Review Article

Immunological Homeostasis for Understanding Inflammatory Bowel Diseases

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Abstract

Inflammatory bowel diseases are thought to be caused by a complex interaction of genetic, immunological, and environmental factors. The involvement of immunological factors in the etiology of inflammatory bowel diseases is suggested by various facts, such as 1) the effectiveness of immunosuppressive agents, steroids, and anti-TNF-α antibody; 2) the presence of autoantibodies, and 3) the spontaneous development of chronic colitis in mice lacking a single immunity-related gene, e.g., IL-2 or TGF-β. In particular, the treatment of Crohn’s disease using anti-TNF-α antibody has been applied to clinical practice with satisfactory clinical results all over the world.

This article describes the immunological pathology of inflammatory bowel diseases as autoimmune diseases, and outlines prospective new treatment methods based on the nature of the disease.

Key words  Regulatory T cells, Inflammatory bowel disease, Colitis, Leukocytapheresis

Inflammatory Bowel Diseases as Autoimmune Diseases

In understanding inflammatory bowel diseases (IBDs) as autoimmune diseases, an important point is the presence of autoantibodies. Unlike systemic lupus erythematosus and myasthenia gravis, autoantibodies involved in ulcerative colitis and Crohn’s disease have not been characterized sufficiently to reveal the nature of these diseases. Autoantibodies in this case refer to the molecules that are specific to epithelial cells and interstitial cells in the intestines. However, these must be proved through various approaches, such as:

1) Identification of autoantibody molecules and epitopes (8 to 10 amino acid residues); 2) Presence of autoantibodies against antigens; 3) Development of animal models for conditions resembling human autoimmune diseases associated with hyperimmunization using purified antigens; 4) Isolation of T-cell receptors (TCR) reacting to autoimmune epitopes; and 5) Experimental development of conditions resembling human IBDs in transgenic mice with TCR genes.

In addition, the pathology of IBDs assuming an autoimmune mechanism is further complicated by the presence of intestinal bacteria.

(1) Several models for chronic colitis (mice and rats) were established in the 1990s, particularly using gene-manipulated mice. These mice models require the presence of indigenous bacterial flora, and do not develop morbidity in a germ-free environment.

(2) Antibiotics are effective in some patients with Crohn’s disease.

These facts suggest the possibility that the autoantigens involved in the autoimmune mechanism for IBD may be the antigens derived from symbiotic intestinal bacteria, rather than the antigens inherent to the human body. In view of the history of symbiosis starting before the evolution of anthropoid apes, intestinal bacterial
A Hypothesis for Autoimmune Mechanism—Central Tolerance and Peripheral Tolerance

Aberrant reactivity (abnormal activation and proliferation) of CD4⁺ helper T cells to self (or intestinal bacteria) is important in the autoimmune mechanism. The immune response in the actual living body consists of the following processes (Fig. 1):

1. Precursor T cells move from bone marrow into the thymus.
2. After entering the thymus, precursor T cells undergo selective removal of the cells (clones) that are reactive to self (negative selection), achieving elimination of autoreactivity and induction of self-tolerance (central tolerance). However, negative selection is not perfect. A small number of autoreactive clones move out of the thymus, mixed in the large majority of clones that recognize non-self.
3. The clones that recognize non-self extensively proliferate in the thymus (positive selection), and move out of the thymus in the form of naïve T cells.
4. There are special clones called regulatory T cells (T_R, Treg), which probably recognize autoantigens in the thymus without being eliminated and differentiate to memory T cells within the thymus. These cells develop following a completely different pathway than that of the clones (naïve T cells) that can recognize self and non-self. Regulatory T cells occur at the rate of 5 to 10% among CD3⁺CD4⁺CD8⁻ cells in the thymus and among peripheral CD4⁺ cells. They normally exert suppressive control over autoreactive clones in the periphery and monitor the aberrant activity of autoreactive clones at the peripheral level (peripheral tolerance).³
5. Once moved into the periphery, the naïve T cells that recognize non-self continuously
monitor the invasion of non-self antigens (foreign antigens), patrolling the blood and dropping in mesenteric lymph nodes.

(6) On the other hand, foreign antigens are taken up by intestinal lymphoid tissues, Peyer’s patches, and isolated lymph follicles, and are processed by the dendritic cells occurring there. In this process, dendritic cells themselves are also activated and migrate to regional mesenteric lymph nodes.

