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Antigen-Specific Blocking of CD4-Specific Immunological Synapse Formation Using BPI and Current Therapies for Autoimmune Diseases

Prakash Manikwar, Paul Kiptoo, Ahmed H. Badawi, Barlas Büyüktimkin, and Teruna J. Siahaan

Department of Pharmaceutical Chemistry, University of Kansas, Lawrence, Kansas 66047

Abstract

In this review, we discuss T-cell activation, etiology, and the current therapies of autoimmune diseases (i.e., MS, T1D, and RA). T-cells are activated upon interaction with antigen-presenting cells (APC) followed by a “bull’s eye”-like formation of the immunological synapse (IS) at the T-cell–APC interface. Although the various disease-modifying therapies developed so far have been shown to modulate the IS and thus help in the management of these diseases, they are also known to present some undesirable side effects. In this study, we describe a novel and selective way to suppress autoimmunity by using a bifunctional peptide inhibitor (BPI). BPI uses an intercellular adhesion molecule-1 (ICAM-1)-binding peptide to target antigenic peptides (e.g., proteolipid peptide, glutamic acid decarboxylase, and type II collagen) to the APC and therefore modulate the immune response. The central hypothesis is that BPI blocks the IS formation by simultaneously binding to major histocompatibility complex-II and ICAM-1 on the APC and selectively alters the activation of T cells from T_{H1} to T_{reg} and/or T_{H2} phenotypes, leading to tolerance.

Keywords

autoimmune diseases; immunological synapse; bifunctional peptide inhibitor (BPI); antigenic peptide

1. IMMUNOLOGICAL SYNAPSE (IS)

A. Mechanism of Formation of IS

T-cell activation is initiated upon interaction of antigen-presenting cells (APC) with T cells via major histocompatibility complex (MHC) and T-cell receptors (TCR) for generating adaptive immune response.^{1,2} Monks et al. were the first to report the formation of a three-dimensional cell–cell contact between a fixed single T cell and an antigen-presenting cell (APC).³ This cell–cell contact is an interaction of surface receptors and intracellular proteins in a well-organized and spatially distributed manner, leading to the formation of two concentric rings termed “supramolecular activation clusters” (SMAC). The inner ring is

referred as the central TCR-SMAC (c-SMAC or Signal-1). It is composed of protein kinase C (PKC- θ) surrounded by an outer or peripheral SMAC (p-SMAC or Signal-2) enriched mainly with leukocyte function-associated antigen-1 (LFA-1) and talin. Initial contact between the T cell and APC involving TCR and MHC-peptide (MHC-p) and other costimulatory molecules is called the “immunological synapse” (IS).⁴ Grakoui et al. have shown that the formation of a nascent IS is initiated by Signal-2 (i.e., intercellular adhesion molecule-1 (ICAM-1) and LFA-1 interactions) at the central junction and by Signal-1 (TCR-MHC-p interactions) at the peripheral junction of the interface between APC and T cells.⁵ Signal-1 and Signal-2 then exchange places (translocate) via actin-based movement to form a stable Signal-1 cluster at the center and a Signal-2 cluster at the peripheral junction (Fig. 1).

Many costimulatory molecules (Signal-2) have been discovered, including CD28/B7-1 (CD80) and CD28/B7-2 (CD86) as positive costimulatory signals and cytotoxic T lymphocyte antigen (CTLA-4)/B7-1 and CTLA-4/B7-2 as negative costimulatory signals (Fig. 2).⁶⁻⁸ The cytoskeletal protein talin and CD2-associated protein as well as intracellular signaling proteins, such as PKC- θ , LcK, ZAP, Fyn, and MEKK2, have also been identified.^{8,9} The roles of negative and positive costimulatory signals are to maintain the balance between the regulatory and effector functions of T cells in the immune system.

