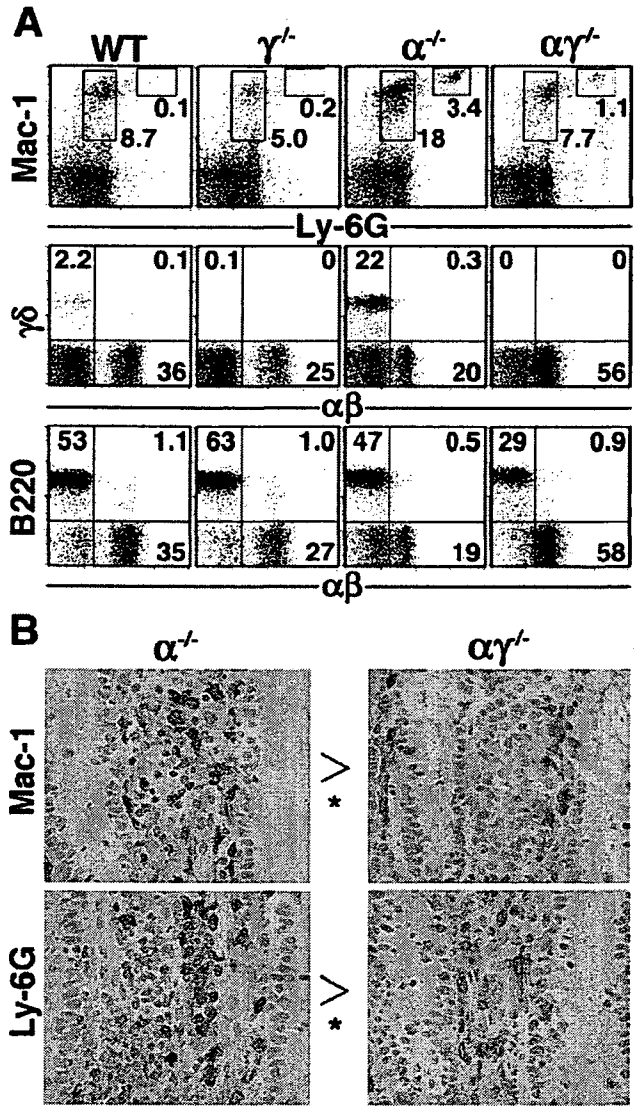

Figure 4. Decrease in colonic Mac-1$^+$Ly-6G$^-$ and Mac-1$^+$Ly-6G$^+$ cells in the absence of γδ T cells. WT (n = 3), $\gamma^{-/-}$ (n = 3), $\alpha^{-/-}$ (n = 4), and $\alpha\gamma^{-/-}$ (n = 4) mice from 28 weeks of age were examined. (*A*) Flow cytometric profiles of colonic LP cells. Absolute numbers of LP cells isolated from these WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ individuals were 5.1 × 10^5, 6.1 × 10^5, 32.3 × 10^5 and 12.0 × 10^5, respectively. (*B*) Representative immunohistochemical verification of the prominent infiltrations of Mac-1$^+$ and Ly-6G$^+$ cells into the inflamed colonic LP of $\alpha^{-/-}$ mice. Five tissue sections prepared from ascending colon to rectum per mouse, namely, 20 sections obtained from inflamed large intestines of $\alpha^{-/-}$ mice and those obtained from inflamed large intestines of $\alpha\gamma^{-/-}$ mice, were examined in a blinded fashion by 5 independent investigators, and the statistical difference in absolute numbers of Mac-1$^+$ and Ly-6G$^+$ cells between large intestinal mucosa from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice were determined by 2-sided Mann–Whitney *U* test. *$P < .05$.

RT-PCR analysis and measurement of the amounts of representative proinflammatory cytokines as well as chemokines were performed to dissect further the role of γδ T cells in the UC-like colitis in $\alpha^{-/-}$ mice.

To this end, messenger RNA (mRNA) and proteins prepared from the large intestines of WT, $\gamma^{-/-}$, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice were examined. Inflamed colonic tissues from $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice contained at least 10 times higher levels of cytokine (Table 1)- and chemokine (Table 2)-specific mRNA than those of WT and $\gamma^{-/-}$ mice except for IL-7 and IL-10 mRNA. In contrast to the mRNA from colonic tissues of $\alpha^{-/-}$ mice, those of $\alpha\gamma^{-/-}$ mice contained significantly smaller amounts of cytokine (TNF-α, IL-1β, IL-6, and TGF-β)- and chemokine (KC and MIP-2)-specific mRNA. With these observations in mind, we measured the amounts of representative cytokines as well as chemokines that had exhibited the differences in mRNA levels between the colonic tissues of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice. First, in situ production of TNF-α, IL-1β, and IL-6 but not TGF-β proteins was significantly downregulated in the inflamed colonic mucosa of $\alpha\gamma^{-/-}$ mice as compared with that of $\alpha^{-/-}$ mice (Table 1). Second, KC and MIP-2 chemokines that are involved in the chemoattract of neutrophils and/or monocytes[23] were significantly decreased in large intestines of $\alpha\gamma^{-/-}$ mice compared with those in large intestines of $\alpha^{-/-}$ mice (Table 2).

To investigate the cell types responsible for the increases in these proinflammatory cytokines and chemokines, real-time RT-PCR analysis of mRNA present in the purified cell subsets from the inflamed colonic LP of $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice was performed (see Supplementary Figure 1 online at www.gastrojournal.org). The IL-1β and MIP-2 mRNA were expressed preferentially by Gr-1$^+$ cells, F4/80$^+$ cells, and CD11c$^+$ cells in the colon, whereas IL-6 mRNA was mainly expressed by Gr-1$^-$F4/80$^-$CD11c$^-$ cell populations. Expression levels of TNF-α- and KC-specific mRNA were comparable between all cell populations (Gr-1$^+$ cells, F4/80$^+$ cells, CD11c$^+$ cells, and Gr-1$^-$F4/80$^-$CD11c$^-$ cells) examined. Finally, the expression levels of these cytokine- and chemokine-specific mRNA in every cell subset were lower in cells from $\alpha\gamma^{-/-}$ mice than those in cells from $\alpha^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org).

Discussion

The $\alpha^{-/-}$ mice spontaneously develop colitis that shares many features with human UC.[16,24] Commensal enteric flora is required for the development of this colitis as indicated by the absence of colitis in $\alpha^{-/-}$ mice that are maintained under germ-free conditions.[22,25] The number of colonic γδ T cells drastically decreases in the $\alpha^{-/-}$ mice under germ-free conditions.[22] However, the study to identify the role of γδ T cells in the UC-like chronic colitis in $\alpha^{-/-}$ mice has been hampered by the difficulty in generating TCRαδ double mutant ($\alpha\delta^{-/-}$) mice because of the genomic organization of these TCR genes.[20] In the present study, we overcame this problem by newly generating $\gamma^{-/-}$ mice and subsequently crossing these mice with $\alpha^{-/-}$ mice to generate TCRα double mutant mice that lacked γδ T cells. By using these $\alpha\gamma^{-/-}$ mice, we herein provide a novel insight into the role of γδ T cells

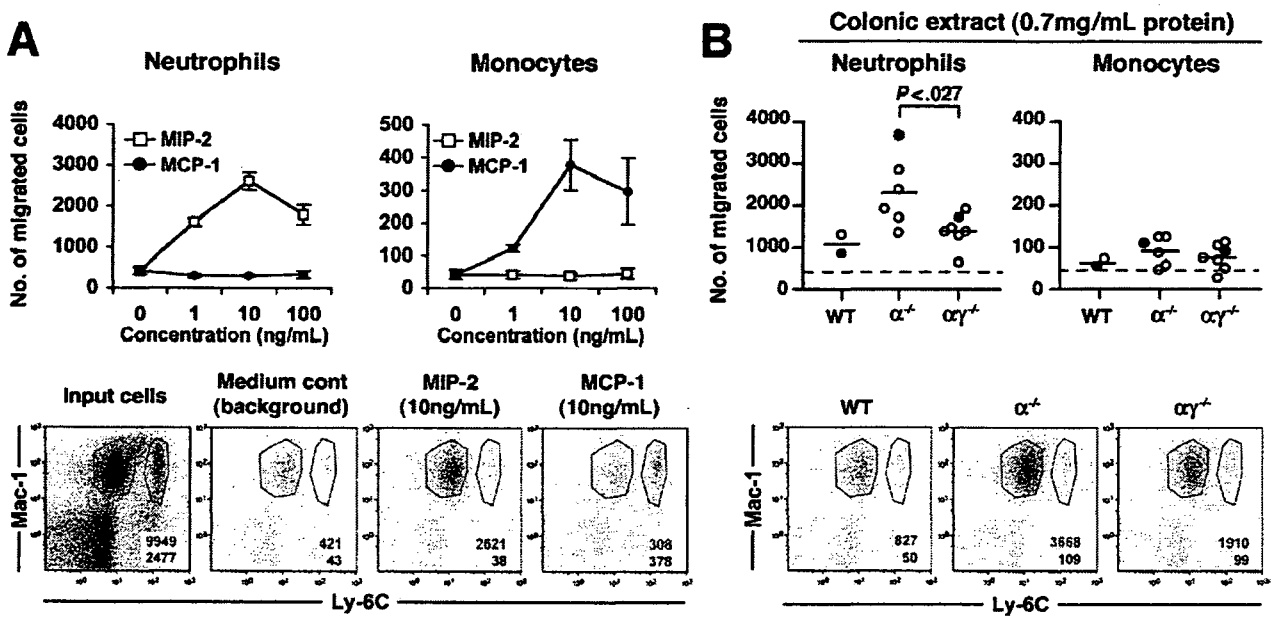

Figure 5. Chemotactic activity of colonic extracts from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice to neutrophils and monocytes. (*A*) The number of neutrophils and monocytes migrated in response to the increasing concentration of MIP-2 (*open square*) and MCP-1 (*solid circle*). The representative flow cytometric profiles are shown in the *lower panels*, and *red* and *blue gates* indicate the neutrophils and monocytes, respectively. *Colored numbers* represent means of the number of cells in each gate. (*B*) Chemotactic responses of neutophils and monocytes to colonic extracts (0.7 mg/mL protein) from WT, $\alpha^{-/-}$, and $\alpha\gamma^{-/-}$ mice, and each *circle* represents an individual mouse. *Horizontal bars* show mean values, and *dotted lines* indicate the number of migrated cells in medium alone. The representative flow cytomeric profiles of 3 individual animals indicated by the *solid red* and *blue circles* (*upper panels*) are shown in the *lower panels*, and *red* and *blue gates* indicate the neutrophils and monocytes, respectively.

that contributes to the exacerbation of UC-like colitis in $\alpha^{-/-}$ mice.

There is growing evidence supporting the fact that γδ T cells are part of the innate immune system and play an active multifaceted immunoregulatory role in the coordinated innate and acquired immune responses that maintain the integrity of many organs containing epithelia.[1,5,26,27] Nevertheless, the details of γδ T-cell functions are still not well understood as compared with those of αβ T cells. γδ T cells might play a defensive role against infections by various pathogenic microorganisms because exaggerated and severe infectious diseases occur in $\delta^{-/-}$ mice.[28–33] However, the same $\delta^{-/-}$ mice have also been demonstrated to display an increased host resistance to infection.[34,35] With regard to this, it is noteworthy that $V\gamma1^{+}$ γδ T cells are reported to eliminate the

Table 1. Real-Time RT-PCR Analysis and ELISA Assay of Cytokines in the Colonic Tissues

	Cytokine							
Mice (n)	TNF-α	IL-1β	IL-6	TGF-β	IFN-γ	IL-7	IL-10	IL-12
RT-PCR (copies per 10^3 HPRT)								
WT (5)	11.4 ± 0.53	6.86 ± 0.55	2.45 ± 0.64	123 ± 14.6	ND	11.7 ± 1.03	2.63 ± 0.28	0.82 ± 0.21
$\gamma^{-/-}$ (5)	9.20 ± 0.76	6.01 ± 0.61	2.14 ± 0.88	106 ± 10.7	ND	10.2 ± 0.78	2.09 ± 0.31	0.33 ± 0.09
$\alpha^{-/-}$ (7)	457 ± 35.7**	260 ± 18.6***	7.06 ± 1.04*	538 ± 48.6*	64.4 ± 8.90	11.0 ± 0.89	4.99 ± 0.72	9.58 ± 1.67
$\alpha\gamma^{-/-}$ (6)	205 ± 51.8**	98.5 ± 21.4***	2.93 ± 0.73*	319 ± 57.4*	45.0 ± 15.6	9.29 ± 1.11	6.42 ± 2.11	6.63 ± 1.01
ELISA (pg/mg protein)								
$\alpha^{-/-}$ (7)	100 ± 12.2**	975 ± 70.1**	8.24 ± 1.39**	2.09 ± 0.11	8.34 ± 1.50			
$\alpha\gamma^{-/-}$ (6)	27.0 ± 8.28**	647 ± 24.6**	2.92 ± 0.52**	2.57 ± 0.31	5.92 ± 2.56			

NOTE. All results are expressed as mean ± SE.
*$P < .05$.
**$P < .01$.
***$P < .001$.
ND, not detected

Table 2. Real-Time RT-PCR Analysis and ELISA Assay of Chemokines in the Colonic Tissues

	Chemokine					
Mice (n)	KC	MIP-2	GCP-2	MCP-1	MIP-1α	MIP-1β
RT-PCR (copies per 10^3 HPRT)						
WT (5)	6.05 ± 1.38	0.23 ± 0.01	12.3 ± 4.11	1.67 ± 0.17	0.78 ± 0.07	1.36 ± 0.22
$\gamma^{-/-}$ (5)	11.8 ± 1.19	0.22 ± 0.03	4.03 ± 1.49	1.77 ± 0.26	0.80 ± 0.04	1.42 ± 0.11
$\alpha^{-/-}$ (7)	382 ± 88.6*	101 ± 12.8**	721 ± 136	23.3 ± 4.37	35.0 ± 3.01	19.8 ± 0.88
$\alpha\gamma^{-/-}$ (6)	65.2 ± 17.4*	28.0 ± 9.80**	307 ± 168	16.0 ± 4.11	23.9 ± 7.34	14.2 ± 3.97
ELISA (pg/mg protein)						
$\alpha^{-/-}$ (7)	144 ± 22.4**	113 ± 21.2*	737 ± 139			
$\alpha\gamma^{-/-}$ (6)	32.4 ± 13.4**	44.9 ± 14.0*	414 ± 219			

