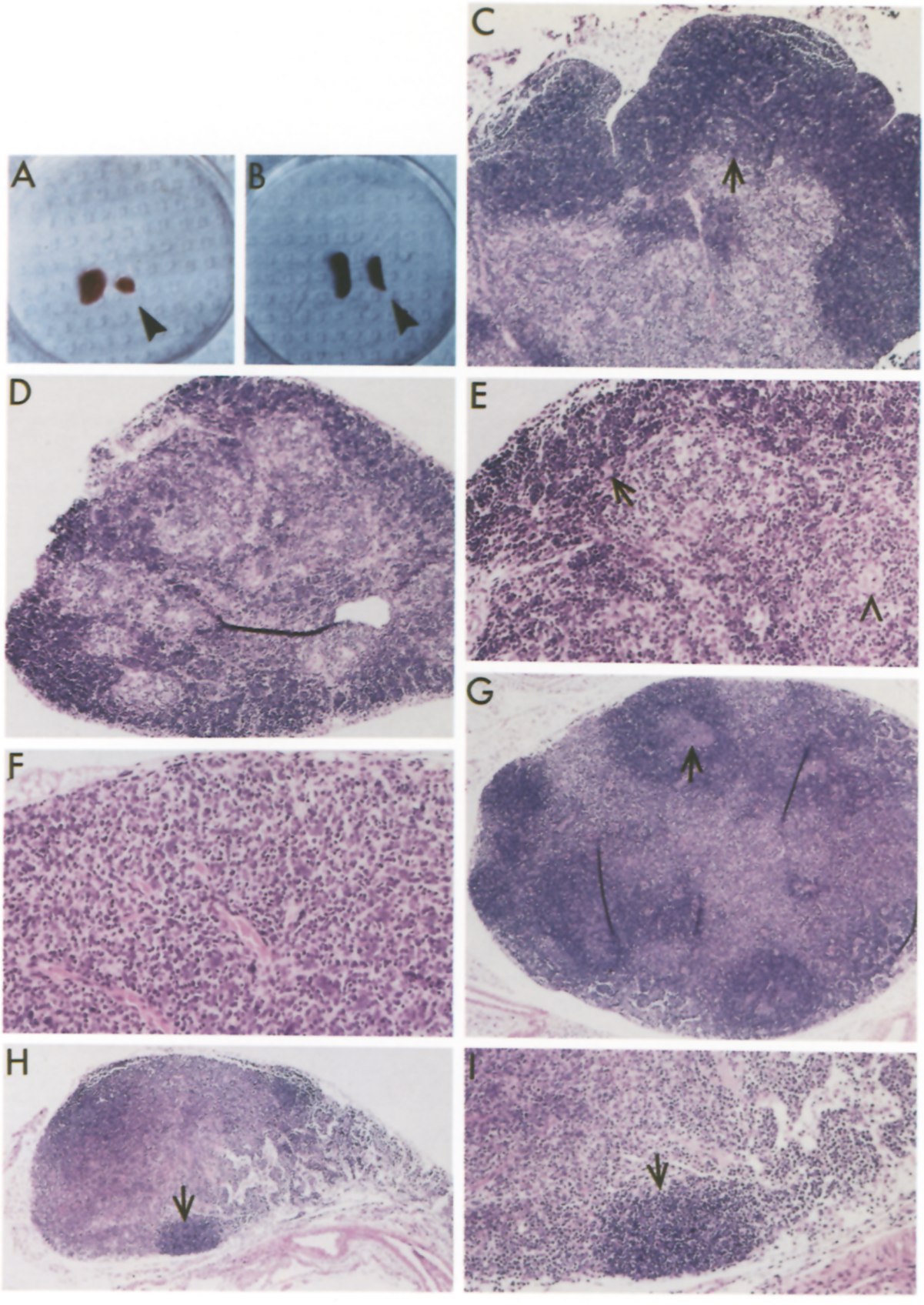
In addition to these histologically defined lesions, $\gamma_c^{-/Y}$ males had a variety of other immunological abnormalities. Based on cell counts and flow cytometric analysis, the absolute numbers of all thymic T cell populations defined by CD4 and CD8 expression were significantly diminished (Table 1; see Figure 4A). Interestingly, there was an increase in the ratio of $CD4^+CD8^-$:$CD4^-CD8^+$ single-positive cells, primarily resulting from an increase in the percentage of the $CD4^+CD8^-$ population. There was an increase in the percentage of thymocytes expressing high levels of CD3ε, T cell receptor β (TCRβ), and CD5, but low levels of heat-stable antigen (see Figure 4A). Specific gating on both $CD4^+CD8^-$ and $CD4^-CD8^+$ thymocytes revealed that they expressed levels of TCR typical of mature single-positive cells. Although TCRαβ T cells developed in $\gamma_c^{-/Y}$ mice, thymocytes expressing TCRγδ were not detected in $\gamma_c^{-/Y}$ mice even when double-negative $CD4^-CD8^-$ cells were specifically examined (data not shown); the presence of TCRγδ cells in $\gamma_c^{-/Y}$ fetuses has not yet been assessed. As expected, no γ_c (TUGm3) expression could be detected. Thus, despite the dramatic decrease in lymphoid mass, it was striking that mature T cells in fact developed in these mice.

At 3 weeks of age, the spleens of $\gamma_c^{-/Y}$ mice were only modestly decreased in size (Figure 2B). Extramedullary hematopoiesis (primarily erythropoiesis), similar to the control mouse (Figures 3A and 3B), was observed in red pulp; however, severe lymphocyte hypoplasia was evident in the white pulp of the $\gamma_c^{-/Y}$ male (Figures 3C and 3D; Table 1). There was a small accumulation of spindle-shaped cells in the periarteriolar lymphoid sheath region; the B cell and marginal zones were absent. Flow cytometric analysis of the splenic T cells revealed an increase in the $CD4^+CD8^-$:$CD4^-CD8^+$ ratio, primarily due to the dramatic decrease in $CD4^-CD8^+$ cells (Figure 4B). The $CD4^+CD8^-$ cells expressed CD3ε and CD28 and did not appear to represent the MHC class I–educated $CD4^+$ population that has been described (Bendelac et al., 1994; Coles and Raulet, 1994; Lantz and Bendelac, 1994), since they were not enriched for TCRVβ8 (Figure 4B) or CD44 (data not shown); in fact, the diminished staining by TCRVβ8 and the absence of $NK1.1^+$ cells were consistent with a decrease in this population of cells. In contrast, the percentages of macrophages/monocytes ($Mac1^+$ cells) and granulocytes ($GR1^+$ cells) were normal or even increased.

Following mitogenic activation with anti-CD3, splenocytes were evaluated for γ_c, IL-2Rβ, IL-2Rα, and CD69 expression (Figure 4C). As expected, no γ_c (TUGm3) expression was observed, and while IL-2Rβ (TMβ1) and IL-2Rα (7D4) were induced, their levels of expression were lower than those seen in the heterozygous female and wild-type male. It is possible that the lower IL-2Rα and IL-2Rβ levels achieved are explained by the lack of IL-2 and IL-4 responsiveness of $\gamma_c^{-/Y}$ mice, since IL-2 (Siegel et al., 1987) and IL-4 (Casey et al., 1992) both up-regulate IL-2Rβ levels and IL-2 (Siegel et al., 1987) up-regulates IL-2Rα. Induction of the activation marker CD69 was also diminished in the $\gamma_c^{-/Y}$ splenocytes (Figure 4C). One of the most remarkable findings in analysis of $\gamma_c^{-/Y}$ spleens was that although splenic mononuclear cells were dramatically decreased in 3-week-old mice, mice ages 4–9 weeks had an apparently age-related increase in splenic lymphocytes (Table 1) and enlarged spleens. Each of these animals exhibited a markedly increased CD4:CD8 ratio (Table 1).