The structure and function of IS are still not well understood because the formation of a mature IS occurs via a dynamic process. In other words, the formation of a mature IS is not merely the formation of protein clusters to sustain TCR signaling; it also requires the involvement of TCR-mediated tyrosine kinase signaling before IS maturation.^{10,11} This suggests that the process of IS formation could be preceded by T-cell activation^{10,11} and possibly the secretion of cytokines or cytotoxic agents by CD4⁺ and CD8⁺ T cells, respectively, upon interaction with APC.¹¹⁻¹⁶ The IS enhances the interaction of CD28/B7-1/2 at the center.¹¹ Lezzi et al.¹⁷ demonstrated that the activation and deletion of either naïve or effector T cells is dependent on the duration of antigenic stimulation. Prolonged antigenic stimulation is required for the activation of naïve T cells, but it causes apoptosis in effector T cells. Celli et al. found that more than 6 hr interaction between naïve CD4⁺ T cells and dendritic cells (DC) is necessary to produce T-cell clonal expansion and inhibition of TCR-MHC interactions that stops the T cell-DC interactions.¹⁸ Furthermore, TCR clustering enhances binding with MHC-p, and the cSMAC is an important site for strong TCR signaling.^{19,20} The internalization of TCR is required for downregulation of TCR signaling.¹⁹ The formation of IS may be required for gene activation to induce and secrete effector molecules and for intercellular transfer of MHC-p complex along with other receptors such as B7-1 from APC to T cell.^{8,21,22} This event may have biological repercussions, allowing T cells to behave as APC, altering the cellular functions and over all controlling the immune response itself.²³

B. Signal-1

A complex of an immunogenic peptide and purified MHC-II (Ia) molecule is recognized by the TCR as a model for Signal-1,^{24,25} and the antigen presentations by MHC-I- or CD1 MHC-like molecules have been described previously.²⁶⁻³⁰ These findings were further

confirmed by solving various crystal structures of peptide–MHC complexes.^{31–39} Later, the specific TCR recognition of MHC–p complex on the surface of APC as a result of antigen processing was termed as Signal-1.^{40,41} TCR consists of an α/β or γ/δ heterodimer connected via a disulfide bond with one variable and one constant region at the extracellular domain.⁴² The variable domain has the greatest diversity as a complementary-determining region 3 (CDR3).⁴³ The sequence of CDR3 is primarily involved in recognizing the antigenic peptide presented by MHC molecules on APC.⁴⁴ MHC class I and class II molecules on APC bind antigenic peptides derived from endogenous and exogenous proteins, respectively. Lipid antigens captured from exogenous antigens bind to CD1 MHC-like molecules.^{30,45}

Upon activation, T cells can differentiate into T_H1 , T_H2 , and T_H17 cells. T_H1 cells produce cytokines such as IFN- γ and lymphotoxin.^{4,46} T_H2 cells produce IL-4, IL-13, IL-5, and IL-6 cytokines,^{4,14,46} and T_H17 cells generate IL-17.⁴⁷ Naïve $CD4^+$ T cells can also be differentiated into an independent lineage called regulatory T cells (T_{reg}), which express fork-head transcription factor Foxp3 and CD25.⁴⁷ $CD4^+$ T cells execute many important effector functions, depending on the cytokines they secrete.⁴⁶ T_{reg} cells are also produced and primed with antigen in the thymus as part of mature cells for controlling immune response.⁴⁸ $CD8^+$ T cells are capable of differentiating into cytotoxic T lymphocytes (CTL);¹² these cells recognize specific antigens presented by MHC class-I on their target cells and perform cell lyses.^{13,15}

There are varieties of MHC-II (polymorphic) expressed on the surface of professional APC (pAPC) (i.e., B cells, DC, and macrophages). MHC-II molecules are generated as heterodimers (α - and β -chains) with $\alpha1$ – $\alpha2$ and $\beta1$ – $\beta2$ domains at the extracellular space.³⁵ The $\alpha1$ - and $\beta1$ -domains bind to the antigenic peptide for presentation to TCR.⁴⁹ Except for the $\alpha1$ domain of MHC-II, all other extracellular domains possess an intradomain disulfide bond. The transmembrane and cytoplasmic domains of MHC-II possess specific sequences required for efficient surface expression,⁵⁰ signaling,⁵¹ antigen presentation,^{52,53} and lateral diffusion of MHC-II molecules on the surface of the APC.⁵⁴

The antigens can be processed and presented by different APC in different ways. For example, the antigen presentation by MHC-II on DC is more effective for activating naïve T cells than that by B cells.⁵⁵ Before the presentation of the peptide fragments of the antigen, the antigen is first internalized via phagocytosis, fluid-phase uptake by macropinocytosis, or receptor-mediated endocytosis via mannose and lectin-like receptors.^{56–58} Then, they are processed via early endosome and then transferred to late endosome before entering lysosomes.^{59–63} Because MHC-II compartments (MIIC) are rich in lysosomal enzymes, proteins are degraded by lysosomal proteases to generate peptide fragments; these peptides are loaded onto MHC-II molecules in the MIIC.^{64–66} The new MHC-II molecules are synthesized and assembled with invariant chain (Ii) in the endoplasmic reticulum to form MHC-II–Ii complexes, which are then transported to the Golgi apparatus and targeted to the endosomal/lysosomal compartments.^{67–70} The portion of Ii in MHC-II–Ii complex is then clipped off by proteases to produce MHC-II molecules with class II-associated invariant chain (CLIP). Then, the CLIP is exchanged for antigenic peptide to form the MHC-II–

peptide (MHC-II-p) complex that is transported to the surface of the APC for presentation to a CD4⁺ T cell.^{28,66}

C. Signal-2

Several roles of costimulatory signals (Signal-2) are to stabilize the physical interactions between TCR and MHC as well as modulating the TCR signal for T-cell activation and tolerance.^{71,72} The positive and negative costimulatory signals can be delivered by CD28/B7 and CTLA-4/B7 interactions (Fig. 2), respectively.^{73–75} Human CD4⁺ T cells and half of CD8⁺ T cells express CD28 with a possible increase in expression upon T-cell activation.^{76,77} A combination of TCR signaling and costimulation via CD2/B7 pathway can upregulate the production of IL-12 receptor,^{78–81} Bcl- χ_L ,⁸² and cytokine transcription.^{83,84} Suppression of the positive costimulatory signal in the presence of TCR–MHC-II–antigen complex generates T-cell anergy, which can be reversed by delivering IL-12.^{85,86} Large and small molecules have been designed to block CD28/B7 signals for therapeutic agents to suppress autoimmune diseases. Therefore, it is necessary to balance the positive costimulatory signal with the negative costimulatory signal (CTLA-4/B7) to maintain tolerance. Normally, CTLA-4 is expressed in activated but not in resting T cells to dampen signals delivered via TCR. CTLA-4 expression is observed at 24–48 hr after priming,⁸⁷ and the strength of expression correlates with stimulus by TCR⁸⁸ upon recruiting Src homology 2 (SH2) domain-containing phosphatase-2 (SHP-2) during T-cell activation.⁸⁹

APC normally express B7-1 (CD80) and B7-2 (CD86); these proteins have V- and C-like domains as well as cytoplasmic domains with phosphorylation sequences for PKC recognition.^{75,90–93} B7-1 and -2 can be upregulated upon activation,⁹⁴ and they are recognized in lower avidity by CD28 than by CTLA-4.^{95,96} Both CD28 and CTLA-4 have a MYPPPY motif that is recognized as the extracellular V-like and C-type domains of B7-1 and -2.^{95,96}

Inducible costimulator (ICOS) and programmed death-1 (PD-1) molecules are additional types of costimulatory molecules in the CD28 family. ICOS has a FDPPPF motif rather than a MYPPPY motif in the CD28 molecule; owing to the difference in this recognition sequence, ICOS does not bind to B7-1 and B7-2.^{97,98} However, ICOS binds to B7-h molecule upon stimulation by TCR activation to increase T-cell proliferation^{97,99–101} as well as IL-4, IL-5, IFN- γ , TNF- α , and GM-CSF production.¹⁰⁰ B7-h also regulates the production of IL-10 and plays a role in induction of tolerance.^{102,103} B cells and macrophages constitutively express B7-h, and its expression can be induced by inflammatory stimuli on fibroblasts as well as nonlymphoid, endothelial, and epithelial cells.

In addition to CTLA-4, PD-1 can deliver negative signals in CD8⁺ T cells to generate tolerance, and both CTLA-4 and PD-1 signals work in an additive fashion upon sustained connection with their respective ligands.¹⁰⁴ The ligands for PD-1 are PD-L1 (B7-H1) (Fig. 2) and PD-L2 (B7-DC).¹⁰⁴ PD-L1 is widely expressed on T cells, B cells, macrophages, monocytes, DCs, and nonlymphoid cells, including endothelial cells, syncytiotrophoblasts in the placenta, muscles, and pancreatic islets,¹⁰⁵ whereas PD-L2 is only found on macrophages and DCs inducible by cytokines.¹⁰⁶ The PD-1 molecule has roles in induction and/or maintenance of peripheral tolerance and acts as a negative regulator of T-cell

responses. Mice with PD-1 deficiency developed a fatal dilated cardiomyopathy with early disease onset¹⁰⁷ and lupus-like disease.¹⁰⁴ PD-1 is expressed during thymic development primarily on CD4⁻ CD28⁻ and γ - δ ⁻ thymocytes and induced on peripheral CD4⁺ and CD8⁺ T cells, B cells, and monocytes upon TCR stimulation.¹⁰⁵ Unlike CTLA-4, PD-1 inhibits the expression of the cell survival gene bcl-X_L,¹⁰⁵ and both CTLA-4 and PD-1 are known to limit glucose metabolism and Akt activation via different pathways. CTLA-4 inhibits Akt activation via protein phosphatase 2a, and PD-1 inhibits Akt activation via CD28-mediated activation of PI3K. Blocking CTLA-4 with antibody reduces CD8⁺ T-cell tolerance upon epitope presentation by resting DC; however, blocking CTLA-4 signal in the presence of PD-1 signal produces weak but constant T-cell priming.¹⁰⁴

LFA-1 and ICAM-1 function as both adhesion and costimulatory molecules (Fig. 2) for T-cell activation.¹⁰⁸ Functionally, LFA-1 has been shown to enhance IL-2 expression, leading to the induction of T-cell proliferation. LFA-1 is exclusively expressed on leukocytes and interacts with its ligands, such as ICAM-1, -2, and -3, to promote a variety of homotypic and heterotypic cell–cell adhesion necessary for normal and pathologic functions of the immune systems. The interaction of LFA-1 with its ligands allows for improved adhesion of leukocytes to vascular endothelium, an essential step in the recruitment and migration of leukocytes into inflamed tissue.^{109–111} Similarly, LFA-1-mediated adhesion is thought to facilitate antigen presentation to T cells, and numerous reports indicate that LFA-1 engagement decreases the minimal stimulatory dose of antigen by 10- to 100-fold.^{112–114}

D. Signal-3

Signal-3, which is provided by the release of inflammatory cytokines by APC or T cells after initiation of Signal-1 and -2, is required for proliferation and differentiation of T cells.^{115,116} Signal-3 can polarize helper T cells to become type 1 helper T cells (T_H1) or type 2 helper T cells (T_H2).¹¹⁷ The “danger theory” proposed by Matzinger indicated that T cells must receive the “danger signal” from Signal-3 and costimulatory signals for their full activation to recognize the danger from pathogens.^{118,119} In contrast, in the absence of the “danger signal,” the immune system induces tolerance instead of an immunizing effect. To determine the effect of the “danger signal” on CD8⁺ and CD4⁺ T-cell proliferation, Mescher et al. utilized microspheres coated with immobilized MHC protein/peptide complexes (artificial APC) to provide Signal-1 and co-immobilized with B7-1 protein for Signal-2.¹¹⁵ IL-12 as Signal-3 was needed along with Signal-1 and -2 to stimulate and proliferate naïve CD8⁺ T cells and, in the absence of IL-12, the T cells did not differentiate into cytolytic effector cells. Similarly, IL-1 but not IL-12 was necessary to proliferate naïve CD4⁺ T cells. These results suggest that Signal-3 from inflammatory cytokines is necessary for proliferation of CD4⁺ and CD8⁺ T cells to prevent tolerance toward a specific antigen.

As a “polarizing” signal, Signal-3 is delivered to naïve helper T cells for their differentiation to either T_H1 or T_H2, and the differentiation depends on the type of cytokine delivered as the Signal-3. If the naïve helper T cells receive IL-12 signal, they differentiate into T_H1. If they receive IL-4, the helper T cells differentiate into T_H2.¹¹⁷ T_H1 cells can produce interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) for directing T-cell immunity against intracellular pathogens. T_H2 cells produce IL-4, IL-5, and IL-13 for optimal self-

proliferation to control humoral immunity as well as fighting against extracellular parasites. The generation of Signal-1 and Signal-2 triggers signal transductions via several different pathways, including calcium–calcineurin, the RAS-mitogen-activated protein (MAP) kinase, and the nuclear factor- κ B pathways (Fig. 2).^{116,120} IL-2 binds to IL-2 receptor (IL-2R) on T cells along with other cytokines to initiate T-cell proliferation through the rapamycin pathway.¹¹⁶ The mechanisms of action of cytokines in triggering the appropriate CD4⁺ and CD8⁺ T-cell responses are still not clear and need further investigation.¹¹⁵

2. ANTIGENIC PEPTIDES

Autoimmune diseases could involve a single organ (e.g., multiple sclerosis (MS), rheumatoid arthritis (RA), and diabetes) or no involvement of specific organs (e.g., systemic lupus erythematosus). T cells and B cells are engaged in inflammatory immune responses, leading to the onset and progression of autoimmune diseases. Activation of both T and B cells depends on antigen presentation. Although antigens are necessary for activation of these lymphocytes, several studies have shown that injections of antigenic peptides/proteins could also suppress autoimmune diseases. The mechanisms of suppression by antigens are thought to work by immune deviation and induction of T_{reg} cells. In the following sections, etiologies and current treatments of various autoimmune diseases are discussed.

A. Multiple Sclerosis

MS is an autoimmune disease that affects the central nervous system; patients suffer from muscle weakness, changes in sensation and speech, vision problems, muscle spasms, and fatigue. In this disease, the autoreactive immune cells (i.e., T cells) attack and cause damage to the axonal via demyelination and inflammation.¹²¹ The myelin sheath damage hampers the communication between nerve cells due to the disruption of the electric impulses along the axons and affects the optic nerves, brainstem, spinal cord, cerebellum, and periventricular white matter. As the result of oligodendrocyte cell death and loss of axons, plaques and lesions can be observed in the brain white matter as well as in the spinal cord. This is a pathological marker of MS.

Nearly 80% of MS patients have clinical relapses referred to as relapsing–remitting MS (RRMS), which is characterized by inflammation of the brain and spinal cord along with white matter lesions.¹²² During the RRMS period, patients have unpredictable relapses followed by long remission with no signs and symptoms of the disease; about 65% of these patients will gradually progress toward a secondary progressive MS (SPMS) typified by an irreversible neurological defect.¹²² Some patients (20%) have a steady neurological decline without remission upon onset of the disease, which is called primary progressive MS (PPMS); however, PPMS is the least common type of MS. Axonal degeneration due to the loss of myelin sheath is commonly observed in PPMS and SPMS.

The cause of MS has not been fully elucidated; however, it is clear that MS patients have a leaky blood–brain barrier (BBB) with brain infiltrations of CD4⁺/CD8⁺ T cells, B cells, and monocytes.^{121,123,124} There is evidence to indicate that proteolipid protein (PLP), myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or some peptide epitopes from these proteins act as self-antigens for T-cell activation (Table I). In the CNS,

naïve CD4⁺ T cells recognize the antigen presented by the local APC called microglia and they differentiate to T_H1 effector cells, which produce IL-2, IFN- γ , and TNF- α .¹²⁵ In addition, T_H17 cells that secrete IL-17 cytokine are also activated by IL-6, IL-23, and TGF- β .^{126,127} Thus, MS patients have elevated levels of IL-17 in the blood and cerebrospinal fluid (CSF).^{128,129} It has been suggested that the BBB of MS patients is leaky and that IL-17 and -22 are responsible for the disruption of the BBB that aids the CNS infiltration of immune cells.¹²² Injection of anti-IL-17 monoclonal antibody (mAb) into SJL/J mice with autoimmune encephalomyelitis (EAE) suppresses the disease development, indicating that IL-17 has an important role in the pathogenesis of EAE and possibly MS.^{127,130} Contrary to this proposal, Haak et al. indicated that IL-17 may not be the major player in pathogenesis of EAE in mouse because IL-17F-deficient mice treated with anti-IL-17 mAb can still develop EAE.¹³¹ Their study indicated that IL-17 may not have a critical role in the pathogenic function of T_H17 cells and T_H1 cells are necessary to initiate CNS inflammation followed by CNS infiltration by T_H17 cells.¹³¹ Haak et al. suggested that although eliminating IL-17 may not be a viable target for treating MS, it is still a good diagnostic marker for MS.¹³¹

Treatment of animal models of EAE with soluble myelin antigens can generate tolerance in diseased animals. For example, oral administration of MBP to Lewis rats before the EAE induction suppresses EAE as a result of clonal anergy or deletion of activated T cells with a reduced number of T_H1 MBP-specific cells.^{132–135} Administration of bovine myelin antigens in a small group of MS patients provided a significant decrease in the number of T cells reactive toward MBP compared with the control group and a lower number of treated patients showing major attacks compared with the untreated control group.¹³⁶ Unfortunately, a subsequent large clinical trial (Phase III) did not show the efficacy of a single oral dose of myelin antigens (MBP and PLP); the previous effects were considered to be a placebo effect. Thus, there are many unanswered questions about the effectiveness of this type of treatment.

Alternatively, intrathecal, intravenous (i.v.), subcutaneous (s.c.), or nasal administration of antigenic peptides has been investigated to induce tolerance in patients. Two phase I trials conducted by Warren et al. demonstrated that administration of MBP peptides, MBP_{75–95} or MBP_{85–96}, in chronic progressive MS patients did not produce any adverse effects.^{137,138} Induction of tolerance was monitored by detecting the reduction of anti-MBP antibodies in CSF. Intrathecal administration resulted in transient neutralization, whereas i.v. administration resulted in long-lasting tolerance or reduction in anti-MBP antibodies for up to 1 year after a second i.v. dose of antigen. However, s.c. administration of the antigenic peptides did not affect the reduction of anti-MBP antibodies in CSF, clearly demonstrating that s.c. injection failed to induce tolerance in these patients.¹³⁸ Unfortunately, these studies failed to relate the clinical significance of the reduction in antibodies against MBP and its effect on the prognosis of the disease in MS patients.

Antigenic peptides derived from MBP were successfully delivered via the nasal route and were effective in inhibiting EAE disease in a murine model.^{139–142} It has been suggested that proliferation of antigen-specific T_{reg} cells in EAE can be triggered by antigenic-peptide presentation by plasmacytoid dendritic cells (pDC) as part of the professional APC.¹⁴³ The mechanism of tolerance in this model is due to MBP-reactive specific T_{reg} cells that produce

IL-10. In addition, these cells were found to express higher levels of IL-4 and TGF- β mRNA.^{141,144} Similarly, altered peptide ligands (APL) are mutated antigenic peptides with increased affinity for MHC-II and/or TCR, and they have been shown to control RA and EAE in animal models. A suggested mechanism of action for APL in inhibiting antigen-specific T-cell activation is increasing the expression of indoleamine 2, 3-dioxygenase (IDO) enzyme in activated T cells. Presumably, the increased amount of IDO converts tryptophan to its metabolites (i.e., 3-hydroxykynurenic acid (3-HKA), picolinic acid (PA), and quinolinic acid (QA)) and these metabolites suppress the EAE relapse by reducing the production of inflammatory cytokines and stimulating the production of IL-4 and IL-10 that are related to T_H2 and T_{reg} cellular differentiation.¹⁴⁵

Another promising approach developed for the treatment of MS involves the use of recombinant T-cell ligands (RTL). A human recombinant T-cell receptor ligand (RTL1000) consisting of DR2 α 1+ β 1 domains of MHC-II molecules linked covalently to MOG₃₅₋₅₅ peptide was shown to reverse clinical and histological signs of EAE¹⁴⁶ and was evaluated for safety in a phase 1 randomized, placebo-controlled, escalating dose study in 34 patients with MS.¹⁴⁷ Preclinical studies of RTL showed that these molecules were effective in ameliorating both active and passive MOG-induced EAE in C57BL/6 mice¹⁴⁶ and chronic EAE in SJL/J mice.¹⁴⁸ One of the constructs, RTL401 (RTL with bound PLP₁₃₉₋₁₅₁), was able to suppress EAE in SJL/J mice immunized with PLP₁₃₉₋₁₅₁ but could not treat MOG₃₅₋₅₅-induced EAE.¹⁴⁸ Furthermore, RTL401 could not treat EAE in mice immunized singly with PLP₁₇₈₋₁₉₁ or MBP₈₄₋₁₀₄ peptides, and the same was also true with other RTL constructs linked to different peptides.¹⁴⁸ This suggests antigen-specific suppression of EAE by RTL modulation of the cognate T-cell specificity. Interestingly, RTL401 was able to ameliorate EAE induced with syngenic whole spinal cord homogenate, indicating that treatment with a single RTL can suppress EAE induced with multiple T-cell specificities provided that one of the immunizing peptides is present in the RTL being used for treatment. This demonstrates that treatment with a single RTL can deviate autoimmune responses of cognate T-cell specificities while inducing bystander suppression that reverses EAE in mice immunized with multiple encephalitogenic peptides.¹⁴⁸ It is thought that the mechanism of action of RTL involves direct reduction of proinflammatory cytokines, such as IL-17 and TNF- α , while concomitantly inducing the production of IL-10, IL-13, and TGF- β and inhibiting the migration of inflammatory cells into the CNS via a reduction in the expression of chemokines/receptors, VCAM-1, and ICAM-1.^{146,148}

Current strategies that are approved for the treatment of MS include two classes of drugs: anti-inflammatory and disease-modifying therapies (DMT). Acute MS relapses are often treated with high-doses of intravenous corticosteroids such as methylprednisolone. This is effective for short-term treatment, but it does not have any effect on long-term recovery. Mitoxantrone (Novantrone[®]), interferon β -1a (IFN β -1a or Avonex[®]/Rebif[®]/CinnoVex[®]), IFN β -1b (marketed as Betaseron[®]/Betaferon[®]), glatiramer acetate (GA, Copaxone[®]), and natalizumab (Tysabri[®]) are current DMT drugs. Mitoxantrone will not cure the disease, but it is effective in slowing the progression of SPMS and delaying the relapse interval time in RRMS.¹⁴⁹ The mechanism of action of mitoxantrone involves disrupting both DNA synthesis and DNA repair. The side effects of mitoxantrone are irreversible cardiomyopathy,

acute leukemia, and bone-marrow suppression; patients receiving this treatment are advised to have regular echocardiograms.

IFN β -1b was first introduced in 1993 for RRMS treatment. In the clinical trials, patients treated with IFN β -1b were free from relapse at the end of 2 years compared with a placebo-treated group.¹⁵⁰ IFN β -1b acts as an anti-inflammatory agent by reducing the production of IFN- γ and TNF- α and inhibits T-cell activation, clonal expansion, and migration into the CNS.¹⁵¹ The mechanism of action of IFN β -1a is thought to be similar to that of IFN β -1b.

Copaxone[®] (glatiramer acetate) is a synthetic peptide polymer (40–100 residues) that consists of Glu, Lys, Ala, and Tyr amino acids in a ratio of 3:7:9:2; it is designed as a decoy for MBP. Copaxone[®] has been suggested to alter the immune response from T_H1- to T_H2-cell differentiation.

Finally, natalizumab (Tysabri[®]) is the first recombinant human mAb approved for the treatment of MS. It inhibits leukocyte adhesion to vascular endothelium by binding to the α_4 -subunit of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins on the surface of leukocytes to prevent leukocyte infiltration into the CNS.¹⁵² Natalizumab has been shown to significantly slow the progress of disability with increasing numbers of disease-free individuals compared with placebo group. It can also reduce disease relapse, loss of vision, and number of lesions in patients.^{153–155} Despite its greater efficacy compared with any other approved MS therapies, its use is limited because of the development of potential fatal adverse effects, such as progressive multifocal leukoencephalopathy (PML), melanoma, hepatotoxicity, opportunistic infections, and primary CNS lymphoma.^{156,157} Recent studies suggest that patients receiving this drug for more than 1 year are at an increased risk for PML.¹⁵⁸ Currently, the FDA has limited the usage of natalizumab to patients not responding to other therapies, and the drug is under a mandatory safety monitoring program.

B. Type-1 Diabetes

Type 1 diabetes (T1D) is marked by insulinitis and the destruction of the pancreatic islet of Langerhans β cells.¹⁵⁹ Although not all-inclusive, studies carried out in nonobese diabetic (NOD) mice show that CD4⁺ and CD8⁺ T cells are partly responsible for the destruction of the β -cells.⁷ Those afflicted with T1D are genetically predisposed, and the progress toward the disease is initiated after encountering an environmental insult, such as diet or microbial infections.⁷ Insulin, glutamic acid decarboxylase (GAD), zinc transporter (ZNT8), and insulinoma antigen (IA-2) are the four major antigens (Table I) for activation of immune cells, and they have been used in assays to detect the progress of T1D.^{160,161} Glutamic acid carboxylase 65 kDa (GAD65) is considered a target for therapy because of its partial role in the activation of certain T cells that recognize it as an antigen.¹⁵⁹

The immunotherapy of T1D focuses on two alternative methods of treatment: prevention of T1D or treating it at its onset. Ever since its discovery, insulin has been considered to be the gold standard of treatment for patients with Type 1 diabetes. Similar to GAD65,¹⁵⁹ insulin has also received attention as a therapeutic method to suppress the activation of T cells.¹⁶¹ By suppressing the activation of T cells, β -cells may be regenerated, and this may lead to the

remission of diabetes.⁷ The administration of autoantigens (GAD65 or insulin) and their peptides has been shown to suppress β -cell autoimmunity.^{7,159}

The role of insulin as an autoantigen is still ambiguous. To date, many epitopes of insulin and proinsulin have been identified.¹⁶¹ Recent findings have shown that insulin auto-antibodies can be induced by insulin B9–23 peptide. NOD mice (H-2d) were immunized with B9–23 peptides, which led to the development of MHC-activated autoantibodies toward insulin.¹⁶² However, it has been shown that the antibodies toward insulin react only with insulin and not with NOD B9-23 peptides, suggesting that insulin antibodies recognize the whole molecule rather than the peptide sequence itself.¹⁶¹ Proinsulin, the precursor of insulin, also has a sequence of amino acids (B24–C36) that are recognized by NOD mice CD4⁺ T cells.¹⁶³ B9–23 has been hypothesized to play an important role in humans as it did in NOD mice because the amino acid sequences are similar in both species. Higashide et al. have shown that the most frequent epitopes recognized by T cells are B10–24, B1–15, and B11–25, with B9–23, B4–18, and B12–26 being identified in some patients.¹⁶⁴ Alternatively, it has been observed in NOD that intranasal vaccines containing proinsulin in a plasmid DNA increased the number of T_{reg} cells and effectively prevented the onset of diabetes.¹⁶⁵

GAD65 is an enzyme that is targeted as an autoantigen by T cells. Anti-GAD65 antibodies can be observed in NOD mice before the onset of T1D. Injection of GAD65, intravenously or intrathymically, to young NOD female mice before any signs of disease has been shown to prevent diabetes and induce CD4⁺ T_{reg} cells.¹⁶⁶ Controlling the activation and increasing the induction of T_{reg} cells by GAD65 antigen may help to suppress T1D.¹⁵⁹ Tisch et al. revealed that peptides derived from GAD65 are able to suppress the progression of T1D in NOD mice before the onset of insulinitis. The peptides used include amino acids 217–236, 247–265, 290–309, and 524–543.¹⁶⁷

C. Rheumatoid Arthritis

RA is a chronic idiopathic disease characterized by persistent inflammation and local destruction in the synovial tissues including the synovium, bone, and cartilage. Although the nature of the antigens responsible for RA pathogenesis has not been clearly elucidated, there is evidence that the disease-associated HLA DR (B1*0401, 0404, 0405, and 0101) molecules, which reside in the MHC and participate in antigen presentation, are associated with the disease.¹⁶⁸ Viral proteins, such as cytomegalovirus and Epstein-Barr virus, as well as several autologous proteins normally expressed in the joints, including gp39, proteoglycan, and type II collagen, have been implicated in the generation of the pathogenic T-cell response in RA (Table I). The driving force behind rheumatoid inflammation is believed to be CD4⁺ T cells responding to an antigenic epitope in the synovium in an HLA-DR-restricted manner. Increased numbers of “memory” CD4⁺ and decreased numbers of “naïve” CD4⁺ T cells in the synovial tissues of RA patients^{169,170} suggest that CD4⁺ T cