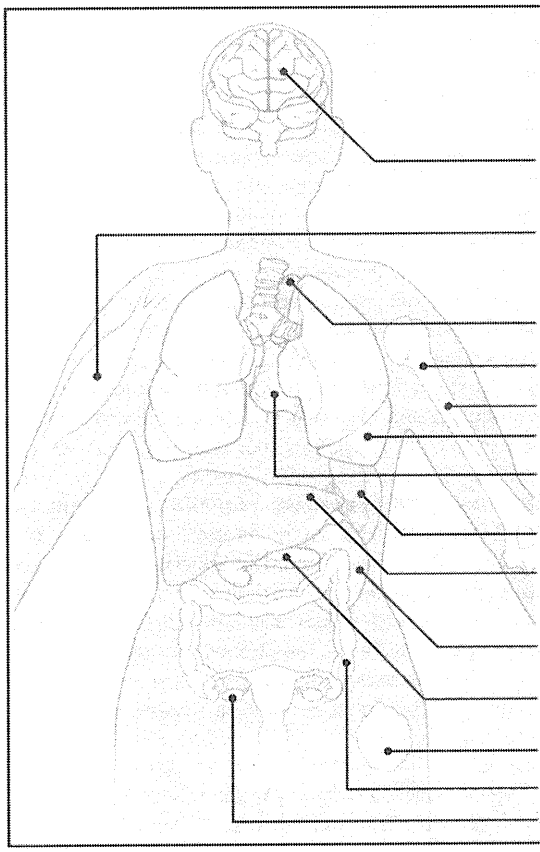

Organ	Roles of Autophagy	Diseases
General function	Starvation-induced amino acid production; constitutive turnover of cytoplasmic contents; selective degradation of p62, damaged mitochondria, microbes, etc.; life-span extension/antiaging	Tumor suppression and progression
Brain	Prevention of aggregate formation; Parkin-dependent mitophagy; regulation of food intake and energy balance	Parkinson disease Alzheimer disease
Muscle	Maintenance of muscle mass	Myopathies : (Danon disease, XMEA, Bethlem myopathies, UCMD) Lysosomal storage diseases
Thymus	Negative selection	
Bone marrow / Hematopoiesis	Erythropoiesis; maintenance of hematopoietic stem cell	
Bone		Paget disease?
Lung	Regulation of airway responsiveness	Cystic fibrosis
Heart	Adaptation to hemodynamic stress; prevention of age-dependent dysfunction	Cardiac hypertrophy
Immune/ lymphoid system	Regulation of cytokine production; development of T cells and B cells	Infection
Liver	Prevention of hepatocellular degeneration; suppression of hepatic tumors; lipid droplet elimination; gluconeogenesis	α_1-antitrypsin deficiency Hepatocellular carcinoma?
Kidney	Maintenance of podocyte and tubular cell integrity	
Pancreas	β-cell adaptation to high-fat diet; prevention of trypsine autoactivation	Diabetes Acute pancreatitis
Adipose tissue	Adipogenesis	Obesity
Intestine	Maintenance of Paneth cell function	Crohn disease
Embryos	Preimplantation development	

Figure 4. Physiological and Pathological Roles of Autophagy
Although this figure is illustrated for a human, these data have been obtained from both mouse and human. XMEA: X-linked myopathy with excess autophagy, UCMD: Ullrich congenital muscular disorder.

thymocytes, which leads to the elimination of some populations of self-reactive T cells (Nedjic et al., 2008) (Figure 4).

Tissue Homeostasis and Renovation in Health and Disease

Liver

Regardless of the nutritional situation, basal autophagy metabolizes cytoplasmic components to prevent accumulation of degenerated proteins and organelles (Figure 4). Loss of *Atg7* in mouse hepatocytes causes marked accumulation of swollen and deformed mitochondria and the appearance of concentric membranous structures consisting of ER. In addition, loss of *Atg7* leads to an increased number of peroxisomes and lipid droplets, as well as the formation of protein aggregates positive for p62 and ubiquitin (Komatsu et al., 2005). These mutant mice exhibit severe hepatomegaly (i.e., an enlarged liver) and hepatocytic hypertrophy, followed by hepatitis (Komatsu et al., 2005). This defective intracellular quality control in the liver appears to be most severe among various types of tissue-specific deletions of autophagy.

What directly causes these abnormalities? Simultaneous deletion of either *p62* or *Nrf2* dramatically attenuates the liver injury resulting from autophagy deficiency (Komatsu et al., 2007a, 2010). These results suggest that persistent activation of Nrf2, due to impaired turnover of p62, can account for most of the pathogenic changes seen in the livers of autophagy-deficient mice.

Autophagy could be a therapeutic target of α_1-antitrypsin deficiency. In affected children, misfolded mutant antitrypsin protein accumulates in the liver and causes liver damage and cirrhosis. Both proteasomal and autophagic pathways have been implicated in degradation of the mutant protein. A recent study demonstrated that an autophagy-activating drug, carbamazepine, decreases the amount of the mutant protein and ameliorates hepatic fibrosis in a mouse model of α_1-antitrypsin deficiency (Hidvegi et al., 2010). Such therapeutic strategies could be applied to a wide range of protein conformation disorders, such as Huntington and prion diseases.

Brain

Even under prolonged fasting conditions, the number of autophagosomes does not increase in nerve cells (Mizushima et al., 2004) probably because nerve cells are supplied with nutrients from peripheral organs. However, recent studies demonstrated that autophagy can be induced in cortical neurons, Purkinje cells, (Alirezaei et al., 2010), and hypothalamic neurons during fasting (Kaushik et al., 2011). This autophagic response in the hypothalamus induces lipophagy and upregulates agouti-related peptide (AgRP) expression, which increases food intake (Kaushik et al., 2011). Accordingly, deleting *Atg7* specifically

in AgRP neurons reduces body weight and adiposity. However, the opposite finding has been also reported; suppressing *Atg7* in the hypothalamus with short-hairpin RNAs causes obesity through the IKK-β/NF-κB pathway (Meng and Cai, 2011). Constitutive suppression of autophagy from the embryonic period versus transient suppression in the adult mice may produce different phenotypes, or distinct types of hypothalamic neurons have different autophagy functions.

In contrast to the rather complicated metabolic role of autophagy, the importance of autophagy as a "housekeeper" is easy to envision because neurons are terminally differentiated and do not divide (Figure 4). Mice lacking autophagy in the central nervous system exhibit neurologic deficits, such as abnormal limb-clasping reflexes, locomotor ataxia, and lack of motor coordination. They also display significant losses of large pyramidal neurons in the cerebral cortex and of Purkinje cells in the cerebellar cortex (Hara et al., 2006; Komatsu et al., 2006; Liang et al., 2010). Polyubiquitinated proteins and p62 accumulate in neurons in almost all regions of autophagy-defective brains, forming inclusion bodies whose size and number increase with aging (Hara et al., 2006; Komatsu et al., 2006, 2007a). Furthermore, mice with autophagy disrupted specifically in Purkinje cells exhibit progressive axonal dystrophy and degeneration of axon terminals, followed by Purkinje cell death and behavioral deficit. These results indicate that autophagy plays a cell-autonomous role in axonal homeostasis (Komatsu et al., 2007b; Nishiyama et al., 2007). Unlike the case of autophagy-deficient hepatocytes, the survival of autophagy-deficient neurons is restored only to a small degree by simultaneous deletion of p62, even though protein aggregates completely disappear in neuronal cells, as they do in hepatocytes (Komatsu et al., 2007a). Thus, neither p62 accumulation nor inclusion body formation explains the neurodegeneration and impaired neurological function of these mutant mice, and it is likely that autophagy plays a more general role in the brain.

Undoubtedly, the housekeeping role of autophagy becomes more evident when neurons are loaded with pathogenic proteins. These proteins include aggregate-prone mutant forms of α-synuclein that underlie Parkison disease and expanded polyglutamine (polyQ)-containing proteins that are responsible for Huntington disease and spinocerebellar ataxia (Figure 4) (Rubinsztein, 2006). Indeed, accumulation of autophagic vacuoles has been demonstrated in human neurodegenerative diseases (Lee et al., 2010; Nixon et al., 2008). To be degraded by the proteasome, substrate proteins must be unfolded prior to delivery into the narrow proteasomal chamber. However, the aggregated proteins are usually resistant to unfolding, and moreover, polyQ fragments could cause malfunction of the proteasome (Bence et al., 2001). Accordingly, clearance of misfolded, aggregate-prone proteins is highly dependent on autophagy. In fact, pharmacologic upregulation of autophagy has been shown to be effective in reducing neuronal aggregates and slowing the progression of neurological symptoms in animal models, such as Huntington disease and tauopathy models (Fleming et al., 2011).

Heart

Constitutively suppressing autophagy in the heart from the embryonic period does not initially cause an obvious phenotype in mice, but these animals spontaneously develop cardiomyopathy and die after 6 months of age (Taneike et al., 2010). Sarcomere structure is impaired and deformed in mice, and dysfunctional mitochondria accumulate in their cardiomyocytes. This housekeeping role of autophagy is more clearly observed when *Atg5* is disrupted specifically in the heart by tamoxifen; sudden ablation of the autophagic function in adult mice immediately causes cardiac hypertrophy and dysfunction, accompanied by the accumulation of ubiquitinated proteins (Nakai et al., 2007). Thus, constitutive basal autophagy is critically important for cardiac homeostasis (Figure 4).

Besides this basal role, autophagy can also be induced in the heart by pressure overload (Nakai et al., 2007; Zhu et al., 2007). Although the animals appear grossly normal at younger ages, constitutive knockout of *Atg5* in the heart sensitizes mice to cardiac dysfunction and dilatation following pressure overload (Nakai et al., 2007). This suggests that autophagy induction in the heart could be an adaptive response to hemodynamic stress. Paradoxically, heterozygous deletion of *Beclin 1*, which partially reduces autophagic activity, improves cardiac function upon pressure overload (Zhu et al., 2007). This suggests that fully activated autophagy could be a maladaptive response, whereas partial, but not complete, suppression of autophagy may be beneficial. Consistent with this hypothesis, partial suppression of autophagy with histone deacetylase (HDAC) inhibitors ameliorates pressure overload-induced cardiac hypertrophy in mice (Cao et al., 2011).

Skeletal Muscle

Mice with *Atg5* (Raben et al., 2008) or *Atg7* (Masiero et al., 2009) disrupted specifically in the skeletal muscle display age-dependent muscle atrophy. Like autophagy-defective cardiomyocytes, their muscle cells exhibit disorganized sarcomeres and the accumulation of p62, ubiquitinated proteins, and deformed mitochondria, confirming the homeostatic role of autophagy in skeletal muscle (Figure 4). Muscle atrophy can be induced by fasting and denervation, but autophagy does not appear to be involved in both of these atrophy processes. Instead, suppression of autophagy exacerbates fasting- and denervation-induced muscle atrophy (Masiero et al., 2009).