NOTE. All results are expressed as mean ± SE.
*$P < .05$.
**$P < .01$.

macrophages infected with *Listeria monocytogenes*, whereas γδ T cells using Vγ elements other than Vγ1 gene appear to lack the ability to control macrophages but possess the ability to protect hosts from the infection-induced tissue injury.[36,37] In contrast to the beneficial function of γδ T cells by virtue of the fact that they can maintain the homeostasis of different types of organs,[1-5,8,27] a deleterious effect of γδ T cells on the regulation of neutrophil-mediated tissue damage after thermal (postburn) injury has been reported.[38] In various chronic and/or autoimmune inflammatory diseases, such as collagen-induced arthritis in mice[39] and murine insulin-dependent diabetes,[40] γδ T cells have been shown to exert a protective effect. Conversely, γδ T cells may directly contribute to autoimmune pathology of murine experimental allergic encephalomyelitis[41] as well as lupus in MRL/lpr mice.[42] Overall, both the beneficial and detrimental roles of γδ T cells in inflammatory process are evident.[43]

In chemically induced acute intestinal inflammation models (2,4,6-trinitrobenzene sulfonic acid- or dextran sulfate sodium-induced colitis), γδ T cells have been reported to play a protective role.[11-14] Depletion of γδ T cells by administration of anti-TCRγδ mAb into $TNF^{\Delta ARE}$ mice with a high frequency of spontaneous ileitis[44] did not lead to any histologic changes of ileitis.[45] However, transfer of bone marrow-derived γδ T cells has been shown to induce CD-like colitis in the bone marrow transplanted CD3εtg colitis model.[46] Although the role of γδ T cells in spontaneous chronic colitis remains to be explored to date, the results of the present study demonstrate the exacerbating effect of γδ T cells on the UC-like chronic colitis in $\alpha^{-/-}$ mice (Figures 3 and 4 and Tables 1 and 2). Interestingly, approximately 20% of $\alpha^{-/-}$ mice during 20 to 60 weeks of age suffered from ARP, whereas none of age-matched $\alpha\gamma^{-/-}$ mice showed ARP (Figure 3*D*). Of note, there were no differences in the age of onset of colitis and in the incidence of colitis (~80%) among 20- to 32-week-old $\alpha^{-/-}$ and $\alpha\gamma^{-/-}$ mice, but much more severe colitis was observed in $\alpha^{-/-}$ mice as compared with $\alpha\gamma^{-/-}$ mice. Therefore, it is possible that ARP may reflect increased severity of colitis and that γδ T cells may participate in the development of ARP.

Absence of γδ T cells in $\alpha\gamma^{-/-}$ mice leads to a significantly reduced production of TNF-α, IL-1β, and IL-6 proteins in the colonic tissues. These findings are consistent with our previous results[24] showing the involvement of TNF-α, IL-1β, and IL-6 in the perpetuation of inflammatory process in $\alpha^{-/-}$ mice. These inflammatory mediators have been shown to be important for host defense and wound repair.[47] Both KC and MIP-2 attract neutrophils to inflamed sites, and, in certain microbial infection, the collection of neutrophils leads to suppuration reflecting an active and vigorous host response against microbes. We also confirmed that colonic extracts from $\alpha\gamma^{-/-}$ mice exhibited the significantly weaker chemotactic activity to neutrophils than those from $\alpha^{-/-}$ mice. KC and MIP-2 mRNA expressions were lower in all purified cell subsets ($Gr\text{-}1^+$, $F4/80^+$, $CD11c^+$, and $Gr\text{-}1^-F4/80^-CD11c^-$ cells) from $\alpha\gamma^{-/-}$ mice than those from $\alpha^{-/-}$ mice. Therefore, in the presence of γδ T cells, many types of immune cells may be triggered to produce more chemokines, followed by infiltration of neutrophils into the colonic mucosa in $\alpha^{-/-}$ mice. γδ T-cell responsiveness that is manifested by recruitment and activation of inflammatory cells in which neutrophils predominate has also been reported.[1,31] In this context, it is of importance to note that the activity and severity of UC patients with increase in γδ T cells in the inflamed mucosa[9,10] (Figure 1) can be judged by the activation state of neutrophils in circulation[48] as well as by regional accumulation of neutrophils in the colonic crypt walls (cryptitis) or in the lumen of crypts (crypt abscess).[49]

The suppressive role of B cells[50] and the aggravating role of $TCR\beta^{dim}$ T cells[22,51] in the pathogenesis of colitis in $\alpha^{-/-}$ mice have been reported. Therefore, it is possible that γδ T cells may contribute to the exacerbation of this colitis by dampening regulatory B-cell function or by cooperating the colitogenic $TCR\beta^{dim}$ T cells. The possible complicated mechanism remains to be explored in the future. Levels of TNF-α and IL-1β mRNA in $F4/80^+$ cells

are higher in $\alpha^{-/-}$ mice compared with $\alpha\gamma^{-/-}$ mice (see Supplementary Figure 1 online at www.gastrojournal.org), suggesting that γδ T cells may activate macrophages to secrete large amounts of proinflammatory cytokines.

In conclusion, although γδ T cells at the inflamed colonic LP of $\alpha^{-/-}$ mice may protect intestinal epithelial injury, proinflammatory cytokines and neutrophil- and/or monocyte-chemoattractant chemokines induced by γδ T cells may directly and/or indirectly contribute to increased severity of UC-like chronic colitis in $\alpha^{-/-}$ mice. Further understanding of the molecular mechanisms of γδ T cell-mediated exacerbation of colitis in $\alpha^{-/-}$ mice will lead us to work out better therapeutic strategies for human UC.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2007.11.056.

References

1. Hayday A, Tigelaar R. Immunoregulation in the tissues by γδ T cells. Nat Rev Immunol 2003;13:233–242.
2. Jameson J, Ugarte K, Chen N, et al. A role for skin γδ T cells in wound repair. Science 2002;296:747–749.
3. Boismenu R, Havran WL. Modulation of epithelial cell growth by intraepithelial γδ T cells. Science 1994;266:1253–1255.
4. Komano H, Fijiura Y, Kawaguchi M, et al. Homeostatic regulation of intestinal epithelia by intraepithelial γδ T cells. Proc Natl Acad Sci USA 1995;92:6147–6151.
5. Mak TW, Ferrick DA. The γδ T-cell bridge: linking innate and acquired immunity. Nat Med 1998;4:764–765.
6. Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002; 347:417–429.
7. Targan SR, Karp LC. Defects in mucosal immunity leading to ulcerative colitis. Immunol Rev 2005;206:296–305.
8. Shiohara T, Moriya N, Hayakawa J, et al. Resistance to cutaneous graft vs host disease is not induced in T-cell receptor δ gene-mutant mice. J Exp Med 1996;183:1483–1489.
9. McVay LD, Li B, Biancaniello R, et al. Changes in human mucosal γδ T cell repertoire and function associated with the disease process in inflammatory bowel disease. Mol Med 1997;3:183–203.
10. Yeung MM-W, Melgar S, Baranov V, et al. Characterization of mucosal lymphoid aggregates in ulcerative colitis: immune cell phenotype and TCR-γδ expression. Gut 2000;47:215–227.
11. Hoffmann JC, Peters K, Henschke S, et al. Role of T lymphocytes in rat 2,4,6-trinitrobenzene sulphonic acid (TNBS) induced colitis: increased mortality after γδ T cell depletion and no effect of αβ T cell depletion. Gut 2001;48:489–495.
12. Inagaki-Ohara K, Chinen T, Matsuzaki G, et al. Mucosal T cells bearing TCR-γδ play a protective role in intestinal inflammation. J Immunol 2004;173:1390–1398.
13. Chen Y, Chou K, Fuchs E, et al. Protection of the intestinal mucosa by intraepithelial γδ T cells. Proc Natl Acad Sci U S A 2002;99:14338–14343.
14. Tsuchiya T, Fukuda S, Hamada H, et al. Role of γδ T cells in the inflammatory response of experimental colitis mice. J Immunol 2003;171:5507–5513.
15. Mizoguchi A, Mizoguchi E, Chiba C, et al. Cytokine imbalance and autoantibody production in T cell receptor-α mutant mice with inflammatory bowel disease. J Exp Med 1996;183:847–856.
16. Mombaerts P, Mizoguchi E, Grusby MJ, et al. Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. Cell 1993;75:274–282.
17. Hokama A, Mizoguchi E, Sugimoto K, et al. Induced reactivity of intestinal $CD4^+$ T cells with an epithelial cell lectin, galectin-4, contributes to exacerbation of intestinal inflammation. Immunity 2004;20:681–693.
18. de Bruijn MF, van Vianen W, Ploemacher RE, et al. Bone marrow cellular composition in *Listeria monocytogenes* infected mice detected using ER-MP12 and ER-MP20 antibodies: a flow cytometric alternative to different counting. J Immunol Methods 1998;217:27–39.
19. Itohara S, Mombaerts P, Lafaille J, et al. T cell receptor δ gene mutant mice: independent generation of αβ T cells and programmed rearrangements of γδ TCR genes. Cell 1993;72:337–348.
20. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395–402.
21. Goren I, Kampfer H, Muller E, et al. Oncostatin M expression is functionally connected to neutrophils in the early inflammation phase of skin repair: implications for normal and diabetes-impaired wounds. J Invest Dermatol 2006;126:628–637.
22. Kawaguchi-Miyashita M, Shimada S, Kurosu H, et al. An accessory role of $TCR\gamma\delta^+$ cells in the exacerbation of inflammatory bowel disease in TCRα mutant mice. Eur J Immunol 2001;31: 980–988.
23. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med 2006;354: 610–621.
24. Mizoguchi A, Mizoguchi E, Bhan AK. Immune networks in animal models of inflammatory bowel disease. Inflamm Bowel Dis 2003; 9:246–259.
25. Dianda L, Hanby AM, Wright NA, et al. T cell receptor-αβ-deficient mice fail to develop colitis in the absence of a microbial environment. Am J Pathol 1997;150:91–97.
26. Tonegawa S, Berns A, Bonneville M, et al. Diversity, development, and probable functions of γδ T cells. Cold Spring Harbor Symp Quant Biol 1989;54:31–44.
27. Havran WL. A role for epithelial γδ T cells in tissue repair. Immunol Res 2000;21:63–69.
28. Mombaerts P, Arnoldi J, Russ F, et al. Different roles of αβ and γδ T cells in immunity against an intracellular bacterial pathogen. Nature 1993;365:53–56.
29. Roberts SJ, Smith AL, Wet AB, et al. T-cell $\alpha\beta^+$ and $\gamma\delta^+$ deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. Proc Natl Acad Sci U S A 1996;93:11774–11779.
30. D'Souza CD, Cooper AM, Frank AA, et al. An anti-inflammatory role for γδ T lymphocytes in acquired immunity to *Mycobacterium tuberculosis*. J Immunol 1997;158:1217–1221.
31. King DP, Hyde DM, Jackson KA, et al. Cutting edge: protective response to pulmonary injury requires γδ lymphocytes. J Immunol 1999;162:5033–5036.
32. Moore TA, Moore BB, Newstead NW, et al. γδ-T cells are critical for survival and early proinflammatory cytokine gene expression during murine *Klebsiella* pneumonia. J Immunol 2000;165: 2643–2650.
33. Selin LK, Santolucito PA, Pinto AK, et al. Innate immunity to viruses: control of vaccinia virus infection by γδ T cells. J Immunol 2001;166:6784–6794.
34. Emoto M, Nishimura H, Sakai T, et al. Mice deficient in γδ T cells are resistant to lethal infection with *Salmonella choleraesuis*. Infect Immun 1995;63:3736–3738.

35. Uezu K, Kawakami K, Miyagi K, et al. Accumulation of γδ T cells in the lungs and their regulatory roles in Th1 response and host defense against pulmonary infection with *Cryptococcus neoformans*. J Immunol 2004;172:7629–7634.
36. Andrew EM, Newton DJ, Dalton JE, et al. Delineation of the function of a major γδ T cell subset during infection. J Immunol 2005;175:1741–1750.
37. Newton DJ, Andrew EM, Dalton JE, et al. Identification of novel γδ T-cell subsets following bacterial infection in the absence of $V\gamma1^+$ T cells: homeostatic control of γδ T-cell responses to pathogen infection by $V\gamma1^+$ T cells. Infect Immun 2006;74: 1097–1105.
38. Toth B, Alexander M, Daniel T, et al. The role of γδ T cells in the regulation of neutrophil-mediated tissue damage after thermal injury. J Leukoc Biol 2004;76:545–552.
39. Peterman GM, Spencer C, Sperling AI, et al. Role of γδ T cells in murine collagen-induced arthritis. J Immunol 1993;151:6546–6558.
40. Harrison LC, Dempsey-Collier M, Kramer DR, et al. Aerosol insulin induces regulatory CD8 γδ T cells that prevent murine insulin-dependent diabetes. J Exp Med 1996;184:2167–2174.
41. Rajan AJ, Gao Y-L, Raine CS, et al. A pathogenic role of γδ T cells in relapsing-remitting experimental allergic encephalomyelitis in the SJL mouse. J Immunol 1996;157:941–949.
42. Peng SL, Madaio MP, Highes DP, et al. Murine lupus in the absence of αβ T cells. J Immunol 1996;156:4041–4049.
43. Hayday A, Geng L. γδ cells regulate autoimmunity. Curr Opin Immunol 1997;9:884–889.
44. Kontoyiannis D, Pasparakis M, Piazzoro TT, et al. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity 1999;10:387–398.
45. Kuhl AA, Loddenkemper C, Westermann J, et al. Role of γδ T cells in inflammatory bowel disease. Pathobiology 2002;70: 150–155.
46. Simpson SJ, Hollander GA, Mizoguchi E, et al. Expression of pro-inflammatory cytokines by $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ T cells in an experimental model of colitis. Eur J Immunol 1997;27: 17–25.
47. Nathan C. Points of control in inflammation. Nature 2002;420: 846–852.
48. Suematsu M, Suzuki M, Kitahora T, et al. Increased respiratory burst of leukocytes in inflammatory bowel diseases: the analysis of free radical generation by using chemiluminescence probe. J Clin Lab Immunol 1987;24:125–128.
49. Simmonds NJ, Allen RE, Stevens TR, et al. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. Gastroenterology 1992;103:186–196.
50. Sugimoto K, Ogawa A, Shimomura Y, et al. Inducible IL-12-producing B cells regulate Th2-mediated intestinal inflammation. Gastroenterology 2007;133:124–136.
51. Takahashi I, Kiyono H, Hamada S. $CD4^+$ T-cell population mediates development of inflammatory bowel disease in T-cell receptor α chain-deficient mice. Gastroenterology 1997;112:1876–1886.

Received April 11, 2007. Accepted November 15, 2007.

Address requests for reprints to: Hiromichi Ishikawa, MD, PhD, Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. e-mail: h-ishika@sc.itc.keio.ac.jp; fax: (81) 3-5360-1508.

Supported in part by a Grant-in-Aid for Creative Scientific Research, the Japan Society for the Promotion of Science (13GS0015); by Special Coordination Funds for Promoting Science and Technology from the Japanese Ministry of Education, Culture, Sports, Science, and Technology; and by Research on Specific Diseases, Japanese Ministry of Health, Labor, and Welfare (to H.I.); by Keio University Special Grant-in-Aid for Innovative Collaborative Research Projects (to T.H.); by National Institutes of Health grants DK47677 (to A.K.B.) and DK064351 (to A.M.); by the Center for the Study of Inflammatory Bowel Disease, Massachusetts General Hospital; and by 21st Century Center-of-Excellence (COE) Program for Life Science from MEXT (to M.S.).

The authors thank Dr Daniel K. Podolsky for his many valuable discussions and comments, Dr Leo Lefrancois for his critical reading of the manuscript, and Dr Atsuhiro Ogawa for his excellent technical assistance.

M.N. and Y.K. contributed equally to this work.

Y.K.'s current location is Department of Immunology, Kinki University School of Medicine, Osaka, Japan.

H.Y.'s current location is Health Research Foundation, Kyoto, Japan.

Conflicts of interest: No conflicts of interest exist.

T.N. was a research fellow supported by 21st Century COE Program for Life Science from MEXT and is now supported by Global COE Program for Human Metabolomic Systems Biology from MEXT (to M.S.).

Hiromichi Ishikawa
Tomoaki Naito
Toshihiko Iwanaga
Hiromi Takahashi-Iwanaga
Makoto Suematsu
Toshifumi Hibi
Masanobu Nanno

Curriculum *vitae* of intestinal intraepithelial T cells: their developmental and behavioral characteristics

Authors' address
Hiromichi Ishikawa[1], Tomoaki Naito[1,2], Toshihiko Iwanaga[3], Hiromi Takahashi-Iwanaga[3], Makoto Suematsu[2], Toshifumi Hibi[4], Masanobu Nanno[5]
[1]Department of Microbiology and Immunology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan.
[2]Department of Biochemistry and Integrative Medical Biology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan.
[3]Laboratory of Cytology and Histology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo, Japan.
[4]Department of Internal Medicine, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan.
[5]Yakult Central Institute for Microbiological Research, Kunitachi, Tokyo, Japan.

Correspondence to:
Hiromichi Ishikawa
Department of Microbiology and Immunology
Keio University School of Medicine
35 Shinanomachi, Shinjuku-ku
Tokyo 160-8582, Japan
Tel.: 81 3 5363 3766
Fax: 81 3 5360 1508
E-mail: h-ishika@sc.itc.keio.ac.jp

Acknowledgements
We would like to thank all members of our laboratories who have contributed to portions of our work embodied in this review paper. The data provided in the preparation of this review were supported in part by a Grant-in-Aid for Creative Scientific Research, the Japan Society for the Promotion of Science (13GS0015), by Special Coordination Funds for Promoting Science and Technology from the Japanese Ministry of Education, Culture, Sports, Science and Technology, by Research on Specific Diseases, Japanese Ministry of Health, Labor and Welfare, and by Keio University Special Grant-in-Aid for Innovative Collaborative Research Projects (T.H.). T.N. is a research fellow supported by the 21st Century Center-of-Excellence Program for Life Science from MEXT (M.S.).

Immunological Reviews 2007
Vol. 215: 154–165

Immunological Reviews
0105-2896

Summary: The alimentary tract has an epithelial layer, consisting mainly of intestinal epithelial cells (IECs), that is exposed to the exterior world through the intestinal lumen. The IEC layer contains many intestinal intraepithelial T cells (IELs), and the total number of IELs constitutes the largest population in the peripheral T-cell pool. Virtually all γδ-IELs and many αβ-IELs in the mouse small intestine are known to express CD8αα homodimers. A wide range of evidence that supports extrathymic development of these CD8αα$^+$ IELs has been collected. In addition, while several studies identified cells with precursor T-cell phenotypes within the gut epithelium, how these precursors, which are dispersed along the length of the intestine, develop into γδ-IELs and/or αβ-IELs has not been clarified. The identification of lymphoid cell aggregations named 'cryptopatches' (CPs) in the intestinal crypt *lamina propria* of mice as sites rich in T-cell precursors in 1996 by our research group, however, provided evidence for a central site, whereby precursor IELs could give rise to T-cell receptor-bearing IELs. In this review, we discuss the development of IELs in the intestinal mucosa and examine the possibility that CPs serve as a production site of extrathymic IELs.

Keywords: CD8αα$^+$-IEL, γδ-IEL, αβ-IEL, extrathymic development of IEL, cryptopatches

Introduction

Surfaces in the body in contact with the outside world include the epidermis and the mucous epithelia. Directly below both the epidermis and the mucous epithelia is an extensive basement membrane (Bm) that serves as a thin wall separating them from the interior of the body, and no capillaries or lymphatic vessels are present in the epidermis and mucous epithelia. Therefore, any lymphomyeloid cells distributed in epidermis and mucous epithelia are extravasated from the postcapillary venules in the interior of Bm, and they must move into the epidermis and mucous epithelia by crossing Bm. T cells and B cells evolved as key players in the immune system of vertebrates, and an infinite number of antigen-specific receptors are produced by a mechanism called somatic gene rearrangement. It has been known for some time that lymphocytes are distributed in the epidermis and mucous epithelia, and in about the middle of the 1970s, it became clear that most intestinal intraepithelial lymphocytes settling in the small intestine of mice are T cells [intestinal

intraepithelial T cells (IELs)] (1). Furthermore, almost all T cells in the epidermis of laboratory mice are those expressing homogenous γδ-type T-cell receptors (TCRs), also known as dendritic epidermal T cells (DETCs). The surprising finding concerning these γδ-DETCs is that they are produced in the thymus at about day 15 of embryonic life and are thus derived from the first wave of fetal γδ thymocytes (2). In this review article, we focus our discussion mainly on findings obtained in mice concerning development of IELs distributed among intestinal epithelial cells (IECs).

Surprising evidence, showed by studies using a monoclonal antibody to TCR, is that almost all mouse IELs are T cells (3–9). IELs are radically different from T cells residing in other sites of the body; most of them are ill-defined T cells with unusual but distinctive characteristics. These cells are located at the front line of defense, at the point which the interior of the body comes in contact with the greatest numbers of antigens from the exterior world.

The interior of the Bm consists of *lamina propria* (LP) that contains abundant immunoglobulin A $(IgA)^+$ B cells, $CD3^+$ T cells (Fig. 1), and various lymphomyeloid cells. In contrast (as discussed later), the exterior of the Bm contains an IEC layer with prominent colonization of CD8α-expressing T cells (Fig. 1). The marked differences between the inside and the outside of the Bm are very important in connection with clarification of *in vivo* physiological functions and development of IELs on the front line of the intestinal mucosa. Research over the past 30 years has shown that IELs in mice and humans, especially those in the small intestine of mice, are a phenotypically and functionally distinctive subpopulation of peripheral T cells that is distinguished from so-called proper T cells, which are distributed in peripheral lymphoid tissues such as the spleen and lymph nodes (LNs) after development in the thymus (10). A vast majority of T cells found in peripheral lymphoid tissues of mice and humans are αβ T cells, while only a few γδ T cells are present. In contrast, IELs in mice and humans include large numbers of cells expressing αβTCRs and those expressing γδTCRs. From a study of IELs in athymic (*nu/nu*) mice, it is clear that many γδ-IELs are present, although the population size is decreased. In addition, in spite of the sharp decrease in αβ-IELs, meaningful numbers of these cells can be detected. Thus, a substantial proportion of γδ-IELs seems to be generated and/or expanded in the absence of the thymus. In contrast, it is well known that both γδ T cells and αβ T cells are virtually undetectable in the spleen and LNs of *nu/nu* mice (11, 12). Functional aspects of IELs have been adequately explained in other reviews in this volume, and this review contains personal insights concerning the past, present, and future of extrathymic development of IELs and where the research is heading. Furthermore, we discuss how IELs settle down in the IEC layer through Bm and emphasize how they behave and survive in the IEC layer in situ.

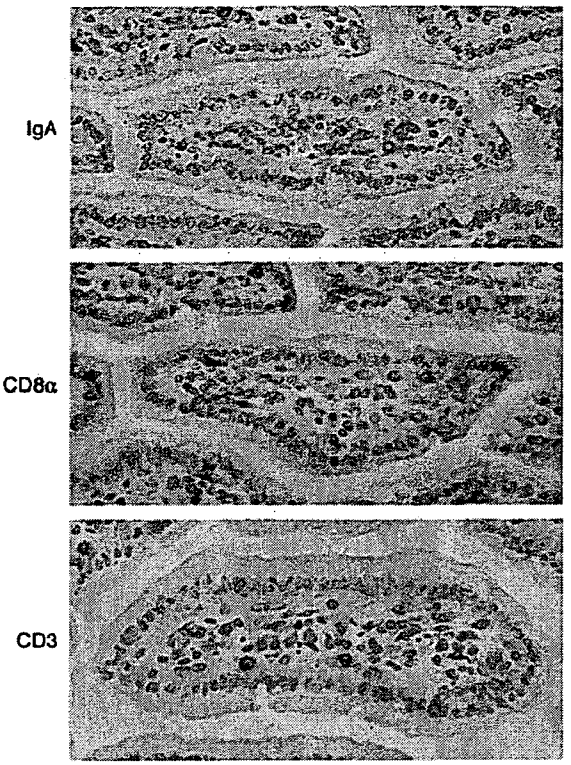

Fig. 1. Immunohistochemical visualization of IgA-, CD8α-, and CD3-expressing cells in jejunal villi. Note that IgA^+ B cells are localized exclusively in the LP, whereas that $CD8\alpha^+$ T cells, namely intestinal IELs, are compartmentalized above the Bm in the IEC layer of the small intestine. In contrast, in addition to numerous IELs in the IEC layer, $CD3^+$ T cells, mostly $CD4^+$ T cells, are also found in the LP of the villi.

IEL development in the intestinal epithelium: evolutionary perspective

The intestine was the first organ to appear when animals became multicellular; even though some multicellular animals lacked brains, there were none without intestines. To defend the intestines against pathogenic microorganisms and harmful substances from the exterior, macrophage-like lymphoid cells developed directly under the intestinal epithelium. The first organ to appear in our living body is the primordial gut, and many organs, including lungs, liver, pancreas, and thyroid gland, are derived from this apparatus. Some marine animals breathe through gills that develop from the upper digestive tract, and pulmonary respiration evolved with the change from marine to terrestrial life. It is well known that the thymus was derived from part of the gill. Therefore, all these organs have

a kindred relation, and latent production of lymphocytes appears possible. In agreement with this argument, fetal liver of mammals including mice is a primary lymphoid organ producing lymphomyeloid cells.

The IEC layer was proposed as a lymphocyte-producing organ as early as 1967 (13). Gut-associated lymphoid tissue (GALT), which contains about 60% of all peripheral lymphocytes, monitors and defends the intestinal mucosa in most vertebrates. T cells and antibodies, the key players in adaptive immunity, have not been found in the jawless fish *Agnatha*, the oldest phylogenic vertebrate lacking a thymus, spleen, and LNs (14, 15). However, GALT, characterized by many lymphoid cells, is found in *Agnatha* such as lampreys and hagfish, and the intestinal mucosa of such animals appears to serve as lymphocyte production sites (14, 15). Furthermore, the bursa of Fabricius, a GALT of birds, and Peyer's patches (PPs) of ruminants are also well known as primary lymphoid tissues responsible for the development of B cells (16). If we consider the large amount of knowledge based on animal evolution, there is nothing remarkable about development of IELs in intestinal mucosa *in situ* in mice and humans.

Findings supporting extrathymic development of murine IELs

Type a and type b IELs

IELs of the murine gut have been identified as ill-defined T cells that lurk in the anatomical front of the intestine (1, 3–9), with a primarily cytotoxic T-cell phenotype (4, 17–19). A large fraction of murine IELs bearing CD8αα homodimers ($CD8\alpha\alpha^+$ IELs) have been proposed to originate locally through a differentiation process initiated in c-kit$^+$IL-7R$^+$lineage marker $(Lin)^-$ gut precursors. Hayday *et al.* (10) have proposed that the functional complexity and phenotype heterogeneity of IELs might be simplified if IELs are classified into just two cell types: 'a' and 'b'. Type a includes $CD4^+$ and $CD8\alpha\beta^+$ αβ-IELs that primarily recognize antigens presented by classical major histocompatibility complex (MHC) class I and class II molecules and are primed within the systemic circulation. Type b IELs include $CD8\alpha\alpha^+$ αβ- and γδ-IELs that respond to antigens not restricted by classical MHC molecules. Although $CD8\alpha\alpha^+$ αβ- and γδ-IELs are clearly different from one another, type b IELs share many 'unconventional' features that distinguish them from type a IELs.

Although dependence of the type a $CD8\alpha\beta^+$ and type b $CD8\alpha\alpha^+$ αβ-IELs but not type b $CD8\alpha\alpha^+$ γδ-IELs on MHC class I molecules was reported using β2-microglobulin (β2m)-deficient mice (20, 21), a recent analysis of gene expression profiles between type b $CD8\alpha\alpha^+$ αβ− and γδ-IELs showed a high degree of similarity (22). These two classes of IELs are not only related functionally but also have a kindred relation.

The total number of αβ-IELs decreased sharply in $\beta2m^{-/-}$ mice due to the disappearance of both $CD8\alpha\beta^+$ (type a) and $CD8\alpha\alpha^+$ (type b) subsets. In β2m/TCR-δ double-mutant mice, which lack β2m and γδ-IELs, the $CD8\alpha\alpha^+$ subset expanded dramatically, while the $CD8\alpha\beta^+$ subset did not (Fig. 2). Thus, in the absence of γδ-IELs, αβ-IELs in β2m-deficient mice outnumbered those in wildtype littermates due to considerable expansion of type b $CD8\alpha\alpha^+$ αβ-IELs (Fig. 2). These results (23) indicate that generation of type b $CD8\alpha\alpha^+$ αβ- and γδ-IELs is essentially β2m independent, while generation of type a $CD8\alpha\beta^+$ αβ-IELs is highly dependent on β2m-MHC class I

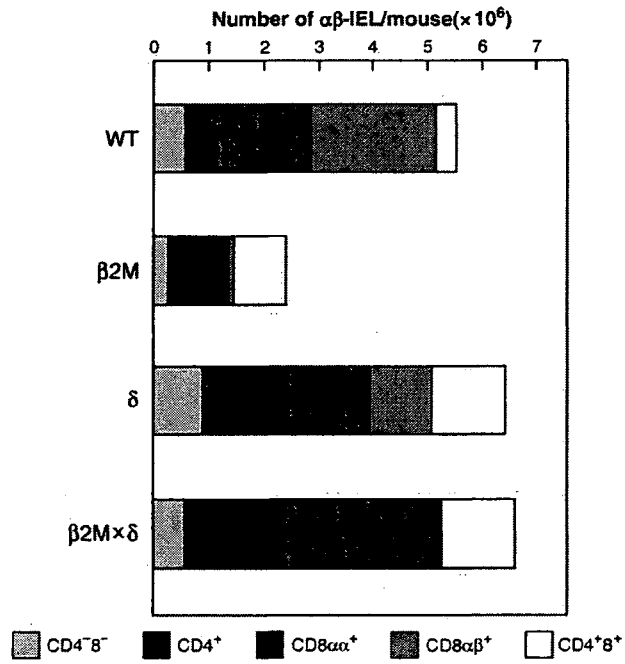

Fig. 2. Composition of αβ-IEL subsets in wildtype (WT), β2m-deficient, TCR-δ mutant (δ), and β2m × TCR-δ double-mutant (β2m × δ) mice. These four different mice were littermates of the F_2 generation of an intercross between $\beta2m^{-/-}$ and $\delta^{-/-}$ mice. IELs isolated from these mutant mice were incubated first with anti-CD8α monoclonal antibody (biotinylated) and then with streptavidin–allophycocyanin. After washing, the IELs were counterstained with two combinations of two phycoerythrin-conjugated monoclonal antibodies (anti-CD4 and anti-CD8β) and two fluorescein-isothiocyanate-conjugated monoclonal antibodies (anti-αβ TCR and anti-γδ TCR, respectively). Absolute numbers of double-negative ($CD4^-CD8^-$), single positive ($CD4^+$, $CD8\alpha\alpha^+$, or $CD8\alpha\beta^+$), and double positive ($CD4^+CD8^+$) subsets in the αβ-IEL population were calculated on the basis of total number of αβ-IELs. Note that $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ αβ-IEL subsets are absent from the small intestine of β2m mutant mice, whereas the $CD8\alpha\alpha^+$ but not $CD8\alpha\beta^+$ αβ-IEL subset expands markedly in the small intestine of double-mutant β2m × δ mice, namely β2m mutant mice that lack γδ-IELs.

molecules expressed by the controlling cells at the type a IEL precursor development site. These findings suggest the possibility that type b IELs, CD8αα$^+$ αβ- and γδ-IELs, develop in the same anatomical site(s). When no β2m-MHC class I molecules are present, development of CD8αα$^+$ αβ-IELs is likely inhibited because development of CD8αα$^+$ γδ-IELs surpasses that of CD8αα$^+$ αβ-IELs competitively.

CD8αα exerts a specific and high affinity for interaction with the non-classical MHC class I molecule thymus leukemia (TL) antigen, which is expressed abundantly by murine thymic stromal cells and by IECs (24). It was also proposed that CD8αα$^+$ TCR-αβ T cells originated from the thymus through agonist-dependent positive selection (25). The mechanism of development of CD8αα$^+$ T cells and *in vivo* physiological functions, including whether or not this scenario is correct, remain to be clarified.

Evidence obtained in a study of athymic *nu/nu* mice

The evidence that most clearly supports thymus-independent development of gut-oriented type b IELs is obtained from a study of T cells in the athymic (nu/nu) mouse. Almost no γδ T cells or αβ T cells are observed in the spleen and LNs of nu/nu mice. A considerable population of γδ-IELs is present in IELs of nu/nu mice, and αβ-IELs can also be detected (Fig. 3). Since these αβ-IELs are CD8αα$^+$ type b IELs and no αβ-IELs are found in TCRβ$^{-/-}$ mice (Fig. 3), it is evident that a few type b αβ-IELs develop independently of the thymus.

Many reports have been published on the thymus-independent development of type b IELs. These findings include the presence of a few CD3$^-$ lymphocytes in the IELs and the resulting possession of precursor T-cell-like properties, i.e. the fact that these tentative precursors observed in mice and humans retain messenger RNA (mRNA) for recombination-activating gene-1 (RAG-1) and RAG-2 and pre-Tα molecules. For details, the reader is referred to previously published articles (8, 9, 26–30).

Here, we introduce our results (31) from screening lymphoid tissues of athymic (nu/nu) RAG-1$^{GFP/+}$ animals, which have the green fluorescence protein (GFP) gene in the RAG-1 locus. Only CD19$^+$ B cells (32) in the bone marrow (BM), spleen, mesenteric lymph nodes (MLNs), and PPs express RAG-1, while in IELs, a meaningful number of CD19$^-$ cells express RAG-1, although the amount of RAG-1 molecules expressed is low (Fig. 4). Since CD19$^-$RAG-1^{low} IELs are CD3$^-$ and are not observed in IELs of RAG-1$^{+/+}$ mice, this finding supports the distribution of small numbers of RAG-1^{low} precursor T cells in IELs. DETCs of wildtype mice that express homogenous γδTCRs (Vγ5Jγ4Cγ1 and Vδ1Jδ2Cδ) are known to be derived from the first wave of fetal γδ thymocytes, which are produced in the thymus at about antenatal day 15 (2). Therefore, even though Vγ5$^+$ DETCs are not present naturally, it has been shown that Vγ1/Vδ6$^+$ DETCs are present in the epidermis of nu/nu mice (33). These findings together with those reported by Matis *et al.* (34) and Yoshikai *et al.* (35) prove that the thymus-dependent type a subset (2) and the thymus-independent type b subset (33–35) such as γδ T cells are present in peripheral anatomical sites other than the IEC layer.

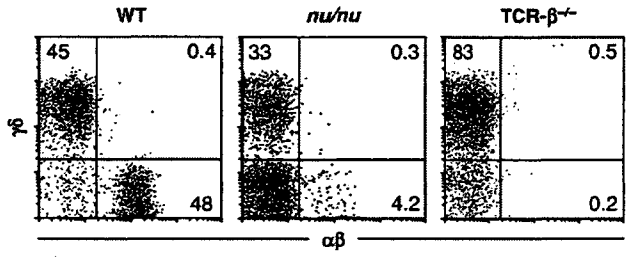

Fig. 3. Composition of αβ- and γδ-IELs isolated from wildtype (WT), athymic (nu/nu) and TCR-β$^{-/-}$ mice. Flow cytometric analysis of IELs isolated from five individuals each of three different strains of mice was performed, and the representative profiles of IELs are presented. In this case, absolute numbers of IELs recovered were 5.4 × 10^6 from WT mice, 2.3 × 10^6 from nu/nu mice, and 5.3 × 10^6 from TCR-β$^{-/-}$ mice. The percentage of αβ- and γδ-IELs in the corresponding quadrants is shown. Note that αβ-IELs are drastically reduced in the athymic condition. Nevertheless, a meaningful number of αβ-IELs are still present in the small intestine of the nu/nu mouse compared with the total absence of αβ-IELs from the TCR-β$^{-/-}$ mouse. In contrast, there are few, if any (<1%), αβ T cells in the spleen and MLNs of this same nu/nu mouse (data not shown).

A new member of GALT: cryptopatches

Discovery of cryptopatches in mouse small intestine as the precursor IEL-producing site

We have shown that multiple tiny lymphoid cell aggregations, filled with about 1000 closely packed c-kit$^+$IL-7R$^+$Thy1$^+$ CD3$^-$B220$^-$ lymphocytes, colonize throughout the small intestinal mucosa of C57BL/6 mice (36). The location is in the crypt LP [cryptopatches (CPs)]. They are first detected in the third week of postnatal life in C57BL/6 mice. In terms of morphogenesis, cellular composition, and fine tissue structure, neither PPs nor isolated lymphoid follicles (ILFs) are identical to CPs (30, 37). CPs contain neither cells undergoing apoptosis nor cells bearing RAG-1 molecules but do contain dendritic stromal cells bearing CD11c/CD18 molecules. The presence of transcripts for germ line TCR genes and mRNA for proteins involved in TCR rearrangement (38) and the ability of c-kit$^+$Lin$^-$ CP cells to generate TCR$^+$ IELs in T-cell-deficient mice (39) indicate that at least some CP cells are committed to the T-cell lineage and are competent for generation of

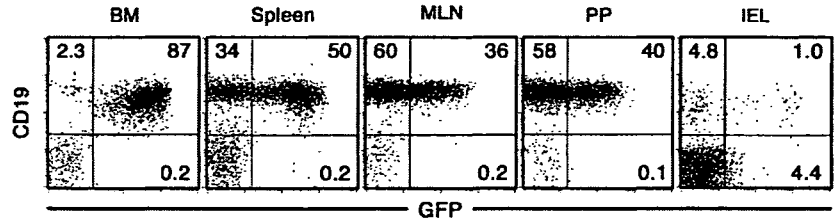

Fig. 4. A small number of RAG-1-expressing lymphocytes are present in the intestinal intraepithelial compartment of nu/nu mice. Lymphocytes from BM, spleen, MLNs, PPs, and IEC layer (IELs) were isolated from nu/nu mice carrying a GFP gene in place of the RAG-1 gene (nu/nu RAG-1$^{GFP/+}$ mice) (31) and then were subjected to flow cytometric analysis. Note that only in the IEL preparation, a meaningful number of GFP-dull-positive cells are present. Importantly, these cells are not B-lineage cells because they are CD19 negative.

thymus-independent type b IELs, especially CD8αα$^+$ γδ-IELs (12).

To obtain direct evidence for generation of type b IELs from the precursors that settle in CPs, cytokine receptor γ chain mutant nu/nu mice that lack a thymus, PPs, CPs, and intestinal T cells (40) were reconstituted with wildtype Ly5.1$^+$ BM cells. BM-derived TCR$^-$ IELs first appeared within villous epithelia of small intestine overlaying regenerated CPs, and these TCR$^-$ IELs subsequently emerged throughout the epithelia. Thereafter, TCR$^+$ IELs increased sluggishly to a number comparable with that in athymic nu/nu mice and consisted of both αβ- and γδ-IELs (38). Taking all these results together, CPs are the first murine GALT to be identified that most likely serve as the site of development of lymphohemopoietic precursors for type b IEL descendents at commencement of weaning (36, 38, 40). Recent data showed a clonal relationship between CP T cells and γδ-IELs (41).

Evidence that CPs are not the precursor IEL-producing site

By characterizing phenotypically distinct lineage-negative populations in the CPs and gut epithelium, Lambolez *et al.* (42) showed that only 3% of CP cells were clearly involved in T-cell differentiation and suggested that these CP structures may have an additional physiological role in the gut. In contrast, Guy-Grand *et al.* (43) clarified the following from an examination of RAG-2 expression using GFP transgenic mice evaluated by GFP (carrying a GFP reporter gene driven by the RAG-2 promoter). In nu/nu mice, T lymphopoiesis occurs mainly in MLNs, less in PPs, and not in CPs. Importantly, this extrathymic T lymphopoiesis is totally repressed in euthymic mice (43). Based on these findings, Guy-Grand *et al.* (43) concluded that in normal euthymic mice, all gut αβ-IELs, including type b CD8αα$^+$ αβ-IELs, are of thymic origin. It has also been shown that thymus transplantation into nu/nu mice results in the appearance of thymus graft-derived αβ- and γδ-IELs in nu/nu recipients (44). To evaluate this important issue, we generated nu/nu *aly/aly* (*alymphoplasia*) double-mutant mice lacking thymus, all LNs, PPs, and ILFs but possessing CPs (12). Substantial colonization by γδ-IELs comprising the major CD8αα$^+$ subset took place, and use of TCR-γ-chain variable gene segments by these γδ-IELs was unaltered (12). These findings indicate that MLNs and PPs are not an absolute requirement for development of γδ-IELs but instead support the notion that gut CPs generate progenitor γδ-IELs, even under athymic conditions. However, absolute numbers of γδ-IELs from nu/nu *aly/aly* mice are smaller than those from the corresponding control nu/nu mice (12). These features indicate that LNs and PPs in fact determine the number of γδ-IELs under the nu/nu conditions. In any event, there would be a hierarchy of T-cell production in terms of anatomical sites, although the detailed mechanism is still not well understood. By a mechanism such as the tropic effect of thyrotropin-releasing hormone on IEL development (45, 46), however, almost all αβ-IELs appear to originate in the thymus in euthymic mice. In nu/nu mice, however, IELs appear to be generated mainly by the MLNs and PPs, and in nu/nu *aly/aly* mice that lack a thymus, all LNs, PPs, ILFs, and αβ- and γδ-IELs appear to be generated from other anatomical sites, probably CPs. In this context, it has been reported that T-cell-committed precursors are distributed not only in the thymus but also in the BM, liver, and spleen (47–49). It is inferred based on these findings that extrathymic T-cell generation has not been proven to be repressed completely in normal euthymic mice. In other words, there is no solid evidence denying a possibility that extrathymic T cells are generated in euthymic conditions.

It has recently been shown that retinoic acid-related orphan receptors (RORγt) detected in fetal lymphoid tissue-inducer (Lti) cells are also expressed in cells within gut CPs and that, by fate mapping of RORγt$^+$ cells, type b IELs, such as γδ-IELs, are not the progeny of RORγt$^+$ CP cells (50). However, it remains an open question whether a small fraction of lymphoid cells in CPs does not express RORγt or all CP cells express RORγt. To investigate this point in detail, we generated nu/+ RORγt$^{GFP/+}$

mice and nu/nu RORγt$^{GFP/+}$ mice and obtained the results shown in Fig. 5.

In agreement with the report of Eberl and Littman (50), many RORγt^{+} (GFP^{+}) cells were present in thymocytes from nu/+ RORγt$^{GFP/+}$ mice, although GFP expression was weak (unpublished observation). Analysis of CP cells from nu/nu RORγt$^{GFP/+}$ mice showed that almost all GFP^{+} cells were interleukin-7 receptor positive (IL-7R)$^{+}$ and CD8α^{-}. However, CD3^{-} cell subsets showing various phenotypes such as IL-7R^{+}GFP^{-}, c-kit^{+}GFP^{+}, c-kit^{+}GFP^{-}, c-kit^{-}GFP^{+}, Thy-1^{+}GFP^{+}, Thy-1^{+}GFP^{-}, Thy-1^{-}GFP^{+}, CD4^{+}GFP^{+}, CD4^{+}GFP^{-}, CD4^{-}GFP^{+}, CD8^{+}GFP^{-}, Lin^{+}GFP^{+} (majority of them CD4^{+}GFP^{+}), Lin^{+}GFP^{-}, and Lin^{-}GFP^{+} were all present but with variable population sizes (Fig. 5A). In addition to the colonization of γδ-IELs and small numbers of αβ-IELs, of particular note was the presence of CD3^{-} IELs showing c-kit^{+}GFP^{+} and IL-7R^{+}GFP^{+} phenotypes in IEC compartments of nu/nu RORγt$^{GFP/+}$ mice (Fig. 5B). These new findings do not necessarily support the conclusion of Eberl and Littman (50) and show distribution of RORγt^{-} c-kit^{+}, IL-7R^{+}, Thy-1^{+}, CD4^{+}, CD8^{+}, and/or Lin^{+} lymphocytes in murine gut CPs. In this context, we previously reported (36) that CPs are not detectable in IL-7R$^{-/-}$ mice. However, although γδ-IELs are absent owing to selective blockage of TCR-γ gene rearrangements (51), we noticed only a slight decrease in development of type b αβ-IEL subsets in IL-7R$^{-/-}$ mice. With these observations in mind, we reinvestigated hundreds of cryosections prepared from small intestines of IL-7R$^{-/-}$ mice by immunohistochemistry and verified that conspicuously emaciated CPs filled with c-kit^{+} cells and decreased by more than 16-fold in number were present in this mutant intestine (40). Similarly, although mice genetically deficient in lymphotoxin α (LTα) have been reported to lack CPs (52), we observed that histogenesis of CPs and intestinal development of αβ- and γδ-IELs remained almost intact in LTα$^{-/-}$ mice (37). In consideration of our research results, i.e. CPs are observed not only in IL-7R$^{-/-}$ mice but also in LTα$^{-/-}$ mice, the conclusion that CP development is not found in RORγt$^{GFP/GFP}$ mice lacking RORγt (50) seems to require careful reexamination. It is easy to ascertain the presence of CPs, but it is quite difficult to conclude that CPs are totally absent. At the same time, we should examine if type b IELs, such as γδ-IELs, develop from the RORγt^{-} c-kit^{+}GFP^{-} or IL-7R^{+}GFP^{-} subset (Fig. 5A) distributed in the CPs of nu/nu RORγt$^{GFP/+}$ mice.

What are these CPs?

Based on the results obtained using RORγt$^{GFP/+}$ mice, Eberl and Littman (50) proposed that the principal function of murine c-kit^{+}Lin^{-} RORγt^{+} CP cells is to induce formation of lymphoid

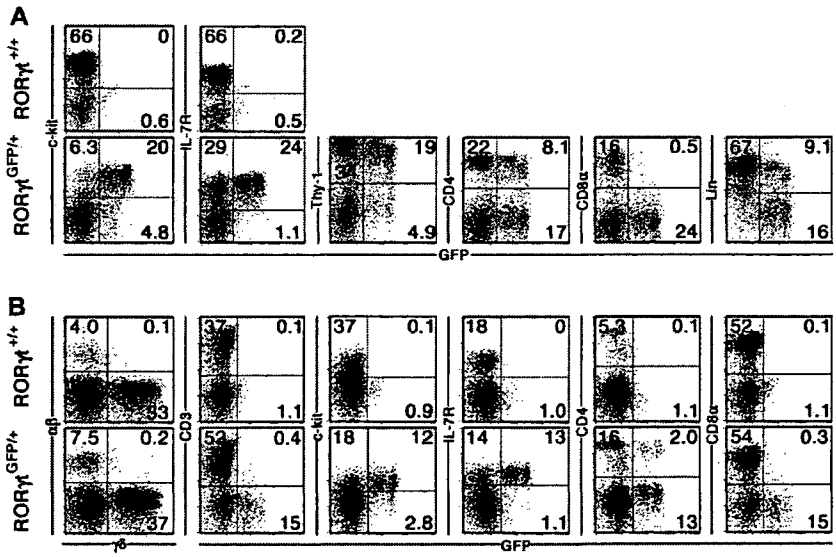

Fig. 5. Flow cytometric analysis of CP cells and IELs isolated from nu/nu mice carrying the wildtype RORγt genes (RORγt$^{+/+}$ mice) and carrying a GFP gene in place of RORγt gene (RORγt$^{GFP/+}$ mice). CP cells were isolated according to the method described previously (37). Although CP cells (A) and IELs (B) isolated from nu/nu RORγt$^{+/+}$ mice do not contain GFP^{+} cells, those isolated from nu/nu RORγt$^{GFP/+}$ mice contain a substantial population of GFP^{+} RORγt-expressing cells. Furthermore, these RORγt-expressing cells from CP and IEL compartments of nu/nu RORγt$^{GFP/+}$ mice appear to be composed of two discrete RORγt^{high} and RORγt^{low} cell subsets. 'Lin' in A represents lineage markers CD3, B220, Mac-1, Gr-1, TER119, CD11c, CD4, and CD8α. Lymphocytes isolated from spleen, MLNs, and PPs of nu/nu RORγt$^{GFP/+}$ mice lack GFP^{+} RORγt-expressing cells (data not shown).

follicles, namely ILFs, in the LP in a manner similar to induction of LNs and PPs by RORγt$^+$ Lti cells. It is also possible that CP cells are precursor cells of gut-oriented lymphomyeloid cells other than IELs (42). The CPs of mice have been discounted as the anatomical site where precursor IELs congregate, as no CP-like lymphoid clusters have been reported in the intestinal mucosa of mammals other than mice. However, since evidence indicates development of gut-oriented T cells, mainly IELs, in the intestinal mucosa of humans (53–59) and rats (60), to determine whether or not CPs and CP-like lymphoid tissues are present in enteric mucosa of animals is an important goal for future experiments. We (61) showed that lymphocyte clusters, just like the structure named lymphocyte-filled villi (LFV) (62), populated predominantly with c-kit$^+$IL-7R$^+$ cells and less with αβTCR cells, were found distributed throughout the length of the small intestine of rats. Nevertheless, we were unable to verify whether these rat LFV, containing undifferentiated lymphocytes, represented clusters of extrathymic precursor T cells. With regard to this same issue, several groups have actively sought evidence of CPs in the human gastrointestinal tract, illuminating distinctive T-cell facets of human fetal gut lymphocytes (58, 59). In agreement with these findings, we have identified multiple tiny c-kit$^+$ lymphoid cell clusters in human fetal intestine at the second trimester of fetal life (unpublished observation). Overall, we take it for granted that the differentiation of type b IELs is not exactly the same among different vertebrates. For instance, if epidermal immune regulation by γδ-DETCs in mice is of considerable physiological importance, how do other mammals including humans cope without γδ-DETCs? In these animals, immunoregulatory function should be provided by some lymphoid cells other than γδ-DETCs.

Based on these findings, precursors of type b IELs that develop extrathymically in humans and rats appear to be produced in CP-like lymphoid tissues of mice during fetal life. Then, the precursors are dispersed throughout the LP or IEC layer, or self-renewal is localized in the IEC layer after expression of the αβTCR or γδTCR and completion of development, as in the case of B-1 B cells. Mouse DETCs (2) expressing homogenous Vγ5/Vδ1$^+$ γδTCRs are produced only in fetal thymus in a very limited period at about day 15 of fetal life. No such Vγ5/Vδ1$^+$ γδ-DETCs are found in the epidermis of athymic nu/nu mice (33), while homogenous Vγ5/Vδ1$^+$ γδ-DETCs of euthymic mice are present throughout life (1.5–2 years). If the supply of Vγ5/Vδ1$^+$ γδ-DETCs occurs only in the first wave of fetal γδ thymocytes development, the life of Vγ5/Vδ1$^+$ γδ-DETCs must be as long as the life of the mouse. Is this really the case? We do not think that it is. It is possible that precursor Vγ5/Vδ1$^+$ γδ-DETCs generated by fetal thymus at about day 15 of fetal life lurk somewhere in the body (such as in the epidermis or dermis), develop at a fixed pace, and produce new Vγ5/Vδ1$^+$ γδ-DETCs. It is also possible that γδ-DETCs expressing homogenous Vγ5/Vδ1-TCRs continuously undergo self-renewal in the epidermis. Indeed, γδ-DETCs are shown to recognize and respond to antigens expressed on damaged, stressed, or transformed keratinocytes by means of their TCRs and produce keratinocyte growth factors (63), and it has been indicated that continuous stimulation of Vγ5/Vδ1-TCR$^+$ γδ-DETCs by the relevant ligand(s) is critical for maintenance of γδ-DETCs throughout the life of animals (64). In this context, it is conceivable that extrathymically generated mature human and rat IELs, which originate from cells settling in CP-like lymphoid tissues of mice during fetal life, undergo continuous self-renewal over the entire postnatal life of these animals by stimulation with relevant gut-associated antigens. In conclusion, although much remains to be learned about the mysterious development of extrathymic T cells in general and also the enigmatic features of CPs before we evaluate them as anatomical sites in which murine type b precursor IELs develop, many aspects of extrathymic T-cell immunobiology are now coming together step-by-step.

Where do these IELs come from?

Many types of cells are present outside the Bm of the small intestine (Fig. 6). Cells other than IELs present in the IEC layer are known to develop from stem cells located in the crypt. As described in the Introduction, IELs must migrate into the IEC layer from LP across the Bm. Immunohistochemical examination has shown that there are at least 2, or many more, IELs for every 10 IECs in mouse small intestine (65, 66). Thus, absolute numbers

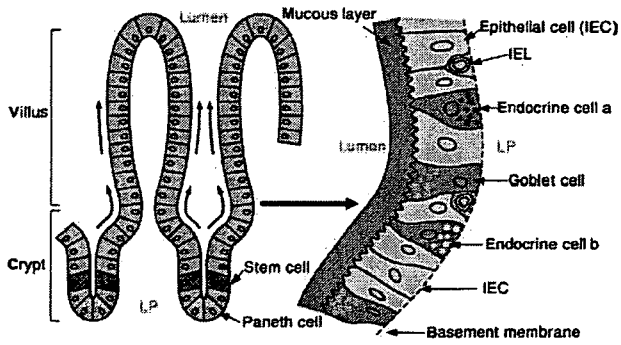

Fig. 6. Schematic illustration of cells that settle in the IEC layer of the small intestine. Every cell that settles in the IEC compartment, except IELs on the right of this figure (magnified), is the progeny of the stem cells shown on the left of this figure. Endocrine cells a and b contain different gut hormones.

of murine IELs are estimated to account for about half of the peripheral T-cell pool (67). However, it is not known if this huge IEL population enters the IEC layer across the Bm at sites such as crypts or villi, as shown in Fig. 6, or if IELs that have entered the IEC layer later cross the Bm and return to the LP. A large fraction of IELs is composed of γδ-IELs, and the fact that γδ T cells are almost absent from the LP compartment of villi in normal mice indicates that once they have entered the IEC layers, IELs and/or precursor IELs might return to LP in rare cases. By electron microscopy, we (39) showed that numerous lymphocytes cross the Bm that comes into contact with the CPs. Furthermore, by analysis of BM-chimeric mice, we (38) verified that donor BM-cell-derived IELs first appeared within the IEC layer of villi around regenerated CPs filled with BM-cell-derived c-kit$^+$ cells. These experimental results, in conjunction with a wide range of evidence showing the presence of small numbers of CD3$^-$ precursor IEL-like lymphocytes in the IEC compartment (8, 9, 26–30, 68), suggest a scenario in which precursor IELs that developed in CPs enter the IEC layer from the Bm that overlays CPs, and then these cells very sluggishly develop into mature type b IELs. In contrast, no definite findings have been obtained on whether type a αβ-IELs, such as CD4$^+$ or CD8αβ$^+$ T cells derived from the thymus, cross the Bm by some route and enter the IEC layer.

Parabiotic C57BL/6 Ly5.1 and Ly5.2 mice sharing circulation have been used by several groups (69, 70) to examine whether c-kit$^+$ stem cells that settle in local organs give rise to lymphoid cells in these organs *in situ*. As anticipated, the partner cells mixed rapidly in the spleen, all LNs, and PPs. In contrast, there were no or very few mixtures of partner cells in the thymus, CPs, and IELs (70). Poussier *et al.* (69) reported that IELs in the murine intestine did not mix together in parabiotic Ly5.1 and Ly5.2 mice. Overall, these findings appear to once again support the notion that IELs arise from their own pre-existing local precursor cells and that neighboring CPs continuously supply these local precursor cells at unknown rates.

What are these IELs?

There are still many riddles concerning the behavior and biological function of IELs that settle in the anatomical front of the IEC compartment *in situ*. Some of these unresolved issues are discussed below. We also discuss the development of research yet to be undertaken and future perspectives.

Behavior

Epithelial stem cells (Fig. 6) proliferate at the base of the crypts. Newly formed cells move upward and differentiate into various types of cells, mostly IECs (Fig. 6), in a process called migration-associated differentiation. The entire process of migration toward the top of the villi takes only several days, and the cells die there, most likely by apoptosis (71).

Electron microscopic examination of immersion-fixed tissue sections shows that IEL interdigitate tightly with IEC at the basolateral faces of IEC (Fig. 7A). Most IELs are terminally differentiated cells or those in the G0/G1 phase of the cell cycle.

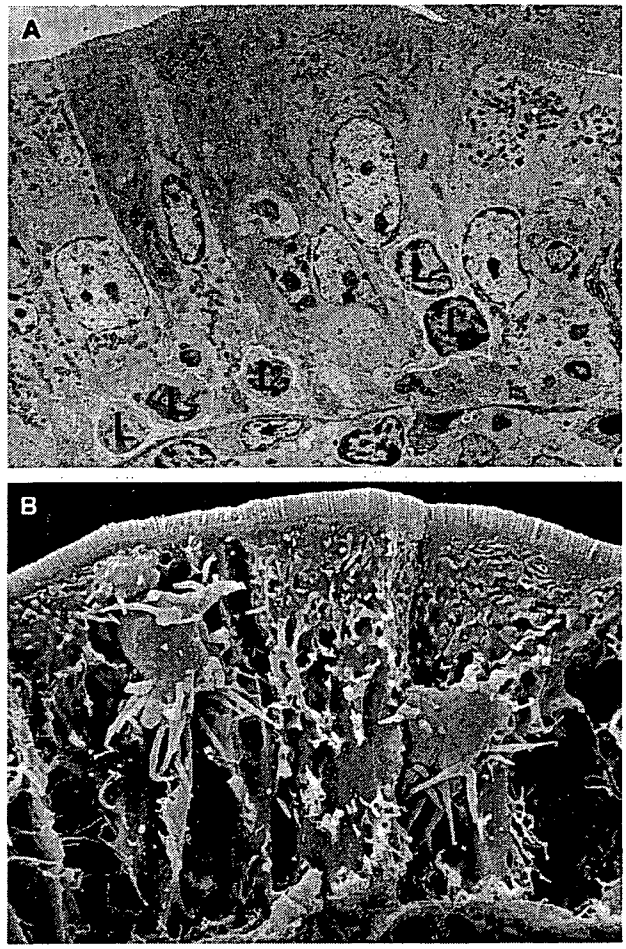

Fig. 7. Electron microscopic analysis of small intestinal epithelium. (A) Transmission electron micrograph showing IELs in intestinal epithelium obtained from an immersion-fixed sample. At least five IELs (L, edged with yellow line) are encased within the epithelium. No free space is visible among epithelial cells in the immersion-fixed samples; IELs appear to be tightly packed in the epithelium. (B) Scanning electron micrograph of IELs in a perfusion-fixed material. Two lymphocytes (yellow) equipped with spine-like processes are detected among epithelial cells. Note the broad free space at the basal region of the epithelium. A perfusion-fixed sample retains more precise and physiological three-dimensional structure of the tissues than that obtained by an immersion-fixed sample because the tissues shrink totally during and after the immersion-fixation. [This image is a modified reproduction of our figure that appeared in *Cell & Tissue Research*, used with kind permission of Springer Science and Business Media (83)].

It has been shown by bromodeoxyuridine labeling experiments that the average half-life of murine IELs is 3 weeks or much longer (72). If we assume that IELs are also migrating to the top of the villi, IECs must continuously get ahead of IELs, suggesting that individual IELs are capable of coming in contact with and/or surveying a significant number of IECs. Are IELs really migrating to the top of the villi? If so, at what speed are they migrating? At present, we do not know whether IELs can actually move upward or whether they maintain a rather stationary position.

Fig. 7B shows a vertically fractured face of perfusion-fixed small intestine by scanning electron microscopy. As is clear from this image, the surface in contact with intestinal lumen (apical surface of IEC) forms a tight junction between the IECs and is covered with microvilli with no gaps. However, there are relatively wide gaps between cells just on the Bm, and IELs are not always fixed by tight interdigitation with the lateral faces of the IECs. They appear to move to and fro relatively freely. IELs are also often in contact with IECs through process-like structures (Fig. 7B). In this image, it looks like the IELs are completely covered with trees, as if it were a mighty jungle viewed from the air, but there are considerable gaps near the ground under the trees where lions (≈ IELs) sleep and animals such as leopards (≈ IELs) lurk and move around (Fig. 8). In any case, clarification of the life and behavior of these mature IELs and the small number of precursor IELs in the IEC compartment are important topics for future study.

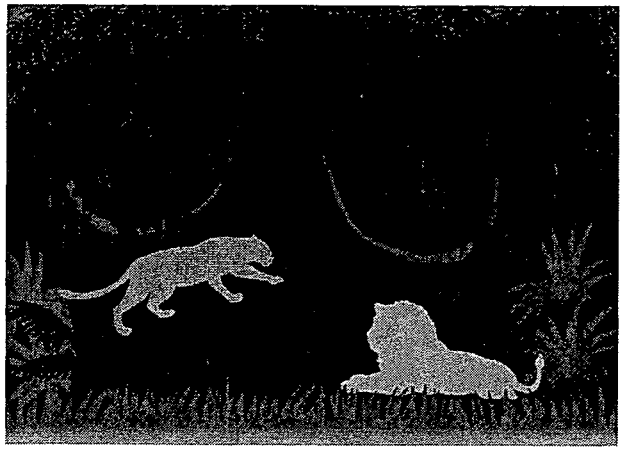

Fig. 8. A pictorial representation of the mighty jungle of the IEC layer. Based on the three-dimensional scanning electron microscopic picture of the IEC layer presented in Fig. 7B, we illustrated a mighty jungle in which a lion (≈IEL, with potentially cytotoxic function?) is resting on the ground (Bm) and a leopard (≈ IEL, like an indiscriminate predator?) is moving around a tree trunk (lateral face of IEC). It is evident that there is considerable room between the tightly packed treetop and the surface of the ground where these animals (≈ IELs) might be freely moving to and fro.

Oligoclonality

Whereas IELs are potentially able to use multiple Vβ TCR genes, both human (73–75) and murine (76) αβ-IELs are known to be derived from a limited number of oligoclonal T-cell clones. This oligoclonality of IELs points to the presence of a restricted set of foreign and/or self-antigens in the gut that may be ligands involved in the stimulation and expansion of these gut T cells. The large numbers of T cells distributed in the spleen and LNs appear to be inexperienced or virgin T cells that have not received antigen stimulation in normal specific pathogen-free mice. In contrast, T cells in the gut of specific pathogen-free mice are activated cells, continuously receiving antigen stimulation. As a result of selection, only competent clones expand and become dominant. Under these circumstances, even though the types of antigens are very diverse and are not limited to a restricted set of foreign and/or self-antigens, the TCR of αβ-IELs eventually results in oligoclonality. One mysterious and unexpected finding is that genetically identical individuals, even from the same litter and housed in the same cage, show distinct and apparently non-overlapping oligoclonal repertoires of both type a and type b αβ-IELs (76). Is the immune response to a diverse range of external antigens in the intestinal lumen really involved in the establishment of the oligoclonality of αβ-IELs? There is no firm experimental evidence to answer this question. In this respect, the finding that αβ-IELs in germ-free mice are also oligoclonal (77) is very intriguing and shows that αβ-IELs are oligoclonal, even when huge numbers of intestinal flora-derived antigens and microbe-associated immunoactive substances are not present.

Cytotoxicity

IELs are mostly terminally differentiated and activated T cells that possess a granular cytoplasmic structure containing perforin and granzyme (78), capable of killing Fc-receptor-bearing target cells after bridging them with anti-CD3, anti-αβTCR, or anti-γδTCR monoclonal antibodies (78–80). However, at most 10^7 IELs per mouse from the small intestine can be isolated for *in vitro* analysis, despite the fact that $5–10 \times 10^7$ IELs have been found to settle in the IEC compartment by immunohistochemistry (67) (Fig. 1). Therefore, we might have studied *in vitro* only a portion of the IELs, which can be isolated easily from intestinal mucosa as a subpopulation maintaining their typical granular cytoplasmic structure and cytotoxic activity. In this context, of great importance in the future is to examine *in vitro* characteristics of the large number of IELs that cannot be recovered using current techniques and/or are lost in the process of purification in a test tube. Such a 'missing' population should be examined,

if the cells maintain their characteristics of terminal differentiation. γδ-IELs from germ-free mice are known to show cytotoxicity (81) in the same way as αβ-IELs in germ-free mice show oligoclonality. Electron and light microscopy shows that murine IELs from *scid/scid* (severe combined immunodeficient) mice that are unable to generate αβ- and γδ-IELs have granulated IELs similar to those in normal mice (82), are $CD3^-CD8\alpha\alpha^+$ IELs are present in *scid/scid* mice and *nu/nu scid/scid* mice (40). In addition, findings showing that $CD3^-CD8\alpha\alpha^+$ IELs are not present in cytokine receptor γ chain mutant *nu/nu* mice lacking CPs (38, 40) suggest that a special but as yet unknown microenvironment in CP and IEC compartments could dictate the ability of precursor IELs entering the IEC layer to possess a granular cytoplasmic structure and to express CD8αα molecules with no relation to presence or absence of a thymus, TCR expression, or intestinal flora.

It is well known that antigen-specific αβ-IELs have a protective role against infection by pathogenic microorganisms, but ligands of cytotoxicity expressed by αβ-IELs and γδ-IELs from specific pathogen-free mice and γδ-IELs from germ-free mice are still not clear. Do these cytotoxic IELs present in the harsh microenvironments at the front line of intestinal mucosa correctly identify target cells by TCR? Even without very strict specificity, are IECs that have been damaged by stress, viral infections, bacterial infections, or transformed IECs rapidly detected and are these dubious IECs eliminated? Are these cytotoxic IELs indiscriminate predators lurking savagely in the jungle of the IEC layer (Fig. 8)? What T cells are these IELs anyway? In any event, elucidation of these conditions is very important to clarify the development and physiological function of IELs.

Conclusion

IELs are known as peripheral T cells with marked specificity and a very large population size, but their development and physiological function remain a mystery. Findings obtained through research over the past 30 years are very important. We view these diverse experimental observations as many tips of an iceberg that must firmly interconnect with its hidden part. Understanding the immunobiology of IELs must begin in the context of intestinal flora, which outnumber the total number of cells in our body and which have evolved with us in a commensal or symbiotic state. Elucidation of the characteristics of immune responses in the intestines, as the frontline defense against pathogens from the outer world, through clarification of the development and physiological function of IELs is essential for manipulation of intestinal immunity for our benefit.

References

1. Guy-Grand D, Griscelli C, Vassalli P. The gut-associated lymphoid system: nature and properties of the large dividing cells. Eur J Immunol 1974;**4**:435–443.
2. Tonegawa S, et al. Diversity, development, ligands, and probable function of γδ T cells. Cold Spring Harb Symp Quant Biol 1989;**54**:31–44.
3. Cerf-Bensussan N, Guy-Grand D, Griscelli C. Intraepithelial lymphocytes of human gut: isolation, characterization and study of natural killer activity. Gut 1985;**26**:81–88.
4. Klein JR. Ontogeny of the Thy-1^-, Lyt-2^+ murine intestinal intraepithelial lymphocyte. Characterization of unique population of thymus-independent cytotoxic effector cells in the intestinal mucosa. J Exp Med 1986;**164**:309–314.
5. Dillon SB, MacDonald TT. Functional characterization of Con A-responsive mouse small intestinal intraepithelial lymphocytes. Immunology 1986;**59**:389–396.
6. Goodman T, Lefrancois L. Expression of the γ-δ T-cell receptor on intestinal $CD8^+$ intraepithelial lymphocytes. Nature 1988;**333**: 855–858.
7. Klein JR. Thymus-independent development of gut T cells. Chem Immunol 1998;**71**: 88–102.
8. Lefrancois L, Puddington L. Basic aspects of intraepithelial lymphocyte immunobiology. In: Ogra PL, Mestecky J, Lamm ME, Strober W, Bienenstock L, McGhee JR, eds. Mucosal Immunology. San Diego: Academic Press, 1999:413–428.
9. Kronenberg M, Cheroutre H. Development, function, and specificity of intestinal intraepithelial lymphocytes. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer L, eds. Mucosal Immunology. San Diego: Academic Press, 2005:565–581.
10. Hayday A, Theodoridis E, Ramsburg E, Shires J. Intraepithelial lymphocytes: exploring the third way in immunology. Nat Immunol 2001;**2**:997–1003.
11. Bandeira A, et al. Extrathymic origin of intestinal intraepithelial lymphocytes bearing T-cell antigen receptor γδ. Proc Natl Acad Sci USA 1991;**88**:43–47.
12. Nonaka S, et al. Intestinal γδ T cells develop in mice lacking thymus, all lymph nodes, Peyer's patches, and isolated lymphoid follicles. J Immunol 2005;**174**:1906–1912.
13. Fichtelius KE. The gut epithelium – a first level lymphoid organ? Exp Cell Res 1968;**49**: 87–104.
14. Matsunaga T. Did the first adaptive immunity evolve in the gut of ancient jawed fish? Cytogenet Cell Genet 1998;**80**:138–141.
15. Du Pasquier L, Flajnik M. Origin and evolution of the vertebrate immune system. In: Paul W, ed. Fundamental Immunology. Philadelphia: Lippincott-Raven Publisher, 1999:605–650.
16. Griebel PJ, Hein WR. Expanding the role of Peyer's patches in B-cell ontogeny. Immunol Today 1996;**17**:30–39.

17. Davies M, Parrott D. The early appearance of specific cytotoxic T cells in murine gut mucosa. Clin Exp Immunol 1980;**42**:273–279.
18. Ernst PB, Clark DA, Rosenthal KL, Befus AD, Bienenstock J. Detection and characterization of cytotoxic T lymphocyte precursors in the murine intestinal intraepithelial leukocyte populations. J Immunol 1986;**136**: 2121–2126.
19. Viney J, Kilshaw P, MacDonald T. Cytotoxic α/β^+ and γ/δ^+ T cells in murine intestinal epithelium. Eur J Immunol 1990;**20**: 1623–1626.
20. Correa I, Bix M, Liao N, Zijlstra M, Jaenisch R, Raulet D. Most $\gamma\delta$ T cells develop normally in β_2-microglobulin-deficient mice. Proc Natl Acad Sci USA 1992;**89**:653–657.
21. Neuhaus O, Emoto M, Blum C, Yamamoto S, Kaufmann S. Control of thymus-independent intestinal intraepithelial lymphocytes by β_2-microglobulin. Eur J Immunol 1995;**25**:2332–2339.
22. Pennington DJ, et al. The inter-relatedness and interdependence of mouse T cell receptor $\gamma\delta^+$ and $\alpha\beta^+$ cells. Nat Immunol 2003;**4**:991–998.
23. Fujiura Y, et al. Development of CD8$\alpha\alpha^+$ intestinal intraepithelial T cells in β_2-microglobulin- and/or TAP1-deficient mice. J Immunol 1996;**156**:2710–2715.
24. Leishman AL, et al. T cell responses modulated through interaction between CD8$\alpha\alpha$ and the nonclassical MHC molecules, TL. Science 2001;**294**:1936–1939.
25. Leishman AJ, et al. Precursor of functional MHC class I- or class II-restricted CD8$\alpha\alpha^+$ T cells are positively selected in the thymus by agonist self-peptides. Immunity 2002;**16**:355–364.
26. Lefrancois L, Puddington L. Extrathymic intestinal T-cell development: virtual reality? Immunol Today 1995;**16**:16–21.
27. Rocha B, Guy-Grand D, Vassalli P. Extrathymic T cell differentiation. Curr Opin Immunol 1995;**7**:235–242.
28. Poussier P, Julius M. Speculation on the lineage relationship among $CD4^-8^+$ gut-derived T cells and their role(s). Semin Immunol 1999;**11**:293–303.
29. Guy-Grand D, Vassalli P. Gut intraepithelial lymphocyte development. Curr Opin Immunol 2002;**14**:255–259.
30. Ishikawa H, Kanamori Y, Hamada H, Kiyono H. Development and function of organized gut-associated lymphoid tissues. In: Mestecky J, Lamm ME, Strober W, Bienenstock J, McGhee JR, Mayer L, eds. Mucosal Immunology. San Diego: Academic Press, 2005:285–405.
31. Kuwata N, Igarashi H, Ohmura T, Aizawa S, Sakaguchi N. Absence of expression of RAG1 in peritoneal B-1 cells detected by knocking into RAG1 locus with green fluorescent protein gene. J Immunol 1999;**163**:6355–6359.
32. Fujimoto M, et al. Complementary role for CD19 and Bruton's tyrosine kinase in B lymphocyte signal transduction. J Immunol 2002;**168**:5465–5476.
33. Ota Y, et al. Extrathymic origin of Vγ1/Vδ6 T cells in the skin. Eur J Immunol 1992;**22**:595–598.
34. Matis LA, Cron R, Bluestone JA. Major histocompatibility complex-linked specificity of γδ receptor-bearing T lymphocytes. Nature 1987;**330**:262–264.
35. Yoshikai Y, et al. Functional T cell receptor δ chain gene messages in athymic nude mice. Eur J Immunol 1988;**18**:1039–1043.
36. Kanamori Y, et al. Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit$^+$IL-7R$^+$ Thy1$^+$ lympho-hemopoietic progenitors develop. J Exp Med 1996;**184**:1449–1459.
37. Hamada H, et al. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. J Immunol 2002;**168**:57–64.
38. Suzuki K, et al. Gut cryptopatches: direct evidence of extrathymic anatomical sites for intestinal T lymphopoiesis. Immunity 2000;**13**:691–702.
39. Saito H, et al. Generation of intestinal T cells from progenitors residing in gut cryptopatches. Science 1998;**280**:275–278.
40. Oida T, et al. Role of gut cryptopatches in early extrathymic maturation of intestinal intraepithelial T cells. J Immunol 2000;**164**:3616–3626.
41. Podd BS, et al. T cells in cryptopatch aggregates share TCR γ variable region junctional sequences with γδ T cells in the small intestinal epithelium of mice. J Immunol 2006;**176**:6532–6542.
42. Lambolez F, et al. Characterization of T cell differentiation in the murine gut. J Exp Med 2002;**195**:437–449.
43. Guy-Grand D, et al. Extrathymic T cell lymphopoiesis: ontogeny and contribution to gut intraepithelial lymphocytes in athymic and euthymic mice. J Exp Med 2003;**197**: 333–341.
44. Lin T, Matsuzaki G, Kenai H, Nomoto K. Progenies of fetal thymocytes are the major source of CD4$^-$CD8$\alpha\alpha^+$ intestinal intraepithelial lymphocytes early in ontogeny. Eur J Immunol 1994;**24**: 1785–1791.
45. Wang J, Klein JR. Thymus-neuroendocrine interactions in extrathymic T cell development. Science 1994;**265**:1860–1862.
46. Wang J, Whetsell M, Klein JR. Local hormone networks and intestinal T cell homeostasis. Science 1997;**275**:1937–1939.
47. Dejbakhsh-Jones S, Jerabek L, Weissman IL, Strober S. Extrathymic maturation of αβ T cells from hemopoietic stem cells. J Exp Med 1999;**155**:3338–3344.
48. Simizu T, et al. The majority of lymphocytes in the bone marrow, thymus and extrathymic T cells in the liver are generated in situ from their own preexisting precursors. Microbiol Immunol 1999;**43**:595–608.
49. Arcangeli M-L, et al. Extrathymic hemopoietic progenitors committed to T cell differentiation in the adult mouse. J Immunol 2005;**174**:1980–1988.
50. Eberl G, Littman DR. Thymic origin of intestinal αβ T cells revealed by fate mapping of RORγt$^+$ cells. Science 2004;**305**:248–251.
51. Maki K, Sunaga S, Ikuta K. The V-J recombination of T cell receptor-γ genes is blocked in interleukin-7 receptor-deficient mice. J Exp Med 1996;**184**:2423–2427.
52. Taylor RT, Lugering A, Newell AN, Williams IR. Intestinal cryptopatch formation in mice requires lymphotoxin α and the lymphotoxin β receptor. J Immunol 2004;**173**: 7183–7189.
53. Deusch K, Luling F, Reich K, Classen M, Wagner H, Pfeffer K. A major fraction of human intraepithelial lymphocytes simultaneously expresses the γ/δ T cell receptor, the CD8 accessory molecule and preferentially uses the Vδ1 gene segment. Eur J Immunol 1991;**21**:1053–1059.
54. Lundqvist C, Baranov V, Hammarstrom S, Athlin L, Hammarstrom ML. Intra-epithelial lymphocytes. Evidence for regional specialization and extrathymic T cell maturation in the human gut epithelium. Int Immunol 1995;**7**:1473–1487.
55. Jarry A, Cerf-Bensussan N, Brousse N, Selz F, Guy-Grand D. Subsets of CD3$^+$ (T cell receptor α/β or γ/δ) and CD3$^-$ lymphocytes isolated from normal human gut epithelium display phenotypical features different from their counterparts in peripheral blood. Eur J Immunol 1990;**20**:1097–1103.
56. Latthe M, Terry L, MacDonald TT. High frequency of CD8$\alpha\alpha$ homodimer-bearing T cells in human fetal intestine. Eur J Immunol 1994;**24**:1703–1705.
57. Lynch S, Kelleher D, McManus R, O'Farrelly C. RAG1 and RAG2 expression in human intestinal epithelium: evidence of extrathymic T cell differentiation. Eur J Immunol 1995;**25**:1143–1147.
58. Koningsberger JC, et al. TCR expression in human fetal intestine and identification of an early T cell receptor β-chain transcript. J Immunol 1997;**159**:1775–1782.
59. Howie D, et al. Extrathymic T cell differentiation in the human intestine early in life. J Immunol 1998;**161**:5862–5872.
60. Ramanathan S, Marandi L, Poussier P. Evidence for the extrathymic origin of intestinal TCRγδ$^+$ T cells in normal rats and for an impairment of this differentiation pathway in BB rats. J Immunol 2002;**168**: 2182–2187.

61. Hitotsumatsu O, et al. Identification and characterization of novel gut-associated lymphoid tissues in rat small intestine. J Gastroenterol 2005;**40**:956–963.
62. Mayrhofer G, Moghaddami M, Murphy C. Lymphocyte-filled villi (LFV): non-classical organized lymphoid tissues in the mucosa of the small intestine. Mucosal Immunol Update 1999;**7**:9–13.
63. Jemeson J, et al. A role for skin γδ T cells in wound repair. Science 2002;**296**:747–749.
64. Jemeson JM, Cauvi G, Deborah A, Witherden DA, Havran WL. A keratinocyte-responsive γδ TCR is necessary for dendritic epidermal T cell activation by damaged keratinocytes and maintenance in the epidermis. J Immunol 2004;**172**:3573–3579.
65. Ferguson A. Intraepithelial lymphocytes of the small intestine. Gut 1977;**18**:921–937.
66. Mysorekar IU, Lorenz RG, Gordon JI. A gnotobiotic transgenic mouse model for studying interactions between small intestinal enterocytes and intraepithelial lymphocytes. J Biol Chem 2002;**277**:37811–37819.
67. Rocha B, Vassalli P, Guy-Grand D. The Vβ repertoire of mouse gut homodimeric α $CD8^+$ intraepithelial T cell receptor α/β^- lymphocytes reveals a major extrathymic pathway of T cell differentiation. J Exp Med 1991;**173**:483–486.
68. Page ST, et al. Intestinal intraepithelial lymphocytes include precursors committed to the T cell receptor αβ lineage. Proc Natl Acad Sci USA 1998;**95**:9459–9464.
69. Poussier P, Edouard P, Lee C, Binnie M, Julius M. Thymus-independent development and negative selection of T cells expressing T cell receptor α/β in the intestinal epithelium: evidence for distinct circulation patterns of gut- and thymus-derived T lymphocytes. J Exp Med 1992;**176**:187–199.
70. Suzuki S, et al. Low level of mixing of partner cells seen in extrathymic T cells in the liver and intestine of parabiotic mice: its biological implication. Eur J Immunol 1998;**28**: 3719–3729.
71. Stappenbeck TS, Wong MH, Saam JR, Mysorekar IU, Gordon JI. Notes from some crypt watchers: regulation of renewal in the mouse intestinal epithelium. Curr Opin Cell Biol 1998;**10**:702–709.
72. Penney L, Kilshaw PJ, MacDonald TT. Regional variation in the proliferative rate and lifespan of αβ TCR^+ and γδ TCR^+ intraepithelial lymphocytes in the murine small intestine. Immunology 1995;**86**: 212–218.
73. Balk SP, et al. Oligoclonal expansion and CD1 recognition by human intestinal intraepithelial lymphocytes. Science 1991;**253**: 1411–1415.
74. Van Kerckhove, et al. Oligoclonality of human intestinal intraepithelial T cells. J Exp Med 1992;**175**:57–63.
75. Blumberg RS, Yockey CE, Grass GG, Ebert EC, Balk SP. Human intestinal intraepithelial lymphocytes are derived from a limited number of T cell clones that utilize multiple Vβ T cell receptor genes. J Immunol 1993;**150**:5144–5153.
76. Regnault A, Cumano A, Vassalli P, Guy-Grand D, Kourilsky P. Oligoclonal repertoire of the CD8αα and the CD8αβ TCR-α/β murine intestinal intraepithelial T lymphocytes: evidence for the random emergence of T cells. J Exp Med 1994;**180**:1345–1358.
77. Regnault A, et al. The expansion and selection of T cell receptor αβ intestinal intraepithelial clones. Eur J Immunol 1996;**26**:914–921.
78. Guy-Grand D, Malassis-Seris M, Briottet C, Vassalli P. Cytotoxic differentiation of mouse gut thymodependent and independent intraepithelial T lymphocytes is induced locally. Correlation between functional assays, presence of perforin and granzyme transcripts, and cytoplasmic granules. J Exp Med 1991;**173**:1549–1552.
79. Goodman T, Lefrancois L. Intraepithelial lymphocytes. Anatomical site, not T cell receptor form, dictates phenotype and function. J Exp Med 1989;**170**: 1569–1581.
80. Ishikawa H, Li Y, Abeliovich A, Yamamoto S, Kaufmann HE, Tonegawa S. Cytotoxic and interferon γ-producing activities of γδ T cells in the mouse intestinal epithelium are strain dependent. Proc Natl Acad Sci USA 1993;**90**:8204–8208.
81. Kawaguchi M, et al. Cytolytic activity of intestinal intraepithelial lymphocytes in germ-free mice is strain dependent and determined by T cells expressing γδ T-cell antigen receptors. Proc Natl Acad Sci USA 1993;**90**: 8591–8594.
82. Croitoru K, et al. Presence of intestinal lymphocytes in mice with severe combined immunodeficiency disease. Eur J Immunol 1990;**20**:645–651.
83. Takahashi-Iwanaga H, Iwanaga T, Sakamoto Y, Fujita T. Ultrastructural and time-lapse observation of intraepithelial lymphocytes in the small intestine of the guinea pig: their possible role in the removal of effete enterocytes. Cell Tissue Res 1995;**280**: 491–497.

GASTROENTEROLOGY 2007;133:559–573

Lamina Propria c-kit$^+$ Immune Precursors Reside in Human Adult Intestine and Differentiate Into Natural Killer Cells

HIROSHI CHINEN,*,‡ KATSUYOSHI MATSUOKA,* TOSHIRO SATO,* NOBUHIKO KAMADA,* SUSUMU OKAMOTO,* TADAKAZU HISAMATSU,* TAKU KOBAYASHI,* HIROTOSHI HASEGAWA,§ AKIRA SUGITA,¶ FUKUNORI KINJO,‡ JIRO FUJITA,‡ and TOSHIFUMI HIBI*

*Division of Gastroenterology and Hepatology, Department of Internal Medicine, School of Medicine, Keio University, Tokyo, Japan; ‡Department of Medicine and Therapeutics, Control and Prevention of Infectious Diseases, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan; §Department of Surgery, School of Medicine, Keio University, Tokyo, Japan; and ¶Department of Surgery, Yokohama Municipal Citizen's Hospital, Yokohama, Japan

Background & Aims: **Recent studies have revealed that murine intestinal mucosa contains several kinds of lineage markers (lin)$^-$ c-kit$^+$ immune precursor cells. However, immune precursors in the human adult intestine have not been studied extensively.** ***Methods:*** **Lamina propria mononuclear cells and intraepithelial lymphocytes from surgically resected human adult intestine were examined for the surface antigen expression and cytokine profile by immunohistochemistry and flow cytometry. The transcriptional profile of these cells was analyzed by reverse-transcription polymerase chain reaction. The phenotypic and functional characterization of the in vitro differentiating cells from the precursors was examined by flow cytometry.** ***Results:*** **We identified lin$^-$ c-kit$^+$ cells scattered throughout lamina propria of the human adult intestine. These intestinal immune precursors expressed CD34, CD38, CD33, interleukin-2Rα, and interleukin-7Rα, and they had much more abundant expression of Id2, PU.1, SpiB1, and lymphotoxin than thymocytes. The lin$^-$ c-kit$^+$ immune precursors mainly differentiated into CD56$^+$ c-kit^{dim} cells during in vitro culture. These in vitro differentiating cells corresponded to intestinal natural killer (NK) cells, which had distinct characteristics from their peripheral counterparts, such as CD83 and integrin α_E expression, less cytotoxic activity, and higher interferon-γ production. Furthermore, both c-kit^{dim} cells and NK cells were increased in lamina propria of Crohn's disease, although there was no change for peripheral blood NK cells.** ***Conclusions:*** **The human intestine may have the unique NK cell differentiation system, which may contribute to maintenance of immune homeostasis in the intestine.**

The cellular components of the immune system, such as T cells, B cells, monocytes, granulocytes, macrophages, dendritic cells, and natural killer (NK) cells, are derived from common hematopoietic stem cells (HSCs) in the bone marrow. As a first step, HSCs differentiate into 2 distinct subsets: common myeloid progenitors and common lymphoid progenitors. Although common myeloid progenitors ultimately differentiate into myeloid cells such as monocytes, granulocytes, macrophages, and dendritic cells,[1] common lymphoid progenitors differentiate into B-cell precursors and common T- and NK-cell precursors (T/NKPs).[2] T/NKPs subsequently differentiate into NKPs and T-cell precursors.[3–5] These steps are assumed to proceed mainly in the bone marrow, which is regarded as the most important site for primary immune cell differentiation.

A unique immune system has developed in the intestine. The intestinal immune system includes Peyer's patches, isolated lymphoid follicles, mesenteric lymph nodes (MLN), lamina propria mononuclear cells (LPMCs), and intraepithelial lymphocytes (IELs). This intestinal immune system maintains immunologic homeostasis against gut luminal antigens. In addition to these components, the intestine has become recognized as a site for differentiation of immune cells. Recent studies have revealed that murine intestinal mucosa contains immune precursor cells, which are lymphoid tissue inducer cells (LTi)[6,7] in the fetus and cryptopatch (CP) cells[8] in the adult. Both LTi and CP cells express c-kit, IL-7 receptor α subunit (IL-7Rα), IL-2Rα, CD44, and CD4^{dim}. These surface phenotypes are similar to those of common lymphoid progenitors in bone marrow, and LTi and CP cells have been reported to develop in situ into Peyer's patches[6,7,9] and extrathymic T cells of IELs,[10,11] respectively. In addition, a recent study suggested that CP cells can function as adult LTi by developing into isolated lymphoid folli-

Abbreviations used in this paper: **CP, cryptopatch; HSC, common hematopoietic stem cell; IENK, intraepithelial natural killer cell; IFN, interferon; IL, interleukin; IL-7Rα, IL-7 receptor α subunit; Lin, lineage markers; LPMCs, lamina propria mononuclear cells; LPNKs, lamina propria natural killer cells; LTi, lymphoid tissue inducer cells; MLN, mesenteric lymph nodes; NK, natural killer; PBL, peripheral blood lymphocytes; PBNKs, peripheral blood natural killer cells; PCR, polymerase chain reaction; pTα, pre-T cell receptor chain α; RAG, recombination activating gene; SEM, standard error of the mean; T/NKPs, common T and natural killer cell precursors; TNF, tumor necrosis factor.**

0016-5085/07/$32.00
doi:10.1053/j.gastro.2007.05.017

cles rather than IELs in normal adult mice.[12] The immune precursor cells in the murine intestine have been investigated extensively; however, only a few reports have referred to immune precursor cells in the human intestine. A recent study showed that $CD3^-$ $CD7^+$ cells in the human fetal intestine express messenger RNA (mRNA) for pre-T-cell receptor chain α (pTα), which is essential for early T-cell differentiation.[13] It also shows that these cells can give rise to $CD3^+$ T cells in vitro and in vivo, using severe combined immunodeficient (SCID) mice engrafted with human fetal intestine.[14] It also has been reported that recombination activating gene (RAG)-1 and RAG-2 mRNA can be detected in the intestinal mucosa of human infants.[15,16] Moreover, $CD3^-$ $CD2^+$ $CD7^+$ cells in the human adult jejunum have been shown to express RAG mRNA as well as pTα mRNA.[16] All these reports have examined intestinal immune precursors in light of extrathymic T-cell differentiation. However, considering the reports on the murine intestine, we assume that more immature immune precursor cells, such as LTi, also may reside in the human adult intestine.

To verify this hypothesis, we first analyzed human adult intestine immunohistochemically, focusing on expression of c-kit, which is a receptor for stem cell factor and is known to be expressed on immune precursor cells such as HSCs.[17] Although intensive analysis did not reveal any $c\text{-}kit^+$ cell clusters such as CP, we found a considerable number of $c\text{-}kit^+$ cells scattered in the lamina propria. We next characterized with flow cytometry these $c\text{-}kit^+$ cells in LPMCs isolated from human adult intestine, which revealed that the $c\text{-}kit^+$ cells in the intestine have phenotypes identical to T/NKPs in the fetal liver[18] and thymus.[19] The $c\text{-}kit^+$ cells mainly were committed to the NK cell lineage in vitro. We also found unique characteristics of mature NK cells residing in the human adult intestine. These results suggest that $c\text{-}kit^+$ cells should differentiate into intestinal NK cells. Furthermore, NK cell differentiation is accelerated in Crohn's disease (CD), indicating that this intestinal NK cell differentiation system may play a role in the pathogenesis of chronic intestinal inflammation. Thus, we were able to show differentiation of intestinal NK cells from $c\text{-}kit^+$ cells in the human adult intestine, which may contribute to maintenance of intestinal immune homeostasis.

Materials and Methods

Tissue Samples

Normal intestinal mucosa and MLN were obtained from macroscopically and microscopically unaffected areas of patients with colon cancer. Intestinal mucosa also was obtained from surgically resected specimens from patients with CD or ulcerative colitis (UC), diagnosed on the basis of clinical, radiographic, endoscopic, and histologic findings, according to established criteria.[20,21] In all samples from patients with CD or UC, the degree of inflammation was histologically moderate to severe. All experiments were approved by the institutional review board and written informed consent was obtained from all the patients.

Histologic Analysis

Tissue sections were treated according to well-established methods. Intestinal specimens were fixed with 4% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) and embedded in paraffin. Sections from paraffin-embedded blocks were deparaffinized and stained with H&E (Sakura Finetech Japan, Tokyo, Japan). For immunohistochemical staining, deparaffinized sections were heated at 100°C for 20 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) in a microwave oven. For the enzyme-labeled antibody method, each section was treated with 3% H_2O_2 (Wako) in 100% methanol and then incubated with normal rabbit serum (Nichirei Biosciences, Tokyo, Japan) for 15 minutes at room temperature to block nonspecific reactions. Thereafter, sections were treated with rabbit anti-human c-kit Ab (Dako Cytommation, Glostrup, Denmark) and incubated at 4°C overnight. Primary antibodies were washed out and sections were incubated with Histofine anti-rabbit Simplestain Max-PO (Nichirei), and visualized with 3-3'-diaaminobenzidine (Nichirei) for peroxidase and counterstained with hematoxylin. Sections incubated with the IgG fraction of normal rabbit serum (Dako) served as negative controls. For identification of mast cells, deparaffinized sections were stained with .05% toluidine blue solution, pH 4.1 (Wako). Mast cells were stained red-purple and other cells were stained blue.

Preparation of LPMCs, IELs, Peripheral Blood Lymphocytes (PBLs), and MLN Cells

LPMCs and IELs were isolated from intestinal specimens using modifications of previously described techniques.[22,23] Briefly, dissected mucosa was incubated in calcium and magnesium-free Hanks' balanced salt solution (Sigma-Aldrich, St. Louis, MO) containing 2.5% heat-inactivated fetal bovine serum (BioSource, Camarillo, CA) and 1 mmol/L dithiothreitol (Sigma-Aldrich) to remove mucus. The mucosa then was incubated in Hanks' balanced salt solution containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) for 60 minutes at 37°C. During this treatment, IELs and epithelial cells were removed from the tissue. Tissues were collected and incubated in Hanks' balanced salt solution containing .02% collagenase type 3 (Worthington Biochemical, Freehold, NJ) for 60 minutes at 37°C. The fraction was pelleted and resuspended in 40% Percoll solution (Amersham Biosciences, Piscataway, NJ), then layered on 60% Percoll before centrifugation at 2000 rpm for 20 minutes at room temperature. Viable LPMCs were recovered from the 40%–60% layer interface. For isolation of IELs, after EDTA treatment the supernatants were

collected and filtered through a glass-wool column to deplete cell debris and sticky cells. Cells were centrifuged over a 40%–60% Percoll solution density gradient. IELs were recovered from the layer interface. PBLs were isolated from heparinized peripheral blood samples by density gradient centrifugation using Lymphoprep (Nycomed Pharma, Oslo, Norway). For isolation of MLN cells, MLN were squeezed and passed through sterile nylon mesh to create single-lymphocyte suspensions.[24]

Giemsa Stain

The lin$^-$ c-kit$^+$ cells and the mast cells in LPMCs were sorted by Epics Altra with the HyPerSort cell sorting system (Beckman-Coulter, Fullerton, CA). The purity of the sorted cells was greater than 98% by postsorting analysis. After spreading the sorted cells on glass slides they were air dried, then the cells were fixed with methanol and stained with pH 6.4 Giemsa solution (Merck, Whitehouse Station, NJ), and they were observed by light microscope.

Flow Cytometric Analysis of c-kit$^+$ LPMCs Differentiation Markers

Cell surface fluorescence intensity was assessed using a FACSCalibur analyzer and analyzed with Cell Quest software (BD Biosciences, San Jose, CA). Dead cells were excluded with propidium iodide staining. The lineage marker monoclonal antibodies that were used were the available Lineage Cocktail 1 (BD Biosciences). Lineage Cocktail 1 included CD3 (SK7), CD14 (MϕP9), CD16 (3G8), CD19 (SJ25C1), CD20 (L27), and CD56 (NCAM16.2). All the antibodies were purchased from BD Biosciences except for CD2, CD20, CD56 (MEM188), and NKG2D, which were purchased from eBiosciences (San Diego, CA); CCR7, CXCR5, and IL-18Rα were purchased from R&D systems (Minneapolis, MN); CD133 was purchased from Miltenyi Biotec (Bergisch, Gladbach, Germany); and CX3CR1 was purchased from Medical & Biological Laboratories (Nagoya, Japan).

Quantitative Real-Time, Reverse-Transcription Polymerase Chain Reaction Analysis

Cells were sorted by Epics Altra with the HyPerSort cell sorting system (Beckman-Coulter). The purity of the sorted cells was always greater than 98% by postsorting analysis. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany), and total thymocyte RNA was purchased from BD Biosciences. Total RNA was treated with Qiagen DNase I to remove any contaminating genomic DNA. Absence of amplification of contaminating genomic DNA was ascertained by polymerase chain reaction (PCR) in which RNA was used as a template. Complementary DNA was synthesized using the Superscript first-strand synthesis system for reverse-transcription PCR (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Semiquantitative real-time, reverse-transcription PCR was performed using TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and on-demand gene-specific primers. The fluorogenic probes were as follows: RAG-1, RAG-2, preTCRα, Id2, PU.1, SpiB1, lymphotoxin α, lymphotoxin β, and β-actin, which all were purchased from Applied Biosystems. Cycling conditions for PCR amplification were 95°C for 10 minutes, followed by 45 cycles of 94°C for 15 seconds, and 60°C for 1 minute. Transcription of mRNA was assessed on a DNA Engine Opticon 2 System and analyzed with Opticon monitor software (MJ Research, Waltham, MA). All samples were analyzed in triplicate.

Lin$^-$ c-kit$^+$ LPMCs In Vitro Culture

CD3$^+$ and CD56$^+$ cells were removed from LPMCs using a magnetic cell-sorting system (MACS; Miltenyi Biotec) according to the manufacturer's instructions. The CD3$^-$ CD56$^-$ LPMCs were cultured at a concentration of 1×10^6/mL in complete medium consisting of 1640 RPMI (Sigma-Aldrich) supplemented with GlutaMAX (Invitrogen), 10% heat-inactivated fetal bovine serum (BioSource), 10 mmol/L HEPES (Invitrogen), 50 μmol/L 2-mercaptoethanol (Wako), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen). The cultures were maintained in a humidified atmosphere at 37°C in 5% CO_2 for 72 hours. All samples were cultured in duplicate.

Isolation of Peripheral Blood NK Cells and Lamina Propria NK Cells

CD56^{dim} (CD3$^-$ CD14$^-$ CD16$^+$ CD56$^+$) peripheral blood NK (PBNK) cells and CD56^{bright} (CD3$^-$ CD14$^-$ CD16$^-$ CD56$^+$) PBNKs were isolated from PBLs by using MACS (Miltenyi Biotec) according to the manufacturer's instructions. Lamina propria NK (LPNK) cells (CD3$^-$ CD14$^-$ CD56$^+$) also were isolated with MACS. The percentage of each isolated NK cell was evaluated by flow cytometry and routinely was greater than 95%.

Cytotoxicity Assay

The cytotoxicity of NK cell subsets against the NK-sensitive K562, a human erythroleukemic cell line (American Type Culture Collection, Rockville, MD), was measured by using a previously described protocol[25] with minor modifications.

NK Cell Cytokine Production

A total of 1×10^6 cells in 1 mL complete RPMI 1640 medium (Sigma-Aldrich) were stimulated with 10 ng/mL IL-12 (Medical & Biological Laboratories) and 100 ng/mL IL-15 (R&D) or 10 ng/mL IL-12 (Medical & Biological Laboratories) and 100 ng/mL IL-18 (Medical & Biological Laboratories) for 8 hours at 37°C. After stimulation, interferon-γ (IFN-γ) or tumor necrosis factor-α