The presence of B cells was analyzed by staining bone marrow and spleen with B220, anti-μ, and anti-immunoglobulin. The $\gamma_c^{-/Y}$ mice exhibited a marked overall decrease in B cells (Table 1). This decrease was most evident in the bone marrow where only rare $B220^+$ and $B220^+\mu^+$ cells were present (Figure 4D; Table 1). Although B cells, as defined by $B220^+\mu^+$ (Figure 4D) or $B220^+\delta^+$ (data not shown) were detected in the spleen, their absolute numbers were markedly reduced as compared with wild-type or heterozygous mice (Figure 4D; Table 1) in all $\gamma_c^{-/Y}$ mice, regardless of age. Despite this overall decrease in B cells, immunoglobulin levels of immunoglobulin M (IgM) were normal in all mice studied. However, in ≥5-week-old mice studied, all other immunoglobulin levels were diminished. The decreased levels of IgG1 and IgE were expected based on the phenotype in the IL-$4^{-/-}$ mice (Kuhn et al., 1991). The low levels of other classes of immunoglobulin as well reflects the greater defect in $\gamma_c^{-/Y}$ mice, consistent with the situation in human XSCID (Puck, 1993).

Cells recovered from the peritoneal cavity were analyzed by staining with B220, CD5, and anti-μ (Figure 4D). The fraction of $B220^+$ cells that were μ^+ was similar in wild-type and $\gamma_c^{-/Y}$ males. Both $CD5^+\mu^+$ and $CD5^-\mu^+$ cells were present in the $\gamma_c^{-/Y}$ males (Figure 4D), consistent with the production of both B-1 and conventional B cell populations (Kantor and Herzenberg, 1993), analogous to the situation in normal mice. B-1 cells are "self-replenishing" B cells and can produce all immunoglobulin isotypes, making major contributions to serum IgM, IgG3, and IgA (reviewed by Kantor and Herzenberg, 1993). Therefore,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these cells may be contributing to the IgM production in the $\gamma_c^{-/Y}$ males. The splenic B cells contained at most a minor population of cells expressing CD5 or Mac1 or both markers (data not shown), consistent with these cells representing conventional B cells, rather than B-1 cells (Kantor and Herzenberg, 1993).

Granulocytes (as indicated by GR1$^+$ cells) were readily detected in bone marrow (data not shown). Histologically, the bone marrow had erythroid hyperplasia, but this was similar to that found in the wild-type male and heterozygous female (data not shown). Analysis of peripheral blood mononuclear cells (PBMC) also revealed greatly diminished B cells and an absence of NK cells, whereas single positive CD4$^+$CD8$^-$ cells and Mac1$^+$ cells were present (data not shown).

Absence of γδ$^+$ DETC and IEL

One of the most striking findings came from the examination of the skin of the $\gamma_c^{-/Y}$ mice (Figure 5). Immunohistochemical studies using antibodies to both the γδ TCR and the Thy1.2 alloantigen demonstrated that DETC were not present in epidermal sheets of 3-, 5-, and 7-week-old $\gamma_c^{-/Y}$ mice (compare Figure 5B with Figures 5A and 5C). There was no general lack of Thy1.2 alloantigens in the epidermis as keratinocytes expressed this antigen on their cell membranes (Figure 5E). Other dendritic epidermal cell populations, such as Langerhans cells, were present in normal density (Figures 5G–5I). The absence of γδ$^+$ cells in the skin prompted us to also examine the intestines for γδ$^+$ IEL. The fact that these cells were also absent in mice lacking γ_c expression (data not shown) was interesting, particularly since in contrast with the γδ$^+$ cells of the skin, a significant proportion of γδ$^+$ IEL are extrathymically derived (reviewed by Lefrançois, 1994).

Immune Function in the γ_c-Deficient Mice

The functional capabilities of T cells were next evaluated. The most striking feature was that thymocytes from $\gamma_c^{-/Y}$ males were capable of responding to phorbol myristate acetate (PMA) plus ionomycin or to anti-CD3 plus anti-CD28 as potently as were age-matched controls (Table 2). Consistent with the absence of γ_c, which is required for IL-4 signaling in T cells (Kondo et al., 1993), the thymocytes from $\gamma_c^{-/Y}$ mice could not proliferate in response to PMA plus IL-4, in marked contrast with wild-type mice and heterozygous females (Table 2). Splenocytes from $\gamma_c^{-/Y}$ mice also responded to PMA plus ionomycin and anti-CD3 plus anti-CD28 (Table 3), but in contrast with the situation for thymocytes, the response was much less potent than that seen with age-matched controls. Splenocytes from $\gamma_c^{-/Y}$ mice also responded to lipopolysaccharide, but again to a substantially lower degree than did normal controls (Table 3). Consistent with the importance of γ_c for IL-4 responses, we were unable to detect a significant response to IL-4 plus anti-μ in $\gamma_c^{-/Y}$ splenocytes (Table 3). In some experiments, a modest response to PMA plus IL-4 was also observed (data not shown), suggesting the possibility of γ_c-independent IL-4-mediated signaling, analogous to the situation in THP-1 cells (Kotanides and Reich, 1993) and in COS-7 cells (Lin et al., 1995). In a preliminary experiment, we also assessed the ability of IL-4 to augment survival of purified T cells. Whereas cells from normal males and heterozygous females exhibited enhanced survival at day 1 and a 5- to 10-fold increase in survival at day 4, cells from a $\gamma_c^{-/Y}$ male did not (data not shown).

We next examined the ability of anti-CD3 plus anti-CD28 to stimulate production of cytokines. In comparison to normal littermates, interferon-γ (IFNγ) production was dramatically reduced (1019.3 ± 432.9 U/ml in controls versus 1.6 ± 0.2 in $\gamma_c^{-/Y}$ males [$p < .0001$]). Both IL-2 and IL-4 production was stimulated, but to only 35%–40% of normal levels (IL-2 production was 13.0 ± 2.7 for controls versus 5 ± 0.9 for $\gamma_c^{-/Y}$ males [$p = .0595$]; IL-4 production was 96.7 ± 13.5 for controls versus 35.5 ± 11.8 for $\gamma_c^{-/Y}$ males [$p = .0223$]). In the normal mice, cytokines were produced at much lower levels by thymocytes than by splenocytes, but no IFNγ, IL-2, or IL-4 were produced by $\gamma_c^{-/Y}$ thymocytes in any experiment. Finally, to assess mast cell/basophil function in the spleen, we examined the ability of plate-bound IgE plus IL-3 plus WEHI supernatant to induce IL-4 production. The $\gamma_c^{-/Y}$ mice exhibited heightened responses, with levels of IL-4 production approximately 10- to 45-fold elevated compared with normal littermates. This may partly be explained by the diminished levels of serum IgE in $\gamma_c^{-/Y}$ mice, resulting in more FcεRI on the surface of cells being available for cross-linking by immobilized IgE; in addition, it is possible that the cells capable of responding represent a higher percentage of splenocytes in $\gamma_c^{-/Y}$ males.

To confirm the absence of NK cells suggested by the absence of NK1.1$^+$ cells, we analyzed the cytolytic activity

Figure 2. Histological Abnormalities in Thymus and Lymph Nodes from $\gamma_c^{-/Y}$ Mice

Photographs of thymuses (A) and spleens (B) from 3-week-old wild-type and $\gamma_c^{-/Y}$ (arrowhead) males.

(C) Thymus of wild-type mouse with normal morphology and a distinct corticomedullary junction (arrow; hematoxylin and eosin, 20×).

(D) Thymus of $\gamma_c^{-/Y}$ mouse with a less well-defined coritcomedullary junction but retaining a subscapsular rim of lymphocytes. A cyst is present. (Hematoxylin and eosin, 20×).

(E) Thymus of $\gamma_c^{-/Y}$ mouse showing small subcapsular lymphocytes (arrow) and a Hassall's corpuscle (arrowhead; hematoxylin and eosin, 50×).

(F) Thymus of SCID/NCr mouse, in which small lymphocytes are not present and there is no corticomedullary junction (hematoxylin and eosin, 50×).

(G) Lymph node of wild-type mouse containing follicles (arrow) with active germinal centers (hematoxylin and eosin, 13.2×).

(H) Lymph node of $\gamma_c^{-/Y}$ mouse with small inactive follicles (arrow) and fewer paracortical lymphocytes than the wild-type mouse (hematoxylin and eosin, 13.2×).

(I) Lymph node of a $\gamma_c^{-/Y}$ mouse with an inactive follicle (arrow) and adjacent spindle-shaped cells that resemble dendritic cells (hematoxylin and eosin, 33×).

Table 1. T and B Cell Populations in Lymphoid Tissues

	T Cells								B cells				
	Thymus					Spleen			Spleen		Bone Marrow		
Sex, genotype, and age	Total × 10^{-6}	DN (Percentage)	DP (Percentage)	CD4 (Percentage)	CD8 (Percentage)	Total × 10^{-6}	CD4 (Percentage)	CD8 (Percentage)	$B220^+$ (Percentage)	$B220^+\mu^{+a}$ (Percentage)	Total × 10^{-6}	$B220^+$ (Percentage)	$B220^+\mu^+$ (Percentage)
Female (+/–)													
3 weeks	87	3	83	10	4	72	20	8	69	64	47	58	16
3 weeks	183	2	88	7	2	33	11	6	59	48	38	33	11
5 weeks	184	2	86	10	3	56	19	12	58	49	37	49	20
6 weeks	216	2	85	11	3	96	17	9	70	57	31	46	18
7 weeks	420	2	85	10	4	70	14	8	56	53	58	68	21
Male (+/Y)													
3 weeks	85	3	84	10	3	68	18	7	68	61	26	57	19
3 weeks	300	2	89	9	2	35	15	8	71	56	38	50	13
3 weeks	97	1	85	11	3	24	10	6	70	56	30	18	7
5 weeks	288	2	78	17	2	56	24	16	51	42	25	44	18
7 weeks	275	3	85	9	3	72	11	5	57	53	62	57	17
9 weeks	244	1	85	11	3	90	14	8	70	65	43	33	10
Male (–/Y)													
3 weeks	5	1	82	16	2	5	10	1	21	17	18	2.9	.8
3 weeks	5	2	80	17	2	5	8	.5	31	20	16	2.0	.5
3 weeks	5	—	—	—	—	5	—	—	—	—	—	—	—
3 weeks	3	2	77	19	2	10	6	.3	30	19	27	1.3	.4
3 weeks	1	1	84	14	1	2	6	.4	—	—	22	0.6	.2
4 weeks	6	6	69	23	3	59	26	1	14	9	20	3.1	.1
5 weeks	2	5	78	16	2	69	20	1	9	4	22	3.1	.1
5 weeks	5	1	77	18	3	90	16	.4	12	9	37	2.4	.1
6 weeks	<.1	74	21	4	1	90	7	.1	5	4	41	0.3	.1
7 weeks	4	16	65	17	3	138	71	6	1	.2	54	3.4	.1
9 weeks	1	2	68	27	2	190	33	1	5	2	30	4.0	.7
9 weeks	3	1	80	18	1	160	38	5	6	2	46	3.6	.5

[a] Only cells with medium to high expression of B220 were counted (see Figure 4D).

[b] This mouse was visibly sick.

T and B cell populations in thymuses, spleens, and bone marrow isolated from heterozygous females, wild-type males, and $\gamma_c^{-/Y}$ males. For the thymuses, shown are total numbers of cells isolated and percentages of $CD4^-CD8^-$ (DN), $CD4^+CD8^+$ (DP), $CD4^+CD8^-$ (CD4), and $CD4^-CD8^+$ (CD8) cells. For the spleens, shown are the total numbers of cells isolated, and percentages of $CD4^+CD8^-$ (CD4), and $CD4^-CD8^+$ (CD8) single-positive cells. For spleens and bone marrow, percentages of $B220^+$ and $B220^+/\mu^+$ cells are shown. Populations not analyzed for a given animal are indicated by a minus. Note the dramatic differences in the spleens isolated from 3-week-old versus 4–9-week-old mice. Dashes, not done.

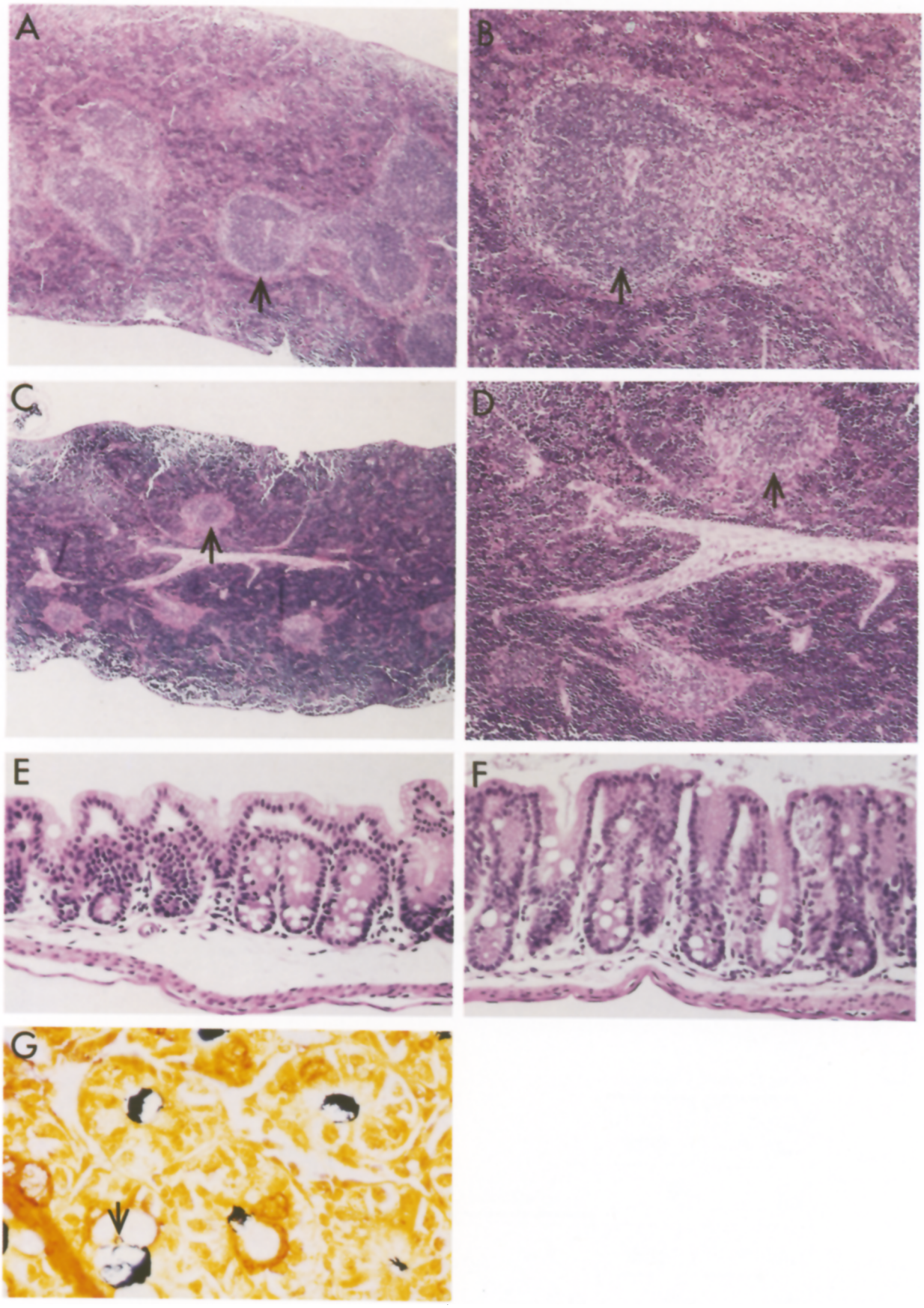


Figure 3. Histological Abnormalities in Spleen and Cecum from $\gamma_c^{-/Y}$ Mice

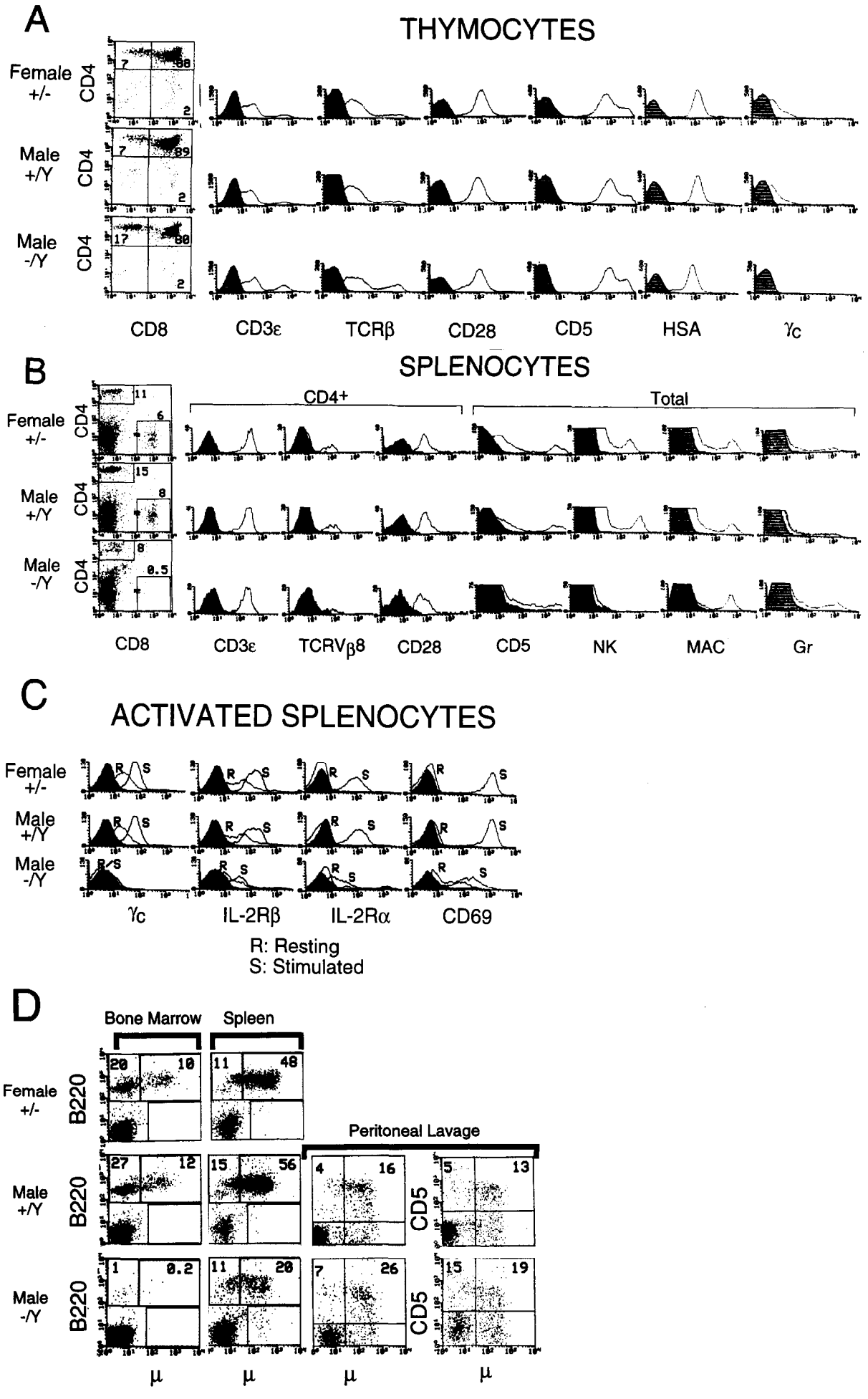
(A and B) Spleen of wild-type mouse. Red pulp has extramedullary hematopoiesis; white pulp (arrow) has normal morphology (hematoxylin and eosin, 13.2×, 33×).

(C) and (D) Spleen of γ_c-deficient mouse. Red pulp has extramedullary hematopoiesis; white pulp (arrow) has severe lymphocyte depletion (hematoxylin and eosin, 13.2×, 33×).

(E) Cecum of wild-type mouse, with normal sized crypts (hematoxylin and eosin, 50×).

(F) Cecum of γ_c-deficient mouse with proliferative typhlitis. Crypt height is increased and there are increased mononuclear cells in the lamina propria (hematoxylin and eosin, 50×).

(G) Cecal crypts of γ_c-deficient mice with tangled mats of helical bacteria (Steiner stain, 160×). Arrow indicates H. hepaticus.


A
THYMOCYTES
Female +/-
Male +/Y
Male -/Y
CD4
CD8
CD3ε
TCRβ
CD28
CD5
HSA
γc
B
SPLENOCYTES
CD4+
Total
CD8
CD3ε
TCRVβ8
CD28
CD5
NK
MAC
Gr
C
ACTIVATED SPLENOCYTES
γc
IL-2Rβ
IL-2Rα
CD69
R: Resting
S: Stimulated
D
Bone Marrow
Spleen
Peritoneal Lavage
B220
CD5
μ

of splenocytes against YAC-1 targets at varying effector-to-target cell ratios. Whereas NK activity in fresh cells from wild-type mice and heterozygous females was 16 ± 4 lytic U/10^7 cells, cytolytic activity in cells from $\gamma_c^{-/Y}$ males was indistinguishable from 0. As expected, IL-2 activated cytolytic activity in the control animals but not in the $\gamma_c^{-/Y}$ males (data not shown), consistent with the requirement of γ_c for IL-2-induced cellular responsiveness.

Inflammatory Bowel Lesions

Proliferative typhlitis (inflammation of the cecum) and colitis with deeper crypts (see Figures 3E and 3F) was observed in $\gamma_c^{-/Y}$ males but not in heterozygous females or wild-type males. In the $\gamma_c^{-/Y}$ males, mitoses were seen higher on the crypts, and mucosal infiltrates of mononuclear cells and neutrophils were present. These lesions were accompanied by the presence of helical bacteria in the crypts and lumen of the cecum and colon (Figure 3G). In this regard, it may be relevant that Helicobacter hepaticus, a newly recognized pathogen of mice that causes chronic active hepatitis (Ward et al., 1994), has been associated with inflammatory bowel lesions in immunocompromised mice including SCID/NCr mice and nude mice (Russell et al., 1995; Ward et al., unpublished data), but not in C57BL/6 mice, which are resistant to development of gut and liver lesions even when the large intestine is colonized with H. hepaticus. There was no evidence of crypt abscesses, mucosal ulcerations, or granulomatous inflammation of the bowel wall in 3-, 5-, and 7-week-old animals as are found in human inflammatory bowel disease. Whether such lesions might become evident later in life, analogous to those of the IL-$2^{-/-}$ mice (Sadlack et al., 1993), is not yet clear.

Discussion

The phenotype of $\gamma_c^{-/Y}$ mice underscores the importance of γ_c in lymphoid development. These mice had small hypoplastic thymuses, with greatly reduced cortical and medullary regions; however, a cortical rim of small lymphocytes was seen and Hassall's corpuscles could be identified. In canine XSCID, in which a 4 bp deletion in the first exon of the γ_c gene prevents production of a functional protein (Henthorn et al., 1994), the thymuses were dysplastic, albeit variable in extent, with Hassall's corpuscles being present in approximately half the animals studied (Snyder et al., 1993). In this sense, canine XSCID initially might appear to be more similar to our $\gamma_c^{-/Y}$ mice than to human XSCID, in which thymic development has traditionally been described as dysplastic, with a complete lack of Hassall's corpuscles. More recently, however, some heterogeneity has been described for humans as well (Gosseye et al., 1983; Nezelof, 1986). However, the extent of thymic development in healthy XSCID humans remains somewhat unclear since few of the human SCID cases represent definitively proven cases of XSCID; the tissue samples are often from autopsy specimens of children who died of overwhelming infection; and even biopsy specimens were presumably obtained from children who came to medical attention because of opportunistic infections. In addition, it is reasonable to assume that thymic development may be influenced by other genetic factors as well, and that humans with different γ_c mutations, which are variable in their extent of inactivation of γ_c function, will be differentially affected. Indeed, an atypical case of XSCID (DiSanto et al., 1994b) and a family with moderate X-linked combined immunodeficiency (Brooks et al., 1990; Russell et al., 1994; Schmalstieg et al., 1995) have peripheral T cell development quite different from that found in classic XSCID.

XSCID dogs consistently exhibited a normal CD4:CD8 ratio in the thymus (Somberg et al., 1994), whereas we reproducibly have found the ratio to be increased. In both dogs and mice, the CD4:CD8 ratio was increased in the periphery. Canine peripheral lymphocytes demonstrated a profoundly diminished phytohemagglutinin response, but the proliferative capacity of thymocytes was not evaluated (Somberg et al., 1994). In the $\gamma_c^{-/Y}$mice, the thymo-

Figure 4. Flow Cytometric Analysis of Thymocytes, Splenocytes, Bone Marrow, and Peritoneal Cells from $\gamma_c^{-/Y}$ Mice

(A) Flow cytometric analysis of thymocytes from 3-week-old mice (heterozygous female, wild-type male, and $\gamma_c^{-/Y}$ male). For two-color histograms, the percentages of $CD4^+CD8^-$, $CD4^+8+$, and $CD4^-CD8^+$ cells are indicated in the appropriate quadrants. For one-color histograms corresponding to surface expression of CD3ε, TCRβ, CD28, CD5, and heat-stable antigen, thymocytes were directly stained with conjugated antibodies; the shaded region represents staining with similarly conjugated control antibodies. Expression of γ_c was assessed by indirect immunofluorescence; the shaded region represents staining with goat anti-rat immunoglobulin–PE alone.

(B) Flow cytometric analysis of splenocytes from the same 3-week-old mice analyzed in (A). For the two-color histograms with anti-CD4 and anti-CD8, the numbers represent the percentage of cells in the boxed regions. To analyze $CD4^+$ cells specifically, splenocytes were stained with anti-CD4 and anti-CD8 and software gating was used to examine expression of CD3ε, TCRVβ8, and CD28 by $CD4^+CD8^-$ ($CD4^+$) cells (left). In addition, ungated total splenocyte populations were assessed for expression of CD5, NK1.1, Mac1, and Gr1 cell surface molecules (right). In both cases, cells were directly stained and shaded regions represent staining of appropriately gated populations of cells with control antibodies.

(C) Expression of γ_c, IL-2Rα, IL-2Rβ, and CD69 on activated splenocytes. Unseparated splenocytes were cultured with soluble anti-CD3ε (145-2C11) and stained with antibodies directed against the indicated cell surface molecules. To assess γ_c and IL-2Rβ expression, cells were cultured for 3 days in the presence (stimulated, S) or absence (resting, R) of anti-CD3ε. Cells were harvested and stained with unconjugated anti-γ_c (TUGm3) or anti-IL-2Rβ (TMβ1), followed by the addition of goat anti-rat immunoglobulin (GαR)–PE. The shaded area represents staining of activated cells with GαR alone. After 3 days, most B cells have died; hence, the majority of cells represent T cells. To assess IL-2Rα and CD69 expression, cells were cultured for 18 hr in the presence or absence of anti-CD3ε and directly stained with anti-IL-2Rα (7D4) or anti-CD69. Histograms represent staining of $Thy1.2^+$ gated T cells. Shaded areas represent staining of equivalently gated activated cells with control antibodies.

(D) B cell development in $\gamma_c^{-/Y}$ mice. Bone marrow and spleen were obtained from the same mice whose profiles are shown in (A–C). Peritoneal cells are representative of those obtained from three independent sets of animals. Cells were stained with antibodies directed against the indicated cell surface molecules (B220, μ, and CD5). The percentages of cells within quadrants of interest are indicated. These percentages do not represent absolute numbers of B cells, as fewer total cells were analyzed from the $\gamma_c^{-/Y}$ mouse.

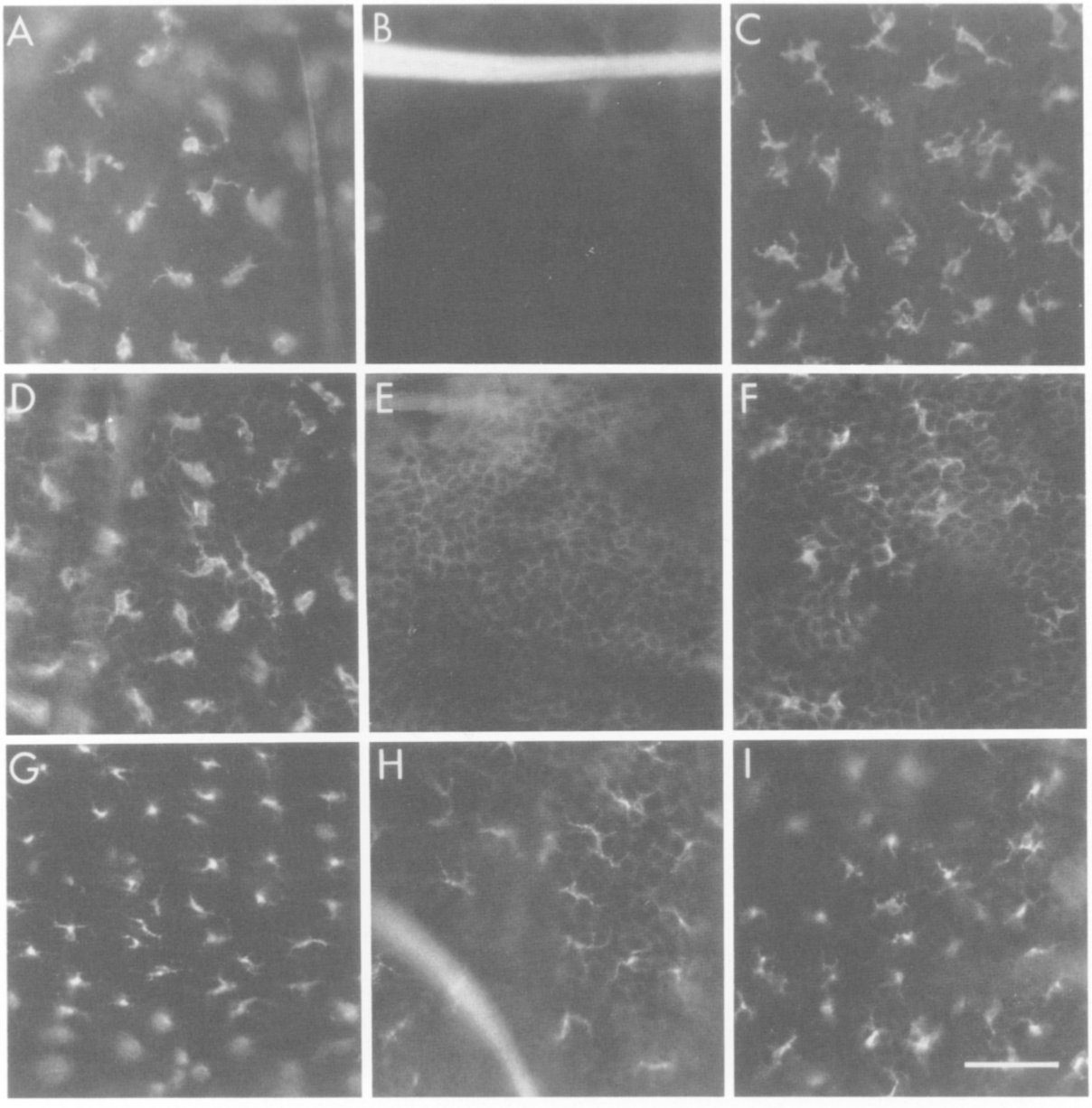


Figure 5. Absence of $\gamma\delta^+$ DETC but Retention of Langerhans Cells in $\gamma_c^{-/Y}$ Mice

DETC are absent in epidermal sheets of γ_c-deficient male mice. Epidermal sheets from wild-type males (A, D, G), from $\gamma_c^{-/Y}$ males (B, E, H), and from heterozygote ($\gamma_c^{+/-}$) females (C, F, I) were assessed for the presence of DETC and Langerhans cells. Normal densities of $\gamma\delta$ TCR$^+$ dendritic cells were seen in the normal (A) and heterozygote (C) mice but no $\gamma\delta$ TCR$^+$ cells were seen in the epidermis of the $\gamma_c^{-/Y}$ mice (B). Anti-Thy1.2 MAb stained keratinocytes in all three specimens, but only stained DETC in normal (D) and heterozygote (F) mice. Normal densities of Langerhans cells are seen in skin from the three different mice (G, H, I). Scale bar, 45 μm.

cytes gave the most reproducible responses to T cell mitogens.

Corresponding to the lymphoid hypoplasia in the thymuses of mice lacking γ_c expression, there was also severe lymphoid hypoplasia in the spleens of the 3-week-old animals examined. The slightly older animals (4–9 weeks of age) had an age-related increase in splenocytes (Table 1). Although the basis for the difference is unknown, it is possible that the retarded populating of the spleen with T cells is explained by the small size of the thymus with a concomitant decreased rate of egress of cells to the periphery. These findings are interesting in view of the normal thymic and T cell development observed in mice lacking production of IL-2 (Schorle et al., 1991), IL-4 (Kuhn et al., 1991), or both IL-2 and IL-4 (Sadlack et al., 1994), indicating that the loss of signaling in response to cytokines besides IL-2 and IL-4 is responsible for the defects in mice lacking γ_c expression. However, it was particularly striking that there was significant T cell maturation in $\gamma_c^{-/Y}$ mice, indicating that pathways not mediated by γ_c-dependent cytokines are sufficient to achieve significant development. In this regard, it is interesting that mice lacking

Table 2. Thymocyte Proliferation[a]

Experiments	Age	Genotype	RPMI	PMA + IL-4	PMA + Ionomycin	Anti-CD3 + Anti-CD28
Experiment 1	3 weeks	+/Y	95 ± 22	15752 ± 3160	22593 ± 2423	
	3 weeks	−/Y	91 ± 56	62 ± 20	33458 ± 424	
	3 weeks	−/Y	182 ± 82	198 ± 28	32630 ± 1532	
	5 weeks	+/−	39 ± 17	21668 ± 880	30294 ± 1722	
	5 weeks	−/Y	25 ± 6	34 ± 23	22359 ± 1471	
Experiment 2	3 weeks	+/Y	68 ± 20	11692 ± 1242	6937 ± 573	
	3 weeks	+/+	101 ± 67	11907 ± 1182	6811 ± 1104	
	3 weeks	−/Y	98 ± 57	62 ± 16	6463 ± 405	
Experiment 3	3 weeks	+/Y	76 ± 6			60288 ± 4750
	3 weeks	−/Y	56 ± 2			66751 ± 412
	5 weeks	+/−	56 ± 9			74868 ± 9058
	5 weeks	−/Y	95 ± 22			63140 ± 3570
Experiment 4	3 weeks	+/Y	36 ± 9			50360 ± 4227
	3 weeks	+/−	46 ± 20			42546 ± 1789
	3 weeks	−/Y	62 ± 22			85148 ± 8376

[a] Data are expressed as mean ± SD.
[^{3}H]thymidine uptake in thymocytes stimulated with PMA plus IL-4, PMA plus ionomycin (Experiments 1 and 2), or anti-CD3 plus anti-CD28 (Experiments 3 and 4). We used 2.5 × 10^4 cells/well in Experments 1 and 2, and 1 × 10^5 cells/well in Experiments 3 and 4. The animals are identified by age and genotype.

IL-7 receptor expression exhibit a more severe phenotype than is seen in the $\gamma_c^{-/Y}$ males, with thymic development being even more blunted with a relative block at the double-negative stage (Peschon et al., 1994). Although the basis for the more severe phenotype in IL-7R$^{-/-}$ mice is unclear, it may be important that a second stromal cell–derived cytokine, denoted thymic stromal-derived lymphopoietin (TSLP) (Peschon et al., 1994) shares a number of actions with IL-7. The TSLP receptor contains the IL-7R (Peschon et al., 1994), and hence both IL-7 and TSLP should be inactive in IL-7R$^{-/-}$ mice. However, it is not yet known whether γ_c is required for TSLP action. If not, then it is conceivable that TSLP (or other γ_c-independent cytokines) could be responsible for the T cell development and T cell proliferation seen in $\gamma_c^{-/Y}$ mice.

The $\gamma_c^{-/Y}$ mice exhibited a marked overall decrease in conventional B cells. In the spleen, while some animal-to-animal variation was found, all exhibited marked decreases in B cells. Moreover, there was consistently a marked decrease in B cells in peripheral blood and bone marrow. These findings are in contrast with the situation in humans (Conley, 1992; Leonard et al., 1994) and dogs (Felsburg and Somberg, 1992) with XSCID, where the B cell numbers are not diminished. The overall decrease in B cells in mice is perhaps explained by the apparently greater role for IL-7 in mice than in humans (discussed by Leonard, 1994), suggested by studies in which mice were treated with a monoclonal antibody (MAb) to IL-7 (Grabstein et al., 1993). IL-7R$^{-/-}$ mice also exhibit depressed levels of peripheral B cells (Peschon et al., 1994), although in these mice the decrease may result from the simultaneous functional inactivation of both IL-7 and TSLP. In any case, the greater severity of the B cell defect in $\gamma_c^{-/Y}$ mice than in humans with XSCID suggests that B cell development is more dependent on γ_c-dependent cytokines in mice than in humans. The defective production of conventional B

Table 3. Splenocyte Proliferation to LPS, IL-4 + Anti-μ, PMA + Ionomycin, and Anti-CD3 + Anti-CD28[a]

Experiments	Age	Genotype	RPMI	LPS	IL-4 + Anti-μ	PMA + Ionomycin	Anti-CD3 + Anti-CD28
Experiment 1	3 weeks	+/−	1114 ± 88	166045 ± 4288	19356 ± 1218		
	3 weeks	+/+	1041 ± 288	149823 ± 3860	28944 ± 1037		
	3 weeks	−/Y	680 ± 62	12009 ± 672	952 ± 99		
	3 weeks	−/Y	842 ± 61	5198 ± 105	817 ± 76		
Experiment 2	3 weeks	+/Y	390 ± 50	122527 ± 116	6405 ± 602		
	3 weeks	+/−	387 ± 57	124898 ± 4966	9288 ± 278		
	3 weeks	−/Y	120 ± 8	19711 ± 2748	189 ± 59		
Experiment 3	3 weeks	+/Y	1124 ± 302			120847 ± 5157	105579 ± 13398
	3 weeks	−/Y	809 ± 107			21436 ± 1535	7357 ± 2407
	3 weeks	−/Y	1698 ± 1109			23028 ± 551	5664 ± 341
	5 weeks	+/+	711 ± 125			98225 ± 5267	84345 ± 12290
	5 weeks	−/Y	613 ± 242			10890 ± 781	3801 ± 764
	6 weeks	−/Y	1124 ± 220			23989 ± 2140	6212 ± 1240

[a] Data are shown as mean ± SD.
[^{3}H]thymidine uptake in splenocytes stimulated with lipopolysaccharide, IL-4 plus anti-μ (Experiments 1 and 2) or with PMA plus ionomycin and anti-CD3 plus anti-CD28 (Experiment 3). Cells (10^5/well) were used in Experiments 1 and 3, and 5 × 10^4 cells/well were used in Experiment 2. The animals are identified by age and genotype.

cells in mice and the presence of peritoneal B-1 B cells lends support to the idea that these populations of B cells emerge from distinct precursors.

The complete absence of Thy-1$^+$ dendritic epidermal T cells was striking. T cells expressing γδ T cell receptors are the earliest appearing TCR$^+$ cells in thymic ontogeny (reviewed by Allison and Havran, 1991). Cells expressing γδ TCR appear at day 14, and until day 18 constitute the major class of TCR$^+$ cells in the thymus. Moreover, they are the dominant lymphoid population in certain epithelial tissues, including the skin (Allison and Havran, 1991). These DETCs are exclusively Vγ3–Vδ1 and, when cultured, are known to produce a variety of cytokines, including IL-1α, IL-2, IL-3, IL-6, IL-7, IFNγ, GM-CSF, tumor necrosis factor α, and tumor necrosis factor β (Bergstresser et al., 1993). IL-2 does not induce proliferation by itself, but it is an important cofactor for proliferation of these cells in vitro (Tschachler et al., 1989). Moreover, IL-2 transgenic mice exhibited an increased number of dendritic epidermal T cells (Ishida et al., 1989), and in utero treatment with a blocking MAb to murine IL-2Rβ completely abrogates their development (Tanaka et al., 1992), but not the development of αβ TCR$^+$ cells nor γδ TCR$^+$ cells in the intestinal epithelia (Tanaka et al., 1992). In this regard, it is interesting that IL-2Rβ expression is largely restricted to γδ cells in day 17 fetal thymus (Tanaka et al., 1992), including the precursors of those cells that target to the skin (Havran and Allison, 1990). This suggests that IL-2 or IL-15 (whose receptor also contains IL-2Rβ [Bamford et al., 1994; Giri et al., 1994]), or both, may be essential for the growth and/or migration of these cells. Furthermore, IL-7 is also an important growth factor for DETC (Matsue et al., 1993). The fact that IL-2, IL-7, and IL-15 signaling requires γ_c (Takeshita et al., 1992; Noguchi et al., 1993b; Kondo et al., 1994; Giri et al., 1994; reviewed by Leonard, 1994) therefore provides a possible explanation for the absence of γδ cells in $\gamma_c^{-/Y}$ mice. In this regard, it is noteworthy that in normal mice, γ_c mRNA is expressed in thymocytes as early as day 13.5 (the earliest point examined; C. Sommers et al., unpublished data), consistent with γ_c being present in the thymus at least as early as the first γδ cells. It is interesting that mice lacking expression of p56^{lck} exhibit a block in development of γδ cells in the thymus, but have normal levels of γδ IEL (Penninger et al., 1993), suggesting a more important role for γ_c than p56^{lck} in the development of IEL.

The fact that the heterozygous females exhibited normal lymphoid development and function was consistent with the normal phenotype of human heterozygous "carrier" females who exhibit strictly nonrandom X chromosome inactivation patterns in their mature T cells, NK cells, and B cells (Conley, 1992). Thus, the mature lymphocytes in heterozygous female mice may be cells in which the X chromosome with the mutated γ_c gene was inactivated, thereby resulting in normal levels of γ_c expression in the cells. However, formal X-inactivation studies in these animals could prove interesting in view of the extent of T cell development in $\gamma_c^{-/Y}$ mice, a finding consistent with the possibility that some murine T cell populations with a mutated γ_c have a greater chance for survival than do the corresponding cells in humans with XSCID.

These $\gamma_c^{-/Y}$ mice provide a valuable in vivo model not only for mapping functional domains of γ_c by establishing γ_c transgenic lines with specifically designed mutations, but also for evaluating gene therapy approaches that have worked in vitro (Qazilbash et al., in press). As compared with SCID mice, $\gamma_c^{-/Y}$ mice have greater T and B cell development. A striking difference, however, is their total absence of NK cells, whereas SCID mice have normal to enhanced NK activity. The absence of NK cells could make these mice extremely valuable as a model system for treating transplanted malignancies in a setting unencumbered by endogenous NK activity.

Experimental Procedures

Sequencing and Mapping of the Murine γ_c Gene

The murine γ_c gene was isolated from a 129 genomic library in λ-FIX (Stratagene). An 802 bp probe corresponding to the 5′ end of the murine γ_c cDNA (Cao et al., 1993) was prepared by PCR using primers corresponding to nucleotides 20–42 (5′-GCACCATGTTGAAACTATTATTG-3′, top strand) and 820–799 (5′-TAATCAACCCCATGGTGCCAAC-3′, bottom strand). One genomic clone (clone 8B) contained a 15 kb NotI fragment spanning the entire gene (see schematic in Figure 1). A 7 kb HindIII subfragment containing all eight exons was subcloned into pBluescript (plasmid denoted pBS-γ_c7.0), and a 5267 bp region spanning the putative promoter, eight exons, and seven introns was sequenced using Sequenase 2.0 (United States Biochemical Corporation).

Preparation of the Targeting Construct

The 2 kb BamHI fragment extending from the BamHI site just 3′ of exon 8 into the plasmid polylinker was excised from the pBSγ_c7.0 (see above section). This fragment was then cloned into pPNT (Tybulewicz et al., 1991) at the BamHI site immediately 3′ to the *neo* gene and 5′ to the *herpes simplex virus–thymidine kinase* (*hsv–tk*) gene. Both the *neo* and *tk* genes in this plasmid are under control of the PGK promoter. The resulting plasmid was denoted pPNTγ_c2.0. pBSγ_c7.0 was then digested with BamHI and EcoRV to liberate a 1.3 kb fragment extending from the BamHI site 5′ of exon 1 to the EcoRV site in exon 3 (see Figure 1). This fragment was cloned into pBluescript SK$^+$ between the BamHI and EcoRV sites (thereby generating the plasmid pBSγ_c1.3). The plasmid was linearized with EcoRV, NotI linkers added and digested with NotI, which cleaved in the linkers as well as in the NotI site 5′ in the polylinker. This NotI fragment was cloned in the NotI site of pPNTγ_c2.0 (upstream of the *neo* gene). The presence of this fragment then allowed sequential digestion with XhoI and NotI (prior to cloning a fragment into the NotI site, the XhoI and NotI are too closely positioned to be simultaneously digested), resulting in the removal of the NotI fragment just cloned, and the NotI to XhoI fragment from pBSγ_c1.3 was then cloned into this position to generate the final pPNTγ_c1.3/2.0 targeting construct that could be linearized with NotI.

ES cells and Transfections

J1 ES cells (passage 12) were propagated on mitomycin C–treated mouse embryonic fibroblasts in culture media supplemented with 1000 U/ml leukemia inhibitory factor (GIBCO BRL). ES cells were trypsinized and resuspended in electroporation buffer. The targeting construct, pPNTγ_c1.3/2.0 (25 μg) was linearized and transfected by electroporation into 1 × 10^7 cells in a .4 cm Gene Pulser (Biorad) cuvette at 400 V, 25 μF. Electroporated cells were plated at a a density of 2.5 × 10^5 cells/60 mm plate on *neo^r* fibroblasts obtained from TCRζ$^{+/-}$ embryos (Love et al., 1993). Positive–negative selection was started 24 hr after transfection using G418 (GIBCO, 350 μg/ml [dry weight]) and gancyclovir (2 μM). Transfection efficiency was estimated by selection in media containing G418 alone.

Identification of γ_c-Deficient ES Clones and Generation of γ_c-Deficient Mice

Surviving ES clones were expanded and frozen, DNA prepared (Laird et al., 1991), digested with HindIII, and subjected to Southern blotting using a probe corresponding to the 1.2 kb 5′ flanking HindIII to BamHI fragment from pBS-γ_c7.0 (Figure 1A, probe A). Filters were hybridized at 42°C with 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 200 μg/ml salmon sperm DNA, 0.1% SDS, 10% dextran sulfate, and 10^6 cpm/ml of probe. This probe hybridized to a 7 kb band in the normal gene and a 2.5 kb band (see Figure 1A) in the clones in which homologous recombination had occurred. Since ES cells are male and γ_c is located on the X chromosome, a single recombination event was sufficient to generate γ_c-deficient ES cells. Out of 341 clones screened, 3 γ_c-deficient clones were identified. Cells from these clones were injected into C57BL/6 blastocysts (15–20 cells/blastocyst), which were implanted into C57BL/6 pseudopregnant foster mothers. Assessed by coat color, 12 chimeric progeny (10 females and 2 males) were mutated to C57BL/6 mice; 1 chimeric female by germline transmission gave birth to a single $\gamma_c^{-/Y}$ male, and chimeric males fathered multiple heterozygous females, which in turn gave birth to additional $\gamma_c^{-/Y}$ males and heterozygous females. $\gamma_c^{-/Y}$ males and heterozygous females were detected by Southern blotting as described or by nested PCR using two sets of primers in which the top strand primer was located in γ_c and the bottom strand primer was located in the *neo* gene. First set, γ_c 5′-CATTCCAGGAGTGCAGTCACTATTTG-3′, *neo*, 5′-GCTGACAGCCGGAACACGGCGG-3′; second set, γ_c 5′-CTCCAA AGAGATTACTTCTGGCTGTC-3′, *neo*, 5′-GATTGTCTGTTGTGCCCAGTCAA GCC-3′. Genomic DNA (200 ng) was used as the template for the first PCR (94°C for 2 min, then five cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, then 30 cycles of 95°C for 1 min, 65°C for 1 min, 72°C for 2 min, then 72°C for 10 min prior to cooling to 4°C). For the second PCR reaction, 2 μl of the first PCR reaction was used as the template (94°C for 2 min, then five cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, then 20 cycles of 95°C for 30 s, 65°C for 30 s, 72°C for 45 s, then 72°C for 10 min prior to cooling to 4°C).

Northern Blotting and Western Blotting

Northern blotting was performed using 5 μg of total RNA by standard methods as previously described (Love et al., 1994). Western blotting was performed using lysates from 1.0×10^6 cells per lane and R878 antiserum to γ_c and enhanced chemiluminescence (Amersham). Although R878 was prepared using a peptide derived from the C terminus of human γ_c (Noguchi et al., 1993b; Russell et al., 1993), it also recognizes murine γ_c.

Pathology

Complete necropsy examinations were performed on three representative 3-week-old mice: a $\gamma_c^{-/Y}$ male, a wild-type male, and a heterozygous female. Tissues were fixed in 10% neutral buffered formalin, processed through alcohols and xylene, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Gut sections were also stained with the Steiner modification of the Warthin–Starry stain for H. hepaticus.

Immunoperoxidase Staining of Frozen Sections

Intestines were dissected, placed in OTC freezing medium (Miles, Elkhart, Indiana) and frozen on dry ice. Sections (10 μm) were placed on silanated slides, dried overnight, and fixed for 15 min in cold acetone. Slides were stored with desiccant at −80°C. All incubations were carried out at room temperature. For immunoperoxidase staining, tissue sections were rehydrated in phosphate-buffered saline (PBS) and nonspecific binding sites blocked by incubating for 30 min in PBS with 2% normal mouse serum and 3% normal goat or rat serum. The blocking solution was removed and the tissue sections were incubated for 60 minutes with hamster anti-mouse δ chain (GL-3, PharMingen) or control MAbs in PBS with 2% goat serum, washed in PBS, and then incubated for 60 min with biotinylated goat anti-hamster IgG (Vector Labs). Alternatively, they were stained with biotinylated rat anti-mouse CD4 (RM4–4, PharMingen). The sections were washed three times for 30 min in PBS, incubated for 30 min with strepatavidin–horseradish peroxidase (Zymed Laboratories), washed six times for 30 min in PBS, and incubated with 0.5% (w/vol) of 3,3 diaminobenzidine (Sigma), 0.05% NaCl, and 0.03% H_2O_2. Sections were rinsed in PBS, counterstained with 5% methyl green (Fisher Scientific) in methanol, rinsed in distilled water, air dried, and mounted with Permount (Fisher Scientific).

Assessment of DETC and Langerhans Cell Densities in Epidermal Sheet Preparations

Ears were removed from 3-, 5-, and 7-week-old mice and dorsal and ventral sides were separated with forceps. Each side was incubated at 37°C in 0.1 M NH_4SCN for 20 min, after which the epidermal sheets were separated from the dermis. The sheets were washed in PBS, fixed in acetone for 30 min at −20°C, and washed again in PBS prior to staining. Sheets were then incubated in a moist chamber at 37°C for 2 hr with one of the following fluorescein isothiocyanate (FITC)-conjugated reagents: hamster anti-γδ TCR, rat anti-Thy 1.2, or their respective isotype controls at 10 μg/ml (PharMingen, San Diego, California) or Y3P (mouse anti-I-A^b) or MK-D6 (mouse anti-I-A^d) at 15 μg/ml (American Type Culture Collection, Rockville, Maryland). The sheets were then washed and mounted on slides and viewed using fluorescence microscopy.

Flow Cytometric Analyses

Cells from thymus, spleen, bone marrow, and PBMC were stained primarily with directly conjugated antibodies and analyzed on a FACScan (Becton Dickinson) using Lysis II software. Data on 5–50 × 10^3 viable cells (as determined by forward versus side scatter) were collected for each sample. For multicolor analyses, cells were first incubated with 2.4G2 MAb to the Fc receptor (Unkeless, 1979) to prevent nonspecific binding. For two- and three-color analyses, cells were sequentially incubated with FITC-conjugated MAb, phycoerythrin (PE)-conjugated MAb, and biotinylated MAbs, and then with Red 670 streptavidin (GIBCO BRL). For two-color analyses, the numbers indicate the percent of cells in each quadrant. For one-color analyses, the shaded regions represent staining with the control antibody. The control antibodies included mouse IgG2a conjugated to FITC or PE (PharMingen, San Diego, California), or Leu-4 conjugated to biotin (Becton Dickinson). For direct staining, the following conjugated antibodies were purchased from PharMingen (San Diego, California): 53-6.7–FITC (anti-CD8), 500.A2–FITC (anti-CD3ε), Gr-1 (specific for granulocyte and monocytes), M1/70 (MAC-1, anti-CD11b), H1.2F3–FITC (anti-CD69), 7D4–FITC (anti-IL-2Rα), PK136–PE (anti-NK1.1), RM4-5–PE (anti-CD4), H57-597–PE (anti-TCRβ), 37.51–PE (anti-CD28), 53-7.3–PE (anti-CD5), M1/69–PE (anti-heat-stable antigen). In additon, Fg.1 (anti-TCRVβ8) was purified and conjugated to FITC in our laboratory. For indirect staining, cells were reacted with unconjugated TUGm3 (provided by Dr. K. Sugamura) or TMβ1 (provided by Drs. T. Tanaka and M. Miyasaka), washed, and stained with goat anti-rat–PE (Southern Biotechnology Associates, Birmingham, Alabama).

Cytotoxicity Assay

Cytotoxicity assays was performed as described (Bloom and Horvath, 1994) against YAC-1 target cells, using doubling dilutions of effector cells beginning with an effector:target cell ratio of 100:1 for unstimulated cells, and a 50:1 for cells stimulated at 2 × 10^6/ml for 44 hr with 1000 U/ml human recombinant IL-2 (Amgen, Thousand Oaks, California). A lytic unit (LU) is the number of effector cells lysing 20% of the target cells. Data are expressed as LU in 10^7 effector cells ± asymptotic SEM (Bloom and Korn, 1983).

Serum Immunoglobulin Levels

Serum IgM, IgG1, IgG2a, IgG2b, IgG3, IgA, and IgE were measured by enzyme-linked immunosorbent assay. Goat anti-mouse coating antibodies and alkaline phosphatase–conjugated second antibodies for IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA were from Southern Biotechnology Associates. Biotinylated second antibodies for IgE were from PharMingen, respectively. Horseradish peroxidase avidin (Vector) and ABTS peroxidase (Kirkegaard and Perry Laboratories) were used to develop the IgE assays. 4–methylumbelliferyl phosphate (2×10^{-4} M) in MUP buffer (0.05 M $NaHCO_3$, 1 mM $MgCl_2$, 01.% NaN_3) was used to develop IgM, IgG1, IgG2a, IgG2b, and IgG3.

Cytokine Production and Cellular Proliferation

For cytokine production, wells of 96-well flat-bottomed plates were coated with anti-CD3 (2C11, 10 μg/ml) overnight at 4°C in PBS, washed, and anti-CD28 ascites (1:500 dilution) added along with thymocytes or unfractionated splenocytes (1–2 × 10^5 cells/well). IFNγ was measured on 48 hr supernatants and IL-2 and IL-4 on 24 hr supernatants. IL-2 and IL-4 were measured by bioassay using the EV clone of CTLL-2 cells and CT4S cells, respectively, using 5000 cells/well and overnight [^{3}H]thymidine pulses in 48 hr assays. IFNγ was measured by enzyme-linked immunosorbent assay. Wells were coated with antibodies to IFNγ (XMG cell line, PharMingen), and developed using a rabbit antiserum to IFNγ (provided by C. Kinzer, National Institute of Allergy and Infectious Diseases), goat anti-rabbit horseradish peroxidase (Southern Biotechnical Associates) and H_2O_2 and PT peroxidase buffer (Kirkegaard and Perry Laboratories). Cytokine levels in control and $\gamma_c^{-/Y}$ mice were expressed as mean ± SEM and values compared using the Student's t test.

For [^{3}H]thymidine incorporation studies in thymocytes, in some experiments, cells were cultured either at 2.5 × 10^4 cells/well in a 96-well flat-bottomed plate and stimulated with RPMI, PMA (10 ng/ml) plus IL-4 (1000 U/ml), or PMA plus ionomycin (1 μM). Alternatively, 10^5 cells/well were stimulated with plate-bound anti-CD3 plus soluble anti-CD28 as described above. Cells were pulsed with [^{3}H]thymidine for the final 12 hr of a 48 hr assay. For [^{3}H]thymidine incorporation studies in splenocytes, in some experiments either 5 × 10^4 or 10^5 cells/well were stimulated with lipopolysaccharide (20 μg/ml) or IL-4 plus anti-μ (5 μg/ml). Alternatively, 10^5 cells/well were stimulated with RPMI, PMA plus ionomycin, or anti-CD3 plus anti-CD28 antibodies, and pulsed with [^{3}H]thymidine as described above.

Mast Cell Assay

Wells were coated with IgE (10 μg/ml) for 2 hr at 37°C in PBS, and 2 × 10^5 unfractionated splenocytes/well added in 10% WEHI 3B conditioned medium and IL-3 (100 ng/ml). IL-4 (U/ml) was measured at 24 hr.

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