Autophagosomes have been frequently recognized as pathognomonic morphological hallmarks of numerous neuromuscular disorders, including Danon disease, X-linked myopathy with excess autophagy (XMEA), and lysosomal storage diseases (Figure 4) (Malicdan et al., 2008). Danon disease with an X-linked dominant inheritance pattern is characterized by cardiomyopathy, myopathy, and variable mental retardation. It is caused by mutations in the coding sequence of Lamp-2, which affects lysosomal function or autophagosome-lysosome fusion (Nishino et al., 2000). Patients with XMEA show similar morphological features to those with Danon disease, implying abnormality of lysosomal function (Malicdan et al., 2008).

Autophagy is also implicated in the pathogenesis of lysosomal storage diseases. Deleting acid alpha-glucosidase in mice is an animal model of Pompe disease, in which glycogen accumulation in lysosomes causes skeletal myopathy and cardiomyopathy. Autophagy may be one route by which cytosolic glycogen is delivered to the lysosome. Suppression of autophagy in the skeletal muscle of this mouse model reduces the glycogen content, and there is a remarkable additive effect

with enzyme-replacement therapy (administration of recombinant human acid alpha-glucosidase) (Raben et al., 2010). This raises the possibility that suppression of autophagy could be a new therapeutic approach for Pompe disease.

Bethlem myopathy and Ullrich congenital muscular disorder are caused by mutations in any of the three genes coding for the extracellular matrix protein collagen-6. Mice deficient in collagen-6 (*col6a1*) provide a model for these diseases. Loss of collagen-6 leads to an activation of Akt, which decreases autophagy through inactivation of the transcription factor FoxO3 and activation of mTOR (Grumati et al., 2010). Consequently, structurally altered organelles, including mitochondria and sarcoplasmic reticulum, accumulate in the muscles of the *col6a1* knockout mice, causing cell toxicity and death. Of note, induction of autophagy by treatment with a low-protein diet, rapamycin, or cyclosporin A clears the abnormal organelles and ameliorates the dystrophic phenotypes in *col6a1* knockout mice (Grumati et al., 2010).

Intestine

Crohn disease is one of the most common inflammatory diseases, and a link to the innate immune system has been established for this disease. However, several studies have identified an unexpected association between Crohn disease and a single-nucleotide polymorphism (SNP) of *Atg16L1* (threonine 300 is replaced by alanine) (Hampe et al., 2007; Rioux et al., 2007; The Wellcome Trust Case Control Consortium, 2007). To date, this is the only case in which a SNP in the core *ATG* genes has been associated with a human disease, and the finding highlights a potential importance of autophagy in intestinal biology (Figure 4). Later studies also identified an association between Crohn disease and IRGM (immunity-related GTPase family, M), which has also been implicated in autophagy against intracellular pathogens (McCarroll et al., 2008; Parkes et al., 2007).

However, the role of autophagy in the intestine and the pathogenesis of Crohn disease are not simple. Threonine 300 is located in the C-terminal WD repeat domain in Atg16L1. Because yeast Atg16 lacks the entire WD repeat domain, the N-terminal half of Atg16L1 may be sufficient for conventional autophagy, and the role of the WD domain remains unknown. One study suggested that the Atg16L1T300A mutant is defective in autophagic sequestration of intracellular bacteria but not in canonical autophagy (Kuballa et al., 2008). Another study, however, suggested that this mutant has intact activity in both canonical autophagy and antibacterial autophagy (Fujita et al., 2009). More experiments are required to determine how the T300A replacement modifies the molecular function of Atg16L1.

Furthermore, evidence from in vivo studies has suggested that autophagy or *ATG* genes have a role in maintaining the normal function of Paneth cells (Cadwell et al., 2008, 2010). Although complete systemic deletion of *Atg16L1* causes neonatal lethality (Saitoh et al., 2008), *Atg16L1* hypomorphic mice, which express a very low level of Atg16L1, are viable and grossly normal. However, these mice exhibit structurally abnormal Paneth cells with cytoplasmic lysozyme staining. In addition, Paneth cells normally possess granules containing antimicrobial peptides, but in the *Atg16L1* hypomorphic mice, these granules are disorganized (Cadwell et al., 2008). Crohn disease patients homozygous for the *ATG16L1* risk allele (T300A) show similar Paneth cell abnormalities as mice with *Atg5* knocked out specifically in the intestine. Furthermore, pathology of the Paneth cell can be dependent on viral infection and probably other environmental factors, as well (Cadwell et al., 2010). Thus, autophagy appears to be important for the integrity of vesicle secretion in Paneth cells.

In addition to these cell-intrinsic mechanisms, a link between autophagy and the immune system can also contribute to the pathogenesis of Crohn disease. This link occurs through a direct interaction between Atg16L1 and NOD2/CARD15 (which has the strongest association with Crohn disease) in the bacterial sensing pathway (Travassos et al., 2010) and through the regulation of inflammatory cytokine production from macrophages (Saitoh et al., 2008).

Pancreas

Constitutive suppression of autophagic activity in pancreatic β cells causes reduction in β cell mass, hypoinsulinemia, and the accumulation of ubiquitinated proteins, p62, and deformed organelles (i.e., mitochondria and ER) (Ebato et al., 2008; Jung et al., 2008). Thus, basal autophagy is important for maintenance of β cell volume and function (Figure 4). Furthermore, autophagy can be activated in β cells by free fatty acids. This process is considered to be an adaptive response, particularly in the presence of insulin resistance (Ebato et al., 2008).

By contrast, autophagy may exert a detrimental effect in the exocrine pancreas. Mice with *Atg5* deleted specifically in acinar cells grow normally and show no significant abnormalities of pancreatic histology and blood biochemistry, suggesting that basal autophagy may be dispensable in pancreatic acinar cells (Hashimoto et al., 2008). However, these mice are resistant to acute pancreatitis induced by cerulein (a cholecystokinin analog). Inappropriate activation of trypsinogen inside acinar cells, which is a cause of acute pancreatitis, is reduced in the absence of autophagy (Hashimoto et al., 2008). Together with the evidence that autophagosomes/autolysosomes accumulate in acute pancreatitis (Hashimoto et al., 2008; Mareninova et al., 2009), these data suggest that trypsinogen could be delivered to the lysosome by autophagy to become the active form in pathogenic settings (Figure 4). However, another study reported an opposite result in which blocking lysosomal function contributed to the pathogenesis of pancreatitis (Fortunato et al., 2009).

This apparent discrepancy could be resolved by the findings of future studies. Activity of cathepsin L, which can completely degrade trypsinogen, is severely reduced in pancreatitis. In contrast, activity of cathepsin B, which converts trypsinogen into active trypsin, seems to be relatively spared in the zymogen granule-containing fraction (Mareninova et al., 2009). This cathepsin "unbalance" could also explain why acute pancreatitis occurs only under special conditions and not following usual starvation, during which trypsinogen should be completely digested even if delivered to the lysosome.

Kidney

Podocytes (visceral epithelial cells) wrap around the capillaries of the glomerulus in the kidney, which help to filter blood. Podocytes display a high level of basal autophagy, implying that they might rely heavily on constitutive autophagic activity for their homeostasis (Mizushima et al., 2004). Consistent with this, podocyte-specific deletion of *Atg5* causes glomerulosclerosis

with an accumulation of intracellular ubiquitinated proteins in aging mice (Hartleben et al., 2010). In addition, these mice show increased susceptibility to proteinuric renal diseases caused by puromycin aminonucleoside and adriamycin, which normally upregulate autophagic activity in intact podocytes (Hartleben et al., 2010).

Autophagy also occurs in renal tubules. Mice with *Atg5* specifically deleted in the proximal tubules exhibit the accumulation of misfolded proteins and deformed organelles, and they are susceptible to ischemia-reperfusion injury (Kimura et al., 2011). These studies suggest that both podocytes and tubular cells require basal autophagy for homeostasis and adaptive autophagy to cope with stresses (Figure 4).

Lung

Deletion of *Atg7* in bronchial epithelial cells leads to the accumulation of p62 and activation of Nrf2. This results in hyper-responsiveness to cholinergic stimuli, suggesting that autophagy has a homeostatic role in lung epithelia (Figure 4) (Inoue et al., 2011). Autophagy also seems to be involved in the development of pulmonary diseases. Cystic fibrosis is a common recessive genetic disease, which is caused by a mutation in cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent chloride channel. A single amino acid deletion, called ΔF508-CFTR, causes the protein to misfold, leading to premature degradation or aggregation. As a result, secretory organs, such as the lung and pancreas, are affected. Airway epithelial cells from cystic fibrosis patients and mouse models of the disease have reduced autophagic activity likely because Beclin 1 is sequestered into cytoplasmic aggregates (Luciani et al., 2010). Impaired autophagy could be pathogenic because restoration of Beclin 1 expression rescues the cystic fibrosis phenotype in mice.

Bone

Although the physiological roles of autophagy in bone remain largely unknown, autophagy may be related to the development of bone diseases. Paget disease of bone is a chronic and metabolic bone disorder that is characterized by increased bone turnover within discrete lesions throughout the skeleton. Mutations in p62, which is a substrate of selective autophagy, have been frequently identified in Paget disease of bone; these mutations are predominantly in the UBA domain of p62 (Goode and Layfield, 2010). The p62 mutations cause increased osteoclastogenesis by activating TRAF6–NF-κB signaling (Figure 3) (Goode and Layfield, 2010). This suggests that the regulation of p62 levels by autophagy is important for bone formation.

Inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD) is a rare inherited disorder that exhibits multiple phenotypic features, including myopathy with rimmed vacuoles and bone disease. This disorder is caused by a mutation in valosin-containing protein (VCP)/p97. Although VCP has multiple functions, such as in ER-associated degradation, it also participates in the maturation of the autophagosome, and immature autophagosomes accumulate in IBMPFD muscles and IBMPFD mutant-expressing mice (Ju et al., 2009).

Tumor Formation and Progression

A tumor-suppressive role of autophagy has been implicated and appears to be particularly important in the liver. Spontaneous benign tumorigenesis is observed in the livers of mice with systemic mosaic deletion of Atg5 (i.e., the *Atg5* gene is deleted in only some populations of cells in various tissues) or with *Atg7* specifically disrupted in hepatocytes (Inami et al., 2011; Takamura et al., 2011). Interestingly, tumors are not formed in other organs in the *Atg5* mosaically deleted mice. Enlarged mitochondria, with at least partially impaired functions, accumulate in hepatocytes of *Atg5*- or *Atg7*-deficient mice (Inami et al., 2011; Takamura et al., 2011). As previously demonstrated in other cell types (Karantza-Wadsworth et al., 2007; Mathew et al., 2007), the oxidative stress responses and genomic damage responses are enhanced in autophagy-deficient livers. Accumulation of p62, at least partially, contributes to tumor growth because the size of the liver tumors in $Atg7^{-/-}$ mice is reduced by the simultaneous deletion of *p62* (Takamura et al., 2011). This may be due to dysregulation of NF-κB signaling (Mathew et al., 2009) and persistent activation of Nrf2 (DeNicola et al., 2011). Mice heterozygous for *Beclin 1*, who have significantly less autophagic activity, are also prone to cancer. These animals develop spontaneous tumors, including hepatocellular carcinoma, and they have an increased incidence of liver tumors after infection with hepatitis B virus (Qu et al., 2003; Yue et al., 2003).

Although autophagy functions as a tumor suppressor in nontumor cells or the early stages of tumor cell development, autophagy becomes important for cancer cell survival once tumors are established. Cancer cells have an increased metabolic demand (in terms of both energy source and building blocks) for proliferation, and they often need to grow under hypoxic conditions until angiogenesis is sufficiently established. Therefore, cancer cells, particularly those with Ras mutations, rely heavily on autophagy and are "addictive" to autophagy (Yang et al., 2011). Growth defects caused by autophagy suppression have also been observed in Myc-induced lymphoma and polyoma middle T-induced mammary tumor cells (Maclean et al., 2008; Wei et al., 2011). Autophagy can also be an adaptive response to chemotherapy (Amaravadi et al., 2007). However, the involvement of Ras is not simple; Ras-induced autophagy contributes to tumor suppression by inducing autophagic cell death and senescence (Elgendy et al., 2011; Young et al., 2009). The Ras-mediated autophagy might have different roles in tumor growth dependent on cellular context or cancer stage.

Given the role of autophagy in tumor progression, autophagy suppression could be a strategy for cancer treatment (Amaravadi et al., 2011; Janku et al., 2011). In fact, tumor cell death can be induced in mouse models using drugs that inhibit autophagy (e.g., the lysosome-inhibitory reagent chloroquine) in combination with conventional chemotherapy (Amaravadi et al., 2007; Degtyarev et al., 2008; Yang et al., 2011). After these basic studies were published, >20 clinical trials of autophagy-inhibiting drugs (i.e., hydroxychloroquine and chloroquine) were initiated (http://clinicaltrials.gov/ct2/results?term=autophagy) (Amaravadi et al., 2011).

One concern could be that, if autophagy is suppressed systemically, it may cause many adverse side effects because autophagy is critically important in almost all tissues. However, thus far, hydroxychloroquine seems to be tolerated (Amaravadi et al., 2011). It is possible that only a partial reduction in autophagic activity, which may show no apparent effect on normal cells,

could be beneficial in human diseases. This may be analogous to the effect of proteasome inhibitors: malignant myeloma cells that secrete immunoglobulin are more dependent on proteasomal degradation than normal cells, and the proteasome inhibitor bortezomib is very effective for the treatment of myeloma (Sánchez-Serrano, 2006).

Antiaging: Renovation of the Whole Organism

As autophagy has many effects on cellular renovation, it would be reasonable to assume that autophagy can contribute to whole-body rejuvenation. As discussed above, suppression of autophagy causes age-dependent dysfunction in various organs. Interestingly, many regimes that promote longevity, including calorie restriction, TOR suppression, sirtuin activation, and spermidine treatment, are able to induce autophagy (Madeo et al., 2010; Rubinsztein et al., 2011). The central question is whether this represents simply a correlation or whether autophagy is indeed one of the key effectors of these regimens. Genetic studies performed in *C. elegans* have shown that some of the autophagy-related genes are required for life-span extension induced by inhibition of insulin/IGF-like signaling and calorie restriction (Hansen et al., 2008; Hars et al., 2007; Jia and Levine, 2007; Meléndez et al., 2003; Tóth et al., 2008), although not all autophagy-related genes have a longevity-promoting effect (Hashimoto et al., 2009). Likewise, autophagy is also required for life-span extension induced by activation of sirtuins (higher eukaryote homologs of the yeast NAD^+-dependent deacetylase Sir2) (Morselli et al., 2010), silencing of TOR (Bjedov et al., 2010; Tóth et al., 2008), spermidine treatment (Eisenberg et al., 2009), and p53 suppression (Tavernarakis et al., 2008). These data indicate that autophagy is a common downstream effector in various life-prolonging signaling pathways. However, as other autophagy-independent pathways are also known to be important, how much autophagy contributes overall to the longevity effects of each regimen needs further investigation.

How can autophagy prolong life span? One obvious mechanism is the cell-autonomous function of autophagy, which avoids accumulation of toxic proteins (e.g., misfolded or aggregation-prone proteins) and organelles (e.g., damaged mitochondria). Additionally, autophagy could reduce inflammatory cytokine secretion and spontaneous tumor incidence, which may also account for its longevity-promoting effect (Madeo et al., 2010).

Conclusion

Cells routinely replace their contents to stay healthy but also to make morphological and functional changes. In this Review, we discussed diverse physiological and pathological processes from the perspective of "autophagy" as an intracellular renovation system. Such a multidisciplinary view is useful to understand why this "self-eating" system has been conserved throughout evolution, how it could participate in normal cellular regulation as well as pathogenesis of human diseases, and how we can take advantage of it for disease therapy. However, many fundamental questions remain. Even with the recent development of sophisticated research tools, such as Cre-mediated conditional knockout techniques, the physiological role of autophagy still remains unknown in some key organs, such as in the bone, skin, and blood vessels. Additionally, although selective autophagy substrates have been identified, the physiological significance of degradation of each substrate, particularly of ubiquitinated proteins, needs to be examined further.

Critical issues also remain with regard to autophagy in therapeutics and diagnostics. Effective indicators or biomarkers for autophagy activity are not currently available. Such markers are important to determine autophagic activity in the disease setting, particularly when monitoring drug effectiveness during autophagy-modulating therapy. Furthermore, the autophagy-modulating drugs currently available are not strictly specific, and the development of more specific drugs will be required. Likewise, although the upregulation of autophagy could be theoretically beneficial for eliminating aggregate-prone proteins, damaged mitochondria, and intracellular bacteria, how these selective autophagic pathways can be stimulated is another challenging issue. Nonetheless, the reality of autophagy-modulating therapy is now closer than was ever expected or predicted.

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Luminal CD4+ T Cells Penetrate Gut Epithelial Monolayers and Egress From Lamina Propria to Blood Circulation

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BACKGROUND & AIMS: The egress of memory T cells from peripheral tissues, such as lung and skin, into the draining lymph nodes requires their expression of CC chemokine receptor 7 (CCR7). In the intestine, resident memory T cells in the intestinal lamina propria (LP) do not express CCR7, indicating that they are tissue bound and do not exit the intestine. **METHODS:** We developed a cell transfer system, using rectal administration of lymphocytes to C57BL/6 mice. Lymphotoxin α-deficient mice were crossed with *RAG-2*$^{-/-}$ (recombination-activating gene-2) mice to generate *lymphotoxin α-deficient* × *RAG-2*$^{-/-}$ mice. **RESULTS:** Severe combined immunodeficient (SCID) or *RAG-2*$^{-/-}$ mice given rectal administration of splenic CD4+ T cells from normal mice developed colitis; the cells proliferated not only in the LP but also in spleen. SCID or *RAG-2*$^{-/-}$ mice given rectal administrations of CD4+ T cells that expressed green fluorescent protein (GFP+CD4+ T cells) localized to the LP within 6 hours but were not found in the spleen until 24 hours after administration. Immunohistochemical and electron microscopic analyses detected CD4+ T cells in the intraepithelial space just 3 hours after intrarectal administration. However, neither CCR7 deficiency nor the sphingosine-1-phosphate receptor agonist Fingolimod impaired the egress of CD4+ T cells from LP to systemic circulation. **CONCLUSIONS: CD4+ T cells not only penetrate from the luminal side of the intestine to the LP but also actively egress from the LP into the circulation. We developed a rectal administration system that might be used to further investigate cell trafficking in intestinal mucosa and to develop enema-based therapeutics for intestinal diseases.**

Keywords: T-Cell Migration; Localization; Mouse Model; Chemokine; Treatment.

BASIC AND TRANSLATIONAL AT

Although accumulated evidence has revealed how effector/memory T cells migrate to peripheral tissues, there are still many enigmas about how these cells egress from peripheral tissues to blood circulation. Egress of immune cells from nonlymphoid peripheral tissues is a critical step in lymphocyte migration as well as lymphocyte homing to these tissues.[1–4] It has been reported that the draining lymphatics of tissues contain substantial numbers of CD4+ and CD8+ lymphocytes, some of which are of memory phenotype,[5,6] but it remains unclear whether these memory lymphocytes are derived from cells that have egressed from peripheral tissues or from the blood via lymph nodes located at closer sites to peripheral tissues.

Recent reports suggest that the egress of effector or memory CD4+ and CD8+ T cells into the draining lymph nodes from the lung[7] and of B cells and naïve or memory CD4+ and CD8+ T cells into the popliteal lymph nodes from the footpad of skin[8] requires the expression of CCR7 on these cells. In the intestine, however, the unique phenotype (CCR9 or integrin $\alpha_4\beta_7$ or $\alpha_E\beta_7$-expressing cells) of the resident memory T cells and the lack of such cells elsewhere suggest that memory T cells in the intestinal lamina propria (LP) and intraepithelial space are tissue bound and do not exit the intestine,[1] but this theory remains unproven experimentally.

The intrarectal administration of cells employed in this study was suggested by the fact that intratracheal instillation of cells in mice can induce their cell migration to the lung and thereafter to the blood systemically.[6] In this paper, we demonstrate that living CD4+ T cells can not only penetrate intestinal barriers from the lumen to the LP but also constantly egress from the LP to the bloodstream constantly in a CCR7- and sphingosine 1-phosphate 1 ($S1P_1$)-independent manner.

Materials and Methods

Please see Supplementary Materials and Methods for more details.

Animals

C57BL/6 mice were purchased from Japan CLEA (Tokyo, Japan). C57BL/6-Ly5.1 and C57BL/6-RAG-2$^{-/-}$ mice were obtained from Taconic Laboratory (Hudson, NY) and Central Laboratories for Experimental Animals (Kawasaki, Japan). CCR7$^{-/-}$ mice were previously generated by M. Lipp (Max-Delbruck Cen-

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cells; GFP, green fluorescent protein; LP, lamina propria; MLN, mesenteric lymph node; NK, natural killer; PB, peripheral blood; SCID, severe combined immunodeficient; SP, spleen; S1P, sphingosine 1-phosphate; T_{EM}, effector-memory T; Tg, transgenic; T_R, regulatory T; UC, ulcerative colitis; WT, wild type.

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ter for Molecular Medicine, Berlin, Germany).[9] Green mice (CAG-green fluorescent protein [GFP] transgenic [Tg] mice) were originally generated by M. Okabe (Osaka University, Japan).[10] Lymphotoxin α (LT α)-deficient ($LT\alpha^{-/-}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and intercrossed into RAG-$2^{-/-}$ mice to generate $LT\alpha^{-/-}$ × RAG-$2^{-/-}$ mice. Mice were maintained under specific pathogen-free conditions in the Animal Care Facility of Tokyo Medical and Dental University. Donors and recipients were used at 6–12 weeks of age. All experiments were approved by the regional animal study committees and were done according to institutional guidelines and Home Office regulations.

Results

Substantial Numbers of Lymphocytes Reside in Crypt Abscesses of Inflamed Mucosa in Patients With Ulcerative Colitis

Accumulating evidence suggests an active moving state of immune compartments between intestinal epithelial cell barriers that separate the inside and outside of the body. For instance, recent reports demonstrated that murine CX3CR1+ dendritic cells (DC) beneath intestinal epithelial cells in small intestine penetrate epithelial barriers by extending dendrites into the luminal side through intraepithelial gaps to capture antigens including commensal bacteria.[11] In addition, it is well-known that large amounts of granulocytes accumulate in the crypt of inflamed mucosa of patients with human inflammatory bowel diseases, suggesting that granulocytes actively penetrate intestinal barriers into the luminal side under inflammatory conditions.[12] This accumulation is pathologically defined as a "crypt abscess," which is one feature of chronic inflammatory diseases of the intestine, such as ulcerative colitis (UC).[12] However, it remains largely unknown whether this is a case with other immune compartments, such as CD4+ T lymphocytes. We therefore re-evaluated immune cell compartments in crypt abscesses in colonic inflamed mucosa of patients with severe UC (Supplementary Table 1). As shown in Figure 1*A* and *B*, in addition to a major compartment, myeloperoxidase-positive granulocytes, we found that substantial numbers of CD4+ and CD8+ T cells and CD20+ B cells, but not CD56+ natural killer (NK) cells, reside in cell aggregates of crypt abscess, suggesting that these lymphocytes can actively commute across the intestinal epithelial barriers.

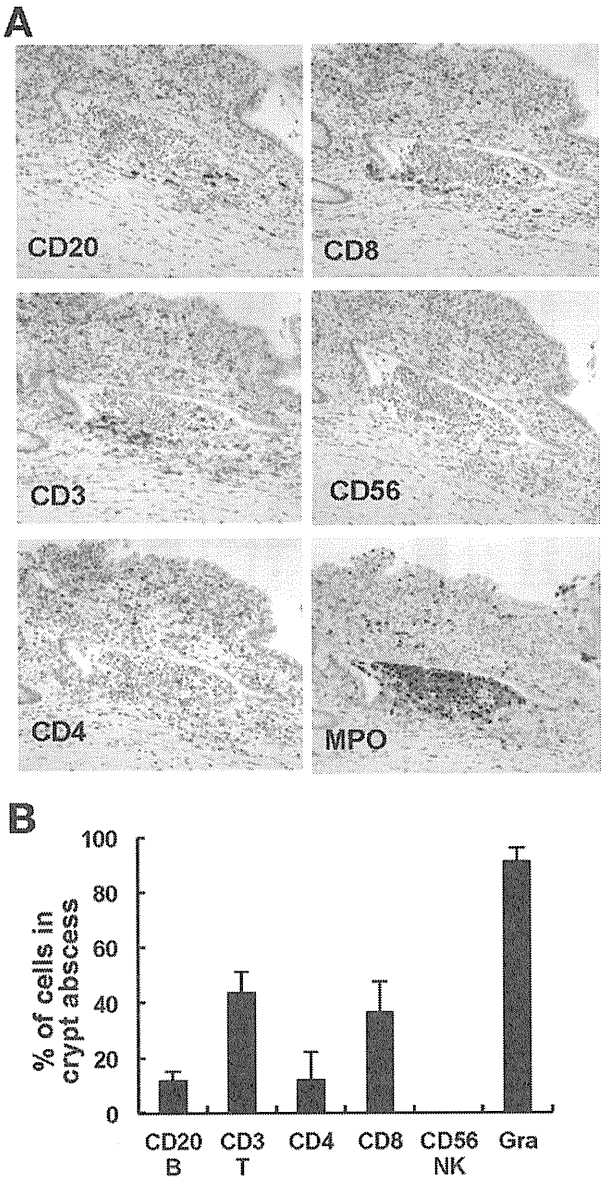

Figure 1. CD4+ T cells reside in crypt abscesses of inflamed mucosa of patients with ulcerative colitis (UC). (*A*) Immunohistochemistry of CD20, CD3, CD4, CD8, CD56, and myeloperoxidase (*MPO*) using samples of patients with severe UC. Four patients with UC (Supplementary Table 1) were examined. Sections were stained brown with a given antibody and counterstained with Mayer's hematoxylin. Representative section of crypt abscess with each stain. Original magnification, ×100. (*B*) Proportion of each compartment per total stained cell number (CD3+ + CD20+ + CD56+ + MPO+) in each crypt abscess.

SCID Mice Intrarectally Administered With Splenic CD4+ T Cells Developed Severe Colitis

Given the above results from human samples of crypt abscesses in inflamed mucosa of UC, we hypothesized that lymphocytes can actively move across intestinal epithelial barriers and therefore that intrarectally administered CD4+ T cells should, conversely, penetrate them from the lumen side. If so, this approach seems to be an ideal strategy to experimentally determine whether intestinal LP CD4+ T cells can thereafter egress LP to the systemic circulation. However, because a huge number of intestinal bacteria, over 10^3 million per mouse constantly occupy the intestinal lumen,[13–15] no attempts to intrarectally administer the cells have yet been reported. To test our hypothesis, we administered splenic CD4+ T cells from normal BALB/c mice intrarectally or intraperitoneally (as a control) into C.B-17 SCID mice (Figure 2*A*). This adoptive transfer system into immunodeficient recipients was adopted because it can be easily assessed whether intrarectally administered cells can penetrate intestinal barriers to the LP by amplifying the cell number of pen-

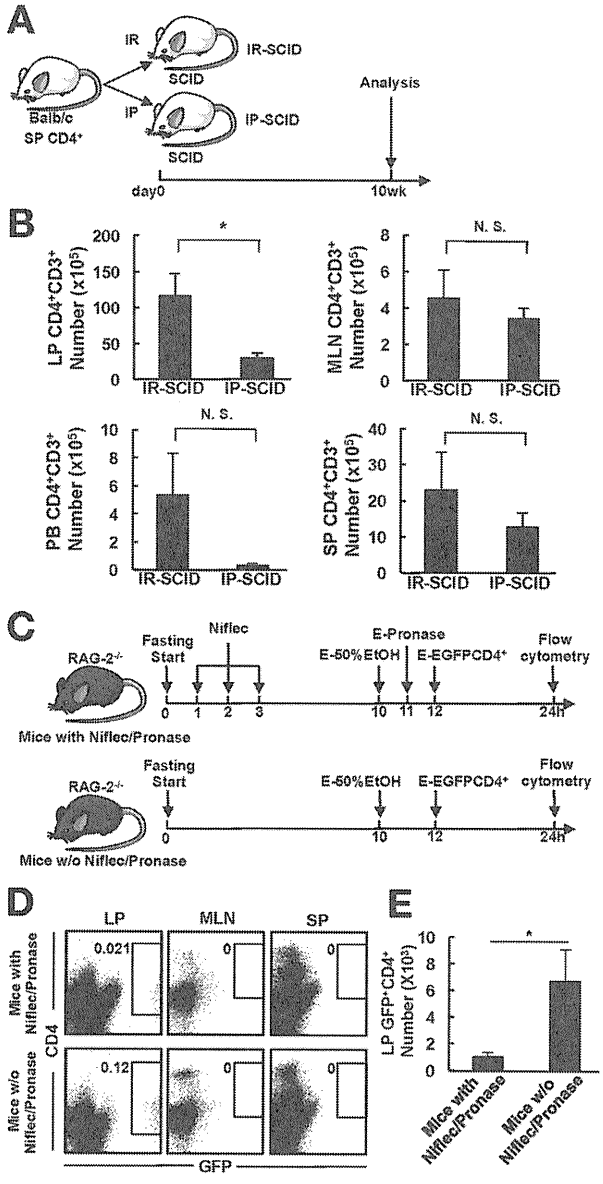

Figure 2. Intrarectally administrated splenic CD4+ T cells can penetrate gut epithelial monolayers and expand not only in colonic lamina propria but also outside the intestine. (*A*) C.B-17 SCID recipient mice were administered with splenic CD4+ T cells from normal BALB/c mice intrarectally (5×10^6, *IR-SCID mice*, n = 9) or intraperitoneally (5×10^5, *IP-SCID mice*, n = 9). (*B*) LP, MLN, and SP $CD3^+CD4^+$ T cells were isolated from the colon at 10 weeks after T-cell administration, and the number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± standard error (SEM) of mean of 9 mice in each group. $^*P < .01$. (*C*) RAG-$2^{-/-}$ mice were pretreated just like protocol of Supplementary Figure 1 (Mice with Niflec/Pronase) or without the pretreatments of Niflec and pronase (*Mice w/o Niflec/Pronase*) before cell administration. RAG-$2^{-/-}$ mice in each group were administered with 5×10^6 $CD4^+$ T cells from EGFP-Tg mice (n = 10 in each group). (*D*) LP, MLN, and SP GFP^+CD4^+ cells were isolated from the colon at 24 hours after cell administration, and the absolute number of GFP^+CD4^+ cells was determined by FACS. *Dot plot* analysis data of FACS is representative one of each group. Representatives of 10 separate samples in each group. (*E*) The absolute number of LP GFP^+CD4^+ cells. Data are indicated as the mean ± SEM of 9 mice in each group. $^*P = .034$.

etrating $CD4^+$ T cells in a mechanism of lymphopenia-driven rapid proliferation in a long-term observation.[16] As a protocol of intestinal preparation, mice were maintained without feeding for 3 hours before cell administration and were given 1 mL of Niflec water (Ajinomoto Pharma Co, Tokyo, Japan) 3 times at intervals of 1 hour by oral catheter to remove the resident stool. Mice were then pretreated with or without 50% ethanol enema and thereafter with 5% pronase enema at 1 hour before cell administration (Supplementary Figure 1).

Consistent with previous reports,[17] SCID mice intraperitoneally administered with splenic $CD4^+$ T cells (hereafter called IP-SCID mice) were healthy throughout the observation period and showed no clinical symptoms of colitis as estimated by clinical score, possibly because of the presence of regulatory T (T_R) cells in the donor $CD4^+$ T-cell preparation (Supplementary Figure 2*A* and *B*). Surprisingly, SCID mice intrarectally administered with $CD4^+$ T cells (IR-SCID mice) developed severe colitis with diarrhea, anorectal prolapses, hunched posture, and weight loss in spite of the presence of T_R cells in the donor $CD4^+$ T cells (data not shown). Ten weeks after cell administration, the colon from IR-SCID mice, but not from IP-SCID mice, was enlarged and had a greatly thickened wall (Supplementary Figure 2*A*). The difference in clinical scores at 10 weeks after administration between the 2 groups was significant (Supplementary Figure 2*B*). Histologic examination showed prominent epithelial hyperplasia and loss of goblet cells with massive infiltration of mononuclear cells in the LP of colon from IR-SCID mice but not from IP-SCID mice (Supplementary Figure 2*C*). The difference in histologic scores between the 2 groups was also significant (Supplementary Figure 2*D*).

A further evaluation of $CD4^+$ T-cell infiltration was made by assessing the absolute number of LP $CD3^+CD4^+$ T cells. No $CD3^+CD4^+$ T cells were recovered from the colonic tissue of SCID mice without cell administration (data not shown). The absolute number of colonic LP $CD4^+$ T cells recovered from IR-SCID mice, but not from IP-SCID mice, far exceeded the number of originally injected cells (Figure 2*B*), indicating penetration of $CD4^+$ T cells from the lumen side to the LP and extensive expansion in the colon in IR-SCID mice during 10 weeks after administration. Furthermore, it was of note that substantial numbers of $CD3^+CD4^+$ T cells were also recovered from mesenteric lymph node (MLN), peripheral blood (PB), and spleen (SP) of IR-SCID mice (Figure 2*B*), suggesting that some of the LP $CD3^+CD4^+$ T cells in the colon of IR-SCID mice could not only penetrate the LP but also egress from LP to systemic circulation. Flow cytometric analysis revealed that $CD3^+CD4^+$ T cells recovered from the LP of IR-SCID or IP-SCID mice are mostly of $CD44^+CD62L^-$ effector-memory T (T_{EM}) phenotype irrespective of the presence or absence of colitis (Supplementary Figure 2*E*). To physiologically adopt this cell transfer system in vivo, we also performed it without ethanol preadministration and recovered a small but substantial number of $CD3^+CD4^+$ T cells from the LP,

MLN, PB, SP, and bone marrow (BM) of IR-SCID mice 10 weeks after administration (Supplementary Figure 3). We also developed simpler, but surprisingly, more efficient intrarectal cell transfer protocol without the pretreatments of Niflec and pronase as demonstrated in Figure 2*C–E*.

The evidence that intrarectal, but not intraperitoneal, administration of splenic $CD4^+$ T cells induced colitis was surprising (Figure 2), although this donor population contains a substantial number of T_R cells (approximately 10% in total $CD4^+$ T cells, data not shown). To assess this issue, we next administered the same number of whole splenic $CD4^+$ T cells or T_R cell-depleted $CD4^+CD25^-$ T cells into SCID mice (Supplementary Figure 4*A*). As expected, the 2 groups of mice similarly developed colitis (data not shown) and had comparable absolute numbers of $CD3^+CD4^+$ T cells in the LP, MLN, PB, SP, and BM at 7 weeks after cell administration (Supplementary Figure 4*B*), suggesting 3 possibilities: (1) naïve $CD4^+$ T, but not T_R cells, penetrate epithelial barriers; (2) both T_R cells and naïve $CD4^+$ T cells penetrate epithelial barriers, but T_R cells cannot suppress the expansion of naïve $CD4^+$ T cells to become colitogenic $CD4^+$ T cells in the LP; and (3) both T_R cells and naïve $CD4^+$ T cells penetrate epithelial barriers, but naïve $CD4^+$ T cells alone egress the LP and are instructed in MLN to become gut-homing receptor-expressing colitogenic $CD4^+$ T cells (see the following section).

We also investigated whether other lymphocytes can penetrate intestinal epithelial monolayer and egress from LP or not. For this purpose, 1×10^8 splenocytes from $Ly5.1^+$ C57BL/6 mice were intrarectally administered to $Ly5.2^+$ RAG-$2^{-/-}$ recipients ($Ly5.1^+$ IR), and 1×10^7 splenocytes from C57BL/6 mice were intravenously administered to RAG-2 recipients ($Ly5.1^+$ IV). Splenocytes were recovered from mice in each group at 4 weeks after cell administration, and the number of $Ly5.1^+CD3^+CD4^+$ ($CD4^+$ T), $Ly5.1^+CD3^+CD8^+$ ($CD8^+$ T), $Ly5.1^+CD3^+NK1.1^+$ (NKT), $Ly5.1^+CD3^-NK1.1^+$ (NK), $B220^+$ (B) cells were determined by fluorescence-activated cell sorter (Supplementary Figure 5*A*). We confirmed that other lymphocytes such as $CD8^+$ T, B, NK, and NKT cells were also detected in the SP of transferred mice, although the numbers of these cells are lower than that of $CD4^+$ T cells (Supplementary Figure 5*B*).

SCID Mice Intrarectally Transferred With Colitogenic $CD4^+$ T_{EM} Cells Developed Severe Colitis

To characterize further the penetration between epithelial barriers and egress from the LP of $CD4^+$ T cells without the impact of the initiation phase of naïve $CD4^+$ T cells, we next intrarectally administered the same number of colitogenic $CD4^+$ T cells obtained from colitic mice previously transferred with $CD4^+CD45RB^{high}$ T cells or splenic $CD4^+$ T cells from normal mice into SCID mice (Figure 3*A*). Both groups developed colitis as evidenced by clinical and histologic scores, and the recovered number of LP $CD4^+$ T cells (Figure 3*B–D*), suggesting that colitogenic memory $CD4^+$ T cells also can penetrate from the intestinal lumen to the LP following intrarectal administration. However, it was of note that the numbers of $CD4^+$ T cells recovered from MLN, PB, and BM of mice intrarectally administered with colitogenic $CD4^+$ T cells were significantly lower than those of mice administered with splenic $CD4^+$ T cells (Figure 3*D*), suggesting that the former cells have a tendency to remain in the LP and not easily egress to afferent lymphatics. Regarding the role of $CD4^+CD25^+Foxp3^+$ T_R cells in their penetration between epithelial barriers and egress from the LP, it was notable that approximately normal proportion of $CD4^+Foxp3^+$ T_R cells resided in the LP and SP of mice administered with splenic $CD4^+$ T cells, but not with colitogenic $CD4^+$ T cells (Figure 3*E*), suggesting that intrarectally administered T_R cells not only penetrate epithelial barriers but also egress the LP.

Short Time Course Analysis of Intrarectrally Administered $CD4^+$ T Cells

To clarify that intrarectally administered $CD4^+$ T cells continue to reside in the intraepithelial space or LP of mice without the impact of cell proliferation in the lymphopenic condition, we next administered splenic $CD4^+$ T cells into RAG-$2^{-/-}$ mice (hereafter referred to as IR-RAG mice) and visualized the localization of $CD4^+$ T cells by immunohistochemistry at the early time points, 3, 12, and 24 hours after administration. As shown in Figure 4*A*, (1) $CD4^+$ T cells (red) adhering to epithelial cells (green) from the lumen side were detected at 3 hours after administration; (2) many $CD4^+$ T cells were found to reside in the intraepithelial space, and a few $CD4^+$ T cells were detected in the LP at 12 hours after administration; and (3), thereafter, $CD4^+$ T cells were as abundant in the LP as in the epithelial space at 24 hours after administration. To determine further whether $CD4^+$ T cells could penetrate to intestinal barriers from the lumen side, we next conducted an electron microscopic analysis using intestinal samples at 6 hours after administration. As expected, we found that small lymphocytes with round nuclei and smooth surfaces resided in the intraepithelial space with normal epithelial structure in IR-RAG mice (Figure 4*B*).

To rule out the possibility that intrarectally administered cells might have directly entered the small blood vessels that were exposed to the damaged intestinal lumen by the ethanol treatment, we next checked the time course of the first emergence of $CD4^+$ T cells from the LP, MLN, and SP. To this end, splenic $CD4^+$ T cells from CAG-GFP Tg mice were administered intravenously or intrarectally to RAG-$2^{-/-}$ mice to precisely determine the absolute number of $CD4^+$ T cells. In RAG-$2^{-/-}$ mice intravenously administered with $CD4^+$ T cells (GFP-IV mice), GFP^+ cells were detected in MLN and SP, but not the LP, at 6 hours or 24 hours after administration and in the LP as well as MLN and SP at 168 hours after administration (Figure 5). In contrast, in RAG-$2^{-/-}$ mice intrarectally administered with $CD4^+$ T cells (GFP-IR mice), GFP^+ cells were found only in the LP or MLN, but not in the SP, at 6 hours after admin-

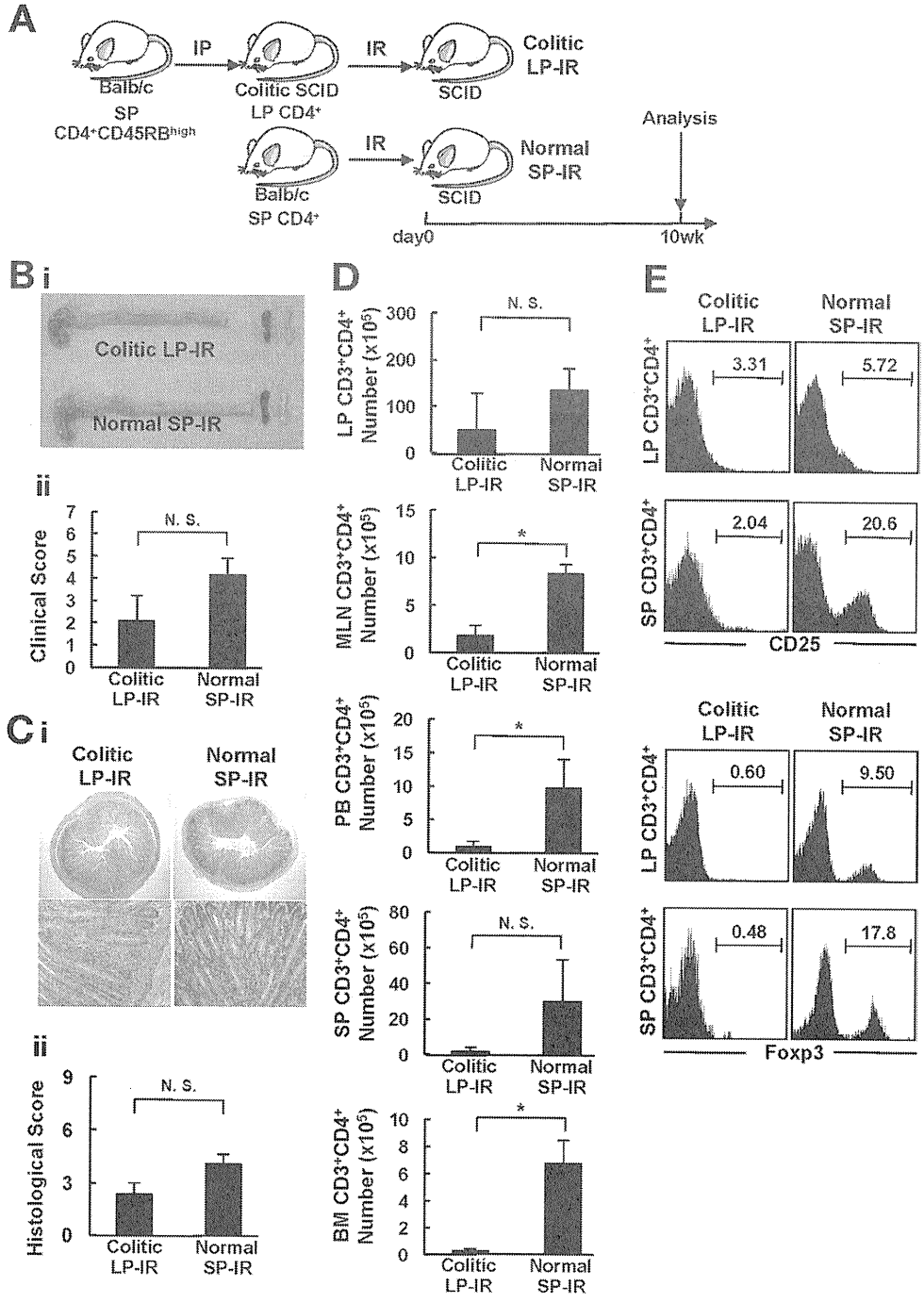

Figure 3. Intrarectally administered colitogenic memory $CD4^+$ T cells not only penetrate intestinal epithelial barriers and reside in the LP but also migrate to MLN and SP. (*A*) SCID recipient mice were intrarectally administered $CD4^+$ T cells from normal BALB/c mice (5×10^6, *Normal SP-IR mice*, n = 7) or $CD4^+$ T cells from colitic SCID mice previously transferred with $CD4^+CD45RB^{high}$ T cells (5×10^6, *Colitic LP-IR mice*, n = 7). (*B-a*) Gross appearance of the colon from *Normal SP-IR* mice and *Colitic LP-IR* mice at 10 weeks after cell administration. (*B-b*) Clinical scores were determined at 10 weeks after administration as described in the Materials and Methods section. Data are indicated as the mean ± standard error of mean (SEM) of 7 mice in each group. $^*P < .01$. (*C-a*) Histologic examination of the colon at 10 weeks after administration. Original magnification, ×40 (*upper panels*) and ×100 (*lower panels*). (*C-b*) Histologic scores were determined at 10 weeks after transfer as described in the Materials and Methods section. Data are indicated as mean ± SEM of 7 mice in each group. $^*P < .05$. (*D*) LP, MLN, and spleen $CD3^+CD4^+$ T cells were isolated from the colon at 10 weeks after cell administration, and the number of $CD3^+CD4^+$ cells was determined by flow cytometry. Data are indicated as the mean ± SEM of 7 mice in each group. $^*P < .01$. (*E*) Phenotypic characterization of $CD3^+CD4^+$-gated T cells expressing $CD25^+$ or $Foxp3^+$ T_R cells in SP and LP of the mice in each group by FACS. Representatives of 7 separate samples in each group.

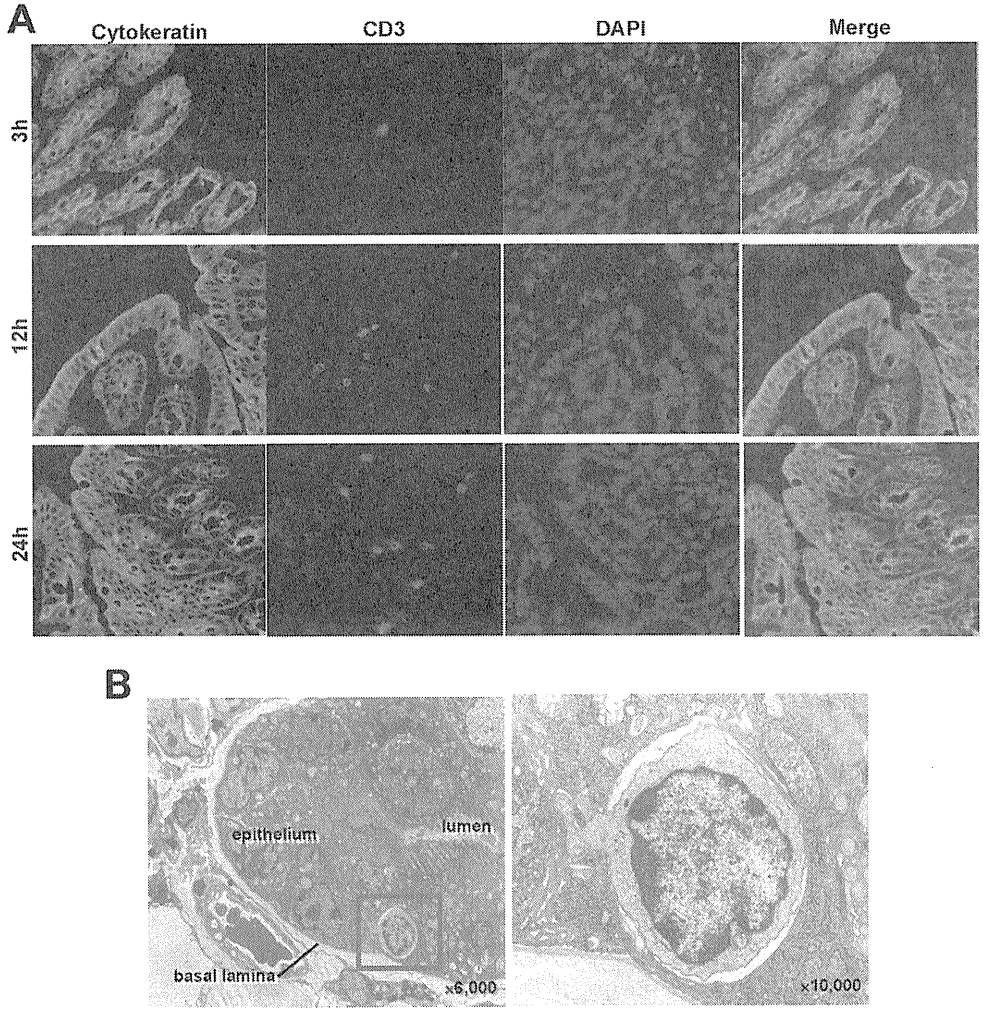

Figure 4. CD4+ T cells penetrate epithelial barriers in colon. Splenic CD4+ T cells from Ly5.1-background C57BL/6 mice were intrarectally administered to Ly5.2-background RAG-$2^{-/-}$ mice. Mice were killed at 3, 12, or 24 hours after administration. (*A*) Immunostaining of cytokeratin (*green*), CD3 (*red*), and 4'6-diamidino-2-phenylindole (*DAPI*) (*blue*) counterstaining. Representative of 4 separate samples in each group. Original magnification, ×100. (*B*) Electron microscopic analysis using intestinal samples at 3 hours after administration. Representative of 2 separate samples. Red square in the left panel was zoomed up to right panel. Original magnification, ×6000 (*left*) and ×10,000 (*right*).

istration and subsequently in the LP, MLN, and SP at 24 and 168 hours after administration (Figure 5). Similar emergence of intrarectally administered CD4+ T cells first in the LP and later in the MLN and SP was confirmed in a similar experiment using immunosufficient C57/BL6 recipient mice (Figure 6). These results suggest that cells administered intrarectally were transferred from the LP to SP via afferent lymphatics to MLN but not directly via blood vessels exposed by ethanol treatment.

We further performed long-term experiment with GFP+ donor/RAG-$2^{-/-}$ recipient system (Supplementary Figure 6*A*) in addition to CD4+ donor/SCID recipient system (Figure 2) because it was possible that the existence of leaky CD4+ T cells in SCID recipient mice affected our previous results. Ten weeks after the transfer, intrarectally, but not intravenously, administered GFP+CD4+ T cells expanded not only in colonic LP but also in the MLN, SP and BM, which led to develop colitis in RAG-$2^{-/-}$ recipient mice (Supplementary Figure 6*B–F*). We further checked cell surface markers of endogenous immune cells in addition to CD4 by fluorescence-activated cell sorter to clarify the accuracy of intrarectal administration of GFP+CD4+ cells. Indeed, CD4+ cells contained both GFP+ and GFP− cells, CD3-gated CD4+ T cells exclusively resided in GFP+ subpopulation, but not in GFP− subpopulation (Supplementary Figure 6*G*), suggesting that GFP-CD4+ cells are non-T cells, such as CD4-expressing macrophages and lymphoid tissue inducer cells. In contrast, NK1.1+, Gr1+, CD11b+, and CD11c+ cells preferentially resided in GFP− cells (Supplementary Figure 6*H*), concluding that GFP+ CD3+CD4+ T cells were all exogenous, but other immune compartments were all endogenous (Supplementary Figure 6*G*).

To further rule out the possibility that CD4+ T cells egress through the colonic lymphoid organ in the intestine such as isolated lymphoid follicle, we performed intrarectally administration of CD4+ T cells using LT$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice, which lack such lymphoid organ. LT$\alpha^{-/-}$ × RAG-$2^{-/-}$ mice or RAG-$2^{-/-}$ mice were intrarectally administered with splenic CD4+ T cells isolated from CAG-GFP Tg mice and were killed at 14 days after administration to assess the localization of CD4+ T cells (Supplementary Figure 7*A*). The absolute numbers of CD4+ T cells recovered from the LP, SP, and BM of

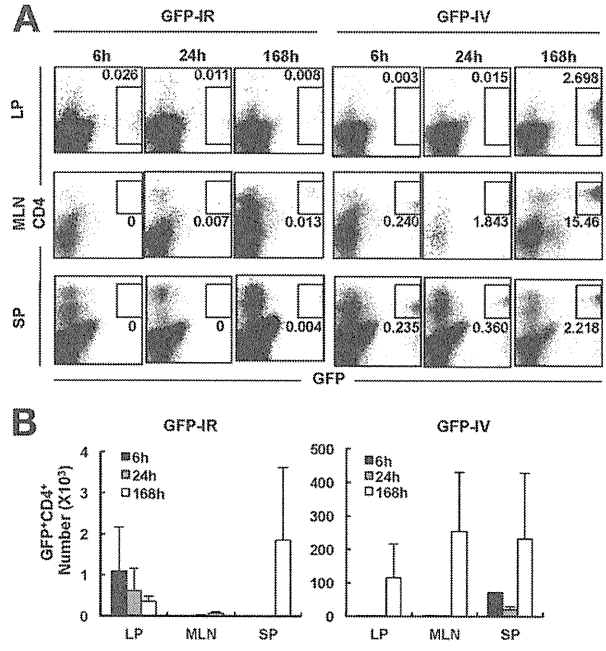

Figure 5. Intrarectally administered cells emerged in the LP and MLN before emerging in SP of recipient mice. (*A*) RAG-2$^{-/-}$ mice were administered CD4$^+$ T cells from CAG-GFP Tg mice intrarectally (1 × 10^7, *GFP-IR mice*, n = 4 at each time point) or intravenously (1 × 10^6, *GFP-IV mice*, n = 4 at each time point) and were killed at 6, 24, or 168 hours after administration. Representatives of 4 separate samples in each group. (*B*) Cells were isolated from LP, MLN, and SP at 6, 24, or 168 hours after administration, and the number of GFP$^+$ cells was determined by flow cytometory. Data are indicated as the mean ± standard error of mean of 4 mice in each group.

LT$\alpha^{-/-}$ × RAG-2$^{-/-}$ mice were equivalent to the paired absolute numbers from RAG-2$^{-/-}$ mice, suggesting that egress of CD4$^+$ T cells from LP is independent of gut-associated lymphoid tissue (GALT).

CD4$^+$ T Cells Egress From the Intestinal LP in CCR7- and S1P$_1$-Independent Manner

Given the evidence that administered CD4$^+$ T cells reside not only in the LP but also in MLN and SP of IR mice, we next attempted to investigate the molecular mechanism of egress of CD4$^+$ T cells from the intestinal LP. Recent reports have demonstrated that CCR7 plays a key role in the return of T cells to lymph node from peripheral tissues, such as lung and skin.[7,8] To assess whether this is the case with the egress of CD4$^+$ T cells from the LP, RAG-2$^{-/-}$ mice were intrarectally administered with splenic CD4$^+$ T cells isolated from wild-type (WT) (WT-IR) or CCR7$^{-/-}$ mice (CCR7-IR) and were killed at 14 days after administration (Figure 7*A*). Consistent with the above results, substantial numbers of CD4$^+$ T cells were recovered from the LP of WT-IR and CCR7-IR mice (Figure 7*B*). Notably, the numbers of CD4$^+$ T cells recovered from the LP, MLN, PB, SP, and BM of CCR7-IR mice were equivalent to paired numbers from WT-IR mice (Figure 7*B*), suggesting that egress from LP is mediated in a CCR7-independent manner. In addition to the above experiment, we performed another experiment to assess the possibility that egress of CD4$^+$ T cells from LP is mediated by S1P, which regulate the trafficking of lymphocytes in secondary lymphoid organs. RAG-2$^{-/-}$ mice were pretreated with phosphate-buffered saline or sphingosine-1-phosphate receptor agonist (FTY720) (1.0 mg/kg), which is the agonist of S1P receptor, daily starting 1 day before the transfer over a period of 2 weeks and were administered 3 × 10^6 CD4$^+$ T cells from WT C57BL/6. They were killed at 14 days after administration (Figure 7*C*). The number of CD4$^+$ T cells recovered from each organ of FTY720-treated mice was equivalent to paired number from control mice (Figure 7*D*), suggesting that CD4$^+$ T cells recovered from FTY720-treated mice could penetrate the gut wall and egress from the LP, and the egress of CD4$^+$ T cells from LP to afferent lymphatics is not mediated by S1P$_1$-dependent manner either.

Discussion

In the present study, by a series of intrarectal administration of living CD4$^+$ T cells into recipient mice, we demonstrated that CD4$^+$ T cells can not only penetrate from the intestinal lumen side to the LP but also egress from

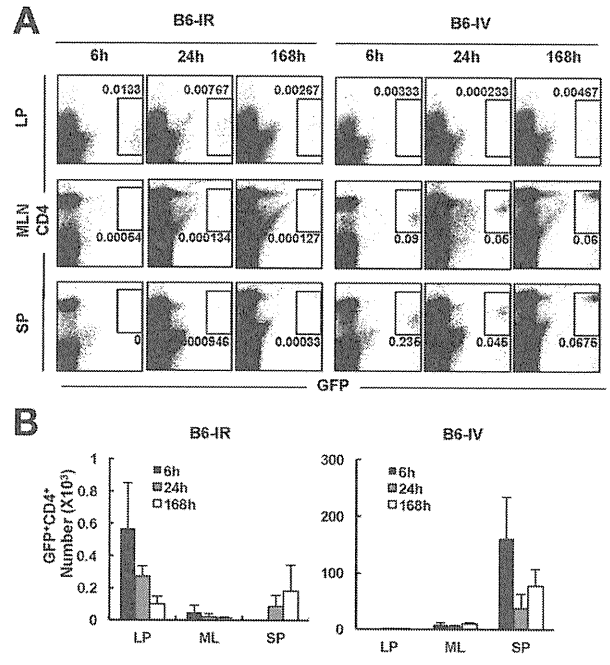

Figure 6. Intrarectally administered cells emerged in the LP and MLN before emerging in SP of immunosufficient CB57/BL6 recipient mice. (*A*) C57/BL6 mice were administered CD4$^+$ T cells from CAG-GFP Tg mice intrarectally (1 × 10^7, *B6-IR mice*, n = 4 at each time point) or intravenously (1 × 10^6, *B6-IV mice*, n = 4 at each time point) and were killed at 6, 24, or 168 hours after administration. (*B*) Cells were isolated from LP, MLN, and spleen at 6, 24, or 168 hours after administration, and the absolute number of GFP$^+$ cells was determined by FACS. Data are indicated as the mean ± standard error of mean of 7 mice in each group.

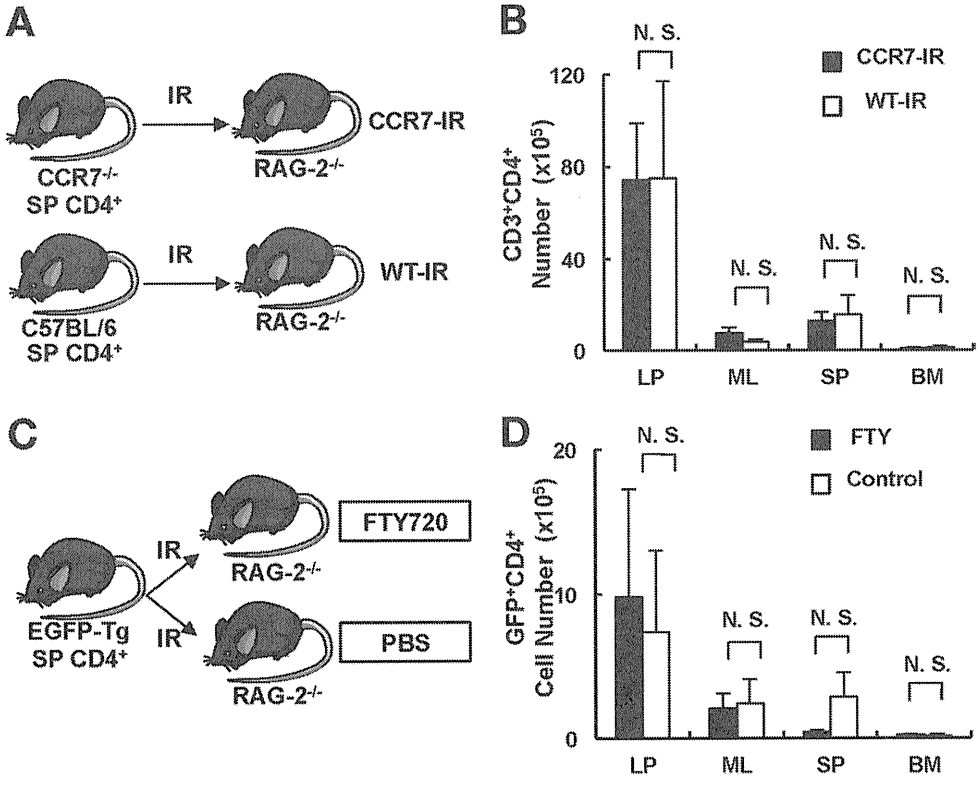

Figure 7. Intrarectally administered cells egress from the LP in a CCR7- and a $S1P_1$- independent manners. (*A*) RAG-$2^{-/-}$ mice were intrarectally administered with 1 × 10^7 $CD4^+$ T cells from WT C57BL/6 mice (*WT-IR mice*, n = 5) or age-matched $CCR7^{-/-}$ mice (*CCR7-IR mice*, n = 5) and were killed at 14 days after administration. (*B*) Cells were isolated from LP, MLN, spleen, and BM at 14 days after administration, and the absolute number of $CD3^+CD4^+$ T cells recovered from each organ was determined by FACS. Data are indicated as the mean ± standard error of mean (SEM) of 7 mice in each group. *N. S.*, not significant. (*C*) RAG-$2^{-/-}$ mice were pretreated with phosphate-buffered saline or FTY720 (1.0 mg/kg) daily starting 1 day before the transfer over a period of 2 weeks and were administered 1 × 10^7 $CD4^+$ T cells from CAG-GFP Tg mice. Mice were killed at 14 days after administration. (*D*) Cells were isolated from LP, MLN, SP, and BM at 14 days after administration, and the number of $CD3^+CD4^+$ T cells was determined by flow cytometory. Data are indicated as the mean ± SEM of 7 mice in each group. *N. S.*, not significant.

the LP to the bloodstream in CCR7- and S1P-independent manners. Although the intestinal LP had been considered to be a "graveyard" of lymphocytes, which possesses a system that suppresses the egress of T cells from this tissue, we here showed experimentally that LP $CD4^+$ T cells are actively returned to systemic circulation.

Pathologically, it is well-known that immune cells, such as granulocytes and macrophages, can penetrate from the intestinal LP to lumen side and accumulate in intestinal crypts to form "crypt abscesses," which are often detected in active stage of UC.[12] In addition, microscopic examination of leukocytes in stool had long been recognized as an important diagnostic tool for patients with acute colitis.[18] Of particular note, Harris et al demonstrated that the stool leukocytes depend on a break in the integrity of intestinal epithelial cells in patients with typhoid fever, in which more than 90% of the white cells were predominantly mononuclear,[19] suggesting that lymphocytes are also able to transude to the intestinal lumen. Indeed, it is also well-known that T lymphocytes physiologically are able to reside in intraepithelial space as intraepithelial lymphocytes.[1,20] Furthermore, recent elegant works demonstrated that $CX3CR1^+$ DC beneath the epithelial cells in small intestine are able to open the tight junctions between adjacent epithelial cells and send dendrites out to sample luminal antigens directly.[11,21] These findings may support the possibility that immune cells actively shuttle between the lumen and the LP side. If so, the present system would be applicable to cell therapy in which protective cells, such as $CD4^+CD25^+Foxp3^+$ T_R cells, are administered intrarectally.

In this study, however, it was surprising that SCID mice intrarectally administered with splenic $CD4^+$ T cells including T_R cells developed colitis. In this regard, it was possible that naïve $CD4^+$ T, but not T_R cells, penetrate epithelial barriers and egress the LP, but this is not likely because we found both T_R and non-T_R cells in the LP and MLN and SP (Figure 3). Rather, it seems more likely that intrarectally administered T_R cells not only penetrate epithelial barriers but also egress the LP but that they cannot suppress the expansion of colitogenic $CD4^+$ T cells in the first initiation site, LP, of naïve $CD4^+$ T cells. In addition, it was also interesting

that the transepithelial migration of $CD4^+$ T cells in the intestine may be a universal phenomenon, as shown in mice of multiple immunological background, such as C.B.-17 SCID, C57BL/6 $RAG^{-/-}$, and C57BL/6 mice, in sharp contrast to the dendrites of $CX3CR1^+$ DC in small intestine are present in C57BL/6 but not in BALB/c mice.[22]

So far, it has been thought difficult to determine whether T cells in intestinal LP can exit the gut to afferent lymphatics. Although an in vivo experiment using a direct injection of cells into the footpad has successfully demonstrated that lymphocytes in peripheral skin tissue can egress in a CCR7-dependent manner,[8] in our hands, it was technically difficult to inject cells that can be distinguished by some molecular markers, such as GFP and Ly5.1, directly into the very thin intestinal wall of mice In addition, even if it were possible to insert a catheter into afferent lymphatics to directly drain cells by using large animals such as sheep, it still remains unclear whether those cells would definitely be derived from the intestinal LP because they could be a mixture of cells from sites including adipose tissues around the intestine and could be drained from the sites of primary LNs that are located closer to the intestine than the draining site.[23] Therefore, we administered cells by the intrarectal route to clarify 2 possible steps: lymphocyte penetration between epithelial barriers and the egress of lymphocytes from the intestinal LP.

This approach may also be open to criticism that cells administered by intrarectal enema would directly enter the small blood vessels, which is exposed to the intestinal lumen via ulcer caused by the ethanol treatment. However, this possibility was not likely because of the following findings: first, a small, but substantial, number of cells administered intrarectally were also found in the LP of recipient mice without ethanol treatment (Supplementary Figure 3); and, second, the first emergent site of cells in recipient mice after intrarectal administration was the LP, but not MLN or SP, in the time course experiment (Figures 5 and 6). Although our results using $CCR7^{-/-}$ mice could not demonstrate the CCR7 dependency of cell egress from the gut, this is not surprising because of the findings of 2 papers using $CCR7^{-/-}$ mice and/or CCR7-Tg mice in skin[8] and lung[7] systems that also demonstrated a CCR7-independent mechanism of cell egress in addition to CCR7-dependent one. In other words, the lack of evidence for CCR7 dependency of cell egress from the gut may explain why it has been so far believed that LP lymphocytes are not able to egress from the gut and thereby die there as if in a "graveyard."[1] Consistent with this, we also observed that intrarectally administered colitogenic $CD4^+CD44^{high}CD62L^-$ T_{EM} cells were subsequently detected in sites outside the intestine, such as MLN and SP (Figure 3).

In conclusion, we have here demonstrated 2 important findings: first, $CD4^+$ T cells are able to migrate from the lumen to the LP side through intraepithelial space; and, second, LP $CD4^+$ T cells are also able to egress from the LP systemically via the bloodstream. This new method may provide a tool for investigation of cell trafficking of intestinal mucosa and also a concept of cell therapy by enema administration for intestinal diseases including inflammatory bowel diseases. Supplementary Figure 8.

Supplementary Material

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.2011.08.035.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Patients

Four patients with ulcerative colitis (UC) undergoing colorectomy at the Tokyo Medical and Dental University Hospital between 1999 and 2006 were enrolled in the study for immunohistochemical study (Supplementary Table 1).

Antibodies

Biotin-conjugated anti-mouse interleukin 7 Receptor alpha chain (A7R34) was obtained from eBioscinece (San Diego, CA). Fc gamma II/III receptor (CD16/CD32)-blocking monoclonal antibodies (mAb) (2.4G2), Phycoerythrin (PE)-, Peridinin chlorophyll protein (PerCP)-, and allophycocyanin (APC)-conjugated anti-mouse CD4 (RM4-5); fluorescein isothiocyanate (FITC)- and PerCP-conjugated anti-mouse CD3 (145-2C11); PE-conjugated anti-mouse CD44 (IM7); FITC-conjugated anti-mouse CD62L (MEL-14); PE-conjugated anti-mouse Ly5.1 (A20); FITC-conjugated anti-mouse B220 (RA3-6B2); FITC-conjugated anti-mouse NK1.1 (PK136); FITC-conjugated anti-mouse CD8a (53-6.7); and PE-conjugated streptavidin were obtained from BD PharMingen (San Diego, CA).

Purification of T-Cell Subsets

$CD4^+$ T cells were isolated from spleen and colon using the anti-CD4 (L3T4)- MACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. To isolate normal lamina propria (LP) $CD4^+$ T cells, the entire length of the colon was opened longitudinally, washed with phosphate-buffered saline (PBS), and cut into small pieces. The dissected mucosa was incubated with Ca^{2+}, Mg^{2+}-free Hank's balanced salt solution containing 1 mmol/L dithiothreitol (Sigma-Aldrich, St. Louis, MO) for 45 minutes to remove mucus then treated with 3.0 mg/mL collagenase (Roshe Diagnosyics GmbH, Germany) and 0.01% DNase (Worthington Biomedical, Freehold, NJ) for 2 hours. The cells were pelleted 2 times through a 40% isotonic Percoll solution and then subjected to Ficoll-Hypaque density gradient centrifugation (40%/75%). Enriched $CD4^+$ T cells from the spleen and the colon (spleen: 94%–97% pure, as estimated by fluorescence-activated cell sorter [FACS] Calibur [Becton Dickinson, Sunnyvale, CA]) were used as donor cells.

Intrarectal Administration of $CD4^+$ T Cells

As a standard protocol of intestinal preparation, recipient mice were maintained without feeding for 1 hour and were given 1 mL of Niflec water (Ajinomoto Pharma Co, Tokyo, Japan) at the concentration of 69 g/L (standard concentration for human) 3 times at intervals of 1 hour by oral catheter. Thereafter, mice were pretreated with 1 mL of ethanol (50% concentration) or distilled water enema and subsequently with 5% pronase enema at 1 hour before cell administration. The method is illustrated in detail in Supplementary Figure 1. Experiment 1 (Figure 2*A* and *B*, Supplementary Figure 2): C.B-17 severe combined immunodeficient (SCID) recipient mice were administered with splenic $CD4^+$ T cells from normal BALB/c mice intrarectally (5×10^6) or intraperitoneally (5×10^5, as a control). Mice were monitored for clinical manifestations. The mice were killed at 10 weeks after administration and assessed for a clinical score that is the sum of 4 parameters as follows: hunching and wasting, 0 or 1; colon thickening, 0–3 (0, no colon thickening; 1, mild thickening; 2, moderate thickening; 3, extensive thickening); and stool consistency, 0–3 (0, normal beaded stool; soft stool; 2, diarrhea; and an additional point was added if gross blood was noted.[1–3] Experiment 2 (Figure 2*C–E*): Recombination-activating gene-2 $(RAG\text{-}2)^{-/-}$ mice were pretreated just like protocol of Supplementary Figure 1 (Mice with Niflec/Pronase) or without the pretreatments of Niflec and pronase (Mice w/o Niflec/Pronase) before cell administration. RAG-$2^{-/-}$ recipient mice in each group were administered with 5×10^6 $CD4^+$ T cells from CAG-GFP transgenic mice (n = 10 in each group). Experiment 3 (Supplementary Figure 3): SCID recipient mice were administered with splenic $CD4^+$ T cells from normal BALB/c mice intrarectally (5×10^6) with ethanol or PBS pretreatment. Mice were monitored for clinical manifestations. The mice were killed at 10 weeks after administration. Experiment 4 (Supplementary Figure 4): SCID mice were intrarectally administered with splenic whole $CD4^+$ T cells (5×10^6) or $CD4^+CD25^-$ T cells (5×10^6) from normal BALB/c mice intrarectally. Mice were monitored for clinical manifestations. Mice were killed at 7 weeks after administration and assessed for a clinical score as mentioned above. Experiment 5 (Supplementary Figure 5): Ly5.2-backgroud RAG-$2^{-/-}$ recipient mice were administered with $Ly5.1^+$ splenocytes intrarectally (1×10^8) or intraperitoneally (1×10^7). Mice were killed at 4 weeks after administration, and the absolute cell numbers of $Ly5.1^+$ donor-derived $CD3^+CD4^+$ T, $CD3^+CD8^+$ T, $CD3^+NK1.1^+$ NKT, $CD3^-NK1.1^+$ NK, and $B220^+$ B cells in spleen of recipient mice were assessed. Experiment 6 (Figure 3): SCID mice were intrarectally administered the same number (5×10^6) of colitogenic $CD4^+$ T cells obtained from colonic LP of colitic mice previously transferred with $CD4^+CD45RB^{high}$ T cells or $CD4^+$ T cells from SP of normal mice. The mice were killed at 10 weeks after administration. Experiment 7 (Figure 4): C57BL/6-background RAG-$2^{-/-}$ mice were administered with splenic $CD4^+$ T cells from CAG-GFP transgenic mice intrarectally (1×10^7) or intravenously (1×10^6, as a control). Mice were killed at 3, 12, or 24 hours after administration for immunohistochemical and electron microscopic analyses. Experiment 8 (Figure 5): C57BL/6-background RAG-$2^{-/-}$ mice were administered with splenic $CD4^+$ T cells from CAG-GFP Tg mice intrarectally (1×10^7) or intravenously (1×10^6, as a