(7) When the naïve T cells recognizing non-self, described in (5) above, incidentally encounter the activated dendritic cells undergoing antigen presentation (6), the naïve T cells are activated quickly, proliferate, and differentiate to effector cells. In this process, the effector cells acquire the expression of homing receptors (integrin α4β7) needed for the movement into the intestinal mucosa. As a result, the effector cells activated in mesenteric lymph nodes re-enter the blood flow via the thoracic duct and eventually enter the intestinal mucosa, where MAdCAM-1, the ligand for integrin α4β7, is expressed.

(8) The lymphocytes in the intestinal mucosa have a special characteristic. Unlike the cells in peripheral blood and lymph nodes, these cells consist of an overwhelmingly large number of memory T cells, express characteristic homing receptors, and are poorly proliferative. Strangely, most lymphocytes in the mucosa do not return to the blood flow and are believed to undergo apoptosis in the mucosa. However, the possibility remains that a small minority of intramucosal lymphocytes may return to the blood flow via an unknown mechanism and contribute to an autoimmune mechanism.

In the light of the development of lymphocytes as outlined above, there are several possibilities regarding the mechanism for autoimmune diseases including IBDs:
1) Abnormal development of regulatory T cells in the thymus;
2) Abnormal maintenance of regulatory T cells in the periphery;
3) Abnormal activation of dendritic cells;
4) Arrival of excessive antigens derived from intestinal bacteria (probably due to failure of epithelial cells and other barriers); and
5) Excessive viability of intramucosal lymphocytes (resistance to apoptosis).

To consider these possibilities, we need to understand the immunological basis for IBDs, focusing on regulatory T cells and the innate immunity system, which is closely related to the abnormal activation of dendritic cells and the abnormality of barrier elements such as epithelial cells. Regulatory T cells and the innate immunity system are hot topics in immunology at the present. These new concepts are expected to find application in the development of new therapies for IBDs.

### Regulatory T cells

In the 1970s, when the study of immunology was promoted energetically focusing on T cells, the group led by Tada and Okumura proposed the presence of suppressor T cells, which are a type of T cell performing negative control over the immune system. While their proposal was based on experimental evidence, it was not possible to identify a gene expression marker using the techniques in molecular biology and immunology at the time, and the enthusiasm dwindled in the early 1980s. However, the group of Sakaguchi in 1995 demonstrated that the functions ascribed to suppressor T cells were found to occur specifically in CD4⁺CD25⁺ (IL-2Rα⁺) cells, which exist at the rate of 5 to 10% among CD4⁺ T cells in healthy organisms.

Following this discovery, there has been a new boom in the study of suppressor T cells, which are now called regulatory T cells. Many types of regulatory T cells have been proposed so far. This article focuses on the relationship between CD4⁺CD25⁺ regulatory T cells and IBDs.

#### CD4⁺CD25⁺ Regulatory T Cells

CD4⁺CD25⁺ cells occur at the rate of 5 to 10% among the CD4⁺ cells in peripheral blood (spleen) of normal mice, rats, and humans.

As mentioned above, CD4⁺CD25⁺ cells are thought to recognize autoantigens in the thymus and undergo selection in a different way from other naïve T cells before they are supplied to the periphery. CD4⁺CD25⁺ regulatory T cells suppress the in vitro proliferation of other CD4⁺CD25⁺ responder cells in a way depending on cell-cell contact. Experimental data concerning the ability of CD4⁺CD25⁺ cells to produce cytokines are inconsistent. Some reports
support and others disprove the production of inhibitory cytokines such as IL-10 and TGF-β in in vitro stimulation systems. However, CD4⁺CD25⁺ regulatory T cells characteristically do not produce IL-2, although they express high-affinity IL-2 receptors. CD4⁺CD25⁺ regulatory T cells are the only cells in the body that constitutively express CTLA-4, a type of co-stimulatory molecule, in cytoplasm. In fact, CD4⁺CD25⁺ regulatory T cells differ from other naïve T cells in that they are CD45RB⁺⁺⁺CD44⁺⁺ memory-type cells, and some of them are CD69 positive and have the phenotype of activated T cells. Recently, a number of groups identified Foxp3 molecules as a specific transcription factor in CD4⁺CD25⁺ regulatory T cells. A mouse strain with Foxp3 gene mutation is known as the scurfy mouse. This mouse has a phenotype (symptoms) closely resembling that of CTLA-4 deficient mice manifested in naïve T cells in that they are CD45RB⁺⁺⁺CD44⁺⁺ regulatory T cells, and develops severe autoimmune disorders including IBDs. Importantly, this mouse shows a decrease in the number of CD4⁺CD25⁺ regulatory T cells. In addition, forced expression of the Foxp3 gene in naïve T cells causes them to act as regulatory T cells and inhibit the development of chronic colitis mediated by transfer of CD4⁺CD45RB⁺⁺⁺CD44⁺⁺ T cells in mice.

CD25 is the alpha chain of IL-2 receptor, which combines with beta and gamma chains to form the high-affinity IL-2 receptor complex. In fact, CD4⁺CD25⁺ cells express high affinity IL-2 receptors and show high affinity to IL-2. Interestingly, IL-2 deficient mice and IL-2 receptor alpha chain deficient mice also develop autoimmune diseases in various parts of the body including chronic colitis. Before the concept of CD4⁺CD25⁺ regulatory T cells was introduced, IL-2 was thought to be a T-cell growth factor inducing autoimmunity. However, IL-2 is now believed to be a factor that is essential to the differentiation, growth, and maintenance of CD4⁺CD25⁺ regulatory T cells. More importantly, CD4⁺CD25⁺ regulatory T cells suppress the development of morbidity in various animal models for autoimmune diseases including the models for IBDs. Although one group has proposed that the CD45RB⁺⁺⁺ cell population consists of regulatory T cells, current theory identifies it with a cell population representing about 30% of the CD4⁺ T cells including CD4⁺CD25⁺ regulatory T cells.

Recent studies have suggested the presence of sub-populations having regulatory function within the CD4⁺CD25⁺ T-cell population. It may be the case that CD4⁺CD25⁺ regulatory T cells consist of many cells other than CD4⁺CD25⁺ regulatory T cells in addition to all regulatory T cells. When CD4⁺CD25⁺CD45RB⁺⁺⁺ cells are isolated from normal mouse spleen cells and transferred to syngeneic immunodeficient SCID mice or Rag deficient mice, chronic colitis develops. The fact that co-transfer of spleen or lymph node CD4⁺CD25⁺ regulatory T cells suppresses the development of chronic colitis indicates that CD4⁺CD25⁺ regulatory T cells possess the suppressive function to exert in vivo inhibition of chronic colitis.

**Regulatory T Cells in the Human Mucosa and Inflammatory Bowel Diseases in Humans**

Despite the large body of knowledge being accumulated concerning the roles of regulatory T cells in the mouse models of chronic colitis, little is known regarding the involvement of regulatory T cells in IBDs in humans. We examined the presence and function of CD4⁺CD25⁺ regulatory T cells localized in various parts of the intestines. In the past, T cells occurring in the intestines were thought to be memory cells, and CD25⁺ cells occurring at low percentages among CD4⁺ and CD8⁺ T cells were regarded as activation markers indicating IL-2 receptor alpha chain. These cells were understood to be pathological cells, particularly in the context of IBDs. After the recent drastic changes in our understanding, we conducted this study as an attempt to answer the fundamental question of whether human CD4⁺CD25⁺ T cells in the mucosa are regulatory T cells or pathogenic T cells in the current perspective.

We confirmed that CD4⁺CD25⁺ cells in normal human intestinal mucosa occurred at a rate of 4 to 8% of CD4⁺ cells. Regulatory T cells in human peripheral blood, unlike those in mice, have been reported to occur predominantly in the CD25⁺ cell population, which is a subclass of CD25⁺ cells characterized by considerably high fluorescence intensity. We, therefore, examined CD4⁺CD25⁺ cells, and found that CD4⁺CD25⁺ T cells in normal human intestinal mucosa occurred at the rate 1 to 3% of CD4⁺ T cells.
The CD4⁺CD25/high cells in the mucosa actually expressed CTLA-4, GITR, and Foxp3 genes. In addition, we clarified that CD4⁺CD25/high T cells in the mucosa are poorly proliferative (anergic) and suppress the proliferation of CD4⁺CD25⁻ responder T cells dependently on cell-cell contact. These results provide the first demonstration that CD4⁺CD25/high T cells in normal human intestinal mucosa possess the regulatory T cell function. This finding will be important in maintaining immunological tolerance in the intestines despite the presence of various antigenic stimuli in the intestines.

The study using the mouse models of chronic colitis suggested that the quantitative decrease in the number and the qualitative decline of the function of CD4⁺CD25⁻ regulatory T cells are the key to developing chronic colitis. However, our results surprisingly show increases in both CD4⁺CD25⁻ cells and CD4⁺CD25/high cells in patients with ulcerative colitis and Crohn’s disease as compared with the mucosa of healthy subjects. The increase in CD4⁺CD25⁻ cells may be explained if we consider that they are pathogenic T cells in the inflammatory mucosa in IBDs, similarly to activation markers such as the transferrin receptor (CD71) and CD69 described in past reports. However, the CD4⁺CD25/high cells in the inflammatory mucosa in IBDs expressed CTLA-4, GITR, Foxp3, etc. in the same manner as those in healthy subjects and at the same time they were anergic, suppressed the proliferation of CD4⁺CD25⁻ cells, and had the same regulatory T cell function as in healthy subjects. Thus, our results show that Tₐ cells in the intestinal mucosa of patients with IBDs increased in number without qualitative decline of function. Although these result seem contradictory, they suggest a possible explanation that the enhancement of cytokine production, including the secretion of IL-6 and IL-15 from activated dendritic cells and the production of IL-2 from pathologic effector cells localized to the site of inflammation, may promote the proliferation and activation of effector cells at the site of mucosal inflammation in IBDs to an extent that cannot be controlled by the increased regulatory T cells. In fact, we confirmed that CD4⁺CD25/high T cells lost their regulatory function after the addition of IL-2. Alternatively, it is also interesting to consider IBDs as a pathology resembling chronic infection like intestinal tuberculosis. The presence of regulatory T cells can be detrimental to the body for the purpose of eliminating particular microorganisms. As the elimination of microorganisms depends on the presence of potent effector cells, the action of regulatory T cells to suppress the effector function may result in the inability to achieve complete elimination of microorganisms, leading to chronic inflammation. In this respect, interesting clinical studies conducted recently by two groups reported that the administration of anti-human CD25 antibodies improved active ulcerative colitis. While the authors of these reports also developed their study protocols on the assumption that IL-2 was a factor aggravating autoimmune diseases, the possibility remains that regulatory T cells may also be the target in ulcerative colitis. Future development of clinical and basic studies in this direction is awaited.

After revealing that regulatory T cells occur locally in the human intestines, we next considered the origin of these regulatory T cells. There are several possibilities:
1) The cells that have moved directly from the thymus to the intestines in the form of regulatory T cells;
2) The cells that have moved from the thymus to the regional lymph nodes (mesenteric lymph nodes in this case), educated there to gain gut-homing receptors, and then moved to the intestines; and
3) The cells that have developed and differentiated locally in the intestines.

Further examination is needed for better understanding of the regulation of intestinal inflammation.

**Leukocytapheresis-assisted Retransfusion of Regulatory T Cells**

In developing a new treatment method for IBDs using regulatory T cells, we considered the use of leukocytapheresis. In Japan, leukocytapheresis is indicated for severe, fulminant, and refractory cases of ulcerative colitis. Three methods are available at present: granulocyte apheresis (GCAP), lymphocyte apheresis (LCAP), and centrifugal leukocytapheresis. Considering the fact that large quantities of lymphocytes are removed in the process of treatment, we devised a new treatment method in which the above-mentioned regulatory CD4⁺CD25⁻ T cells are
isolated from the removed lymphocytes and retransferred. However, in the mainstream of clinical immunology, development of this kind of treatment is focused on collecting a small quantity of peripheral blood from the patient, separating CD4^+CD25^+ regulatory T cells, culturing them in vitro, and retransferring them to the patient. Such methodology has already entered the stage of clinical application in the field of tumor immunology. However, it cannot be applied to IBDs unless we solve significant problems, including: the applicability to benign diseases in younger patients; the safety of the use of various factors and cells needed for culturing (IL-2, anti-CD3 antibodies, and allogeneic cells); the possibility of carryover of these factors and cells during retransfer; and biohazard management against serious infections associated with long-term culturing.

We consider that the best approach at present is to develop a method that does not require a cell culture system, like the one using leukocytapheresis being developed in Japan. Leukocytapheresis is performed in a closed system and its safety has already been acknowledged. Through a joint study with Dynal, we developed Basiliximab-ClinExVivo, which is a preparation of anti-human CD25 chimeric antibodies (Basiliximab; Simulect, Novartis, Switzerland), approved for clinical use, bound to clinically applicable ClinExVivo (Dynal, Norway) separation beads. In addition, we are using the separation system of Dynal to ensure execution of the recovery process in a complete closed circuit. In this system, the recovered leukocytes are directly reacted with Basiliximab-ClinExVivo and subjected to magnetism while they were kept in the leukocyte bag, and the closedness during separation can be ensured easily. At present, the development of this revolutionary therapy (Leukocytapheresis-assisted Retransfusion of CD4^+CD25^+ T_h cells, LART-25) is promoted under the support of Center for Cell Therapy, Tokyo Medical and Dental University. The purpose of this therapy is to correct the decrease in peripheral regulatory T cells occurring in patients with active ulcerative colitis, aiming at the final result of increasing the supply of regulatory T cells to the site of inflammation and the lymphoid tissues responsible for inflammation.

Evidence concerning the use of various immunosuppressive agents for the treatment of IBDs is being accumulated, and these agents are used widely. However, they are also causing serious side effects such as damage to the kidneys and pancreas. Regulatory T cells are the living body’s own immunosuppressive agent, and the use of them is not expected to cause such side effects. On the other hand, retransfer of large quantities of regulatory T cells may cause excessive suppression of immunity, potentially causing problems of carcinogenesis and infection. The new therapy must be performed only after sufficient informed consent, absolute confirmation of safety based on in vitro analysis, animal experiments, etc., and the establishment of consensus between immunologists and the public.

**Innate Immunity**

Innate immunity is an immune mechanism that is contrasted with acquired immunity.\(^\text{15}\) It has long been known that lipopolysaccharide (LPS) acts as an endotoxin and induces dramatic immune reaction, sometimes causing shock and other responses. It was found in 1997 that the true nature of this response is the reaction mediated by toll-like receptors (TLR). Innate immunity is the immune mechanism working in the important early stage before the characteristic establishment of acquired immunity. It is a defense system against infection consisting of non-lymphatic immune cells such as neutrophils, macrophages, and dendritic cells. TLR is the receptor for pathogen-associated molecular patterns (PAMP) occurring in pathogens such as bacteria, fungi, and viruses. The subtypes from TLR1 to TLR10 have been identified in humans. For example, TLR2 recognizes the peptidoglycan (PGN) of Gram-positive bacteria, TLR2 recognizes viral dsRNA, TLR4 recognizes lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria, TLR5 recognizes a structural protein of bacterial flagella called flagellin, TLR7 recognizes imidazoquinolines, which are synthetic compounds with antiviral activity (no ligands of biological origin have been identified), and TLR9 recognizes CpG DNA of microorganisms.

On the other hand, acquired immunity is an immune mechanism that exists only in vertebrate animals. It depends on antigen-specific reactions
of T cells via TCR and B cells via B cell receptors and antibodies (immunoglobulin). The ability of acquired immunity to recognize a great variety of antigens derives from the use of receptors with randomly rearranged VDJ sequences. Acquired immunity is thought to be mainly involved in antigen recognition at the time of re-infection and the elimination of pathogens in the later stages of infection. In mammals, innate immunity and acquired immunity cooperate to constitute a secure and economically effective defense system.

As mentioned above, intestinal bacteria are essential to the development of chronic colitis. However, it has not been determined whether the antigen protein of intestinal bacteria, TLR signal, or both is important. Recently, Takeda and colleagues found that the development of chronic colitis is inhibited in the double knockout mouse produced from the STAT3 knockout mouse, a model for chronic colitis, and the MyD88 knockout mouse lacking the innate immunity system. On the other hand, we found that the MyD88 knockout mouse surprisingly shows aggravation of DSS-induced colitis in comparison with normal control mice. We hope to see further developments in study related to the role of innate immunity in colitis, including the hot topic of the therapeutic application of probiotics.

**Therapy for Inflammatory Bowel Diseases Using Probiotics**

As discussed above, the presence of intestinal bacteria is an indispensable condition for the development of colitis. However, not all bacteria aggravate chronic colitis. The species in the category of probiotics (e.g., *lactobacilli, bifidobacteria, streptococci*, and non-pathogenic *Escherichia coli*) may act beneficially to the body. In fact, the effectiveness of the administration of probiotics has been demonstrated in several mouse models for IBDs. In addition, studies have shown the effectiveness of probiotics in preventing pouchitis after surgical treatment of ulcerative colitis, as well as the effectiveness in inducing and maintaining remission in mild to moderate ulcerative colitis. On the other hand, the value of the use of probiotics in Crohn’s disease has not been established. The proposed mechanisms for the action of probiotics include the antagonism against pathogenic bacteria through competitive binding to epithelial cells and the promotion of the production of secretory IgA and suppressive cytokines. Expectations are running high for probiotic therapy, as it poses little risk of side effects and it is closely related to intestinal bacteria, which are suggested to be involved in the early stages of disease. Even unexpected developments are anticipated, such as a suppressive TLR signal.

**Conclusion**

Due to advances in immunology, the treatment of IBDs is making tremendous progress both in Japan and in the rest of the world. The development of unexpected new therapies may be realized within years. To this end, we hope that the readers gain a better grasp of IBDs based on an accurate understanding of immunology in general and immunology of the mucosa, including the recent remarkable breakthroughs in immunological studies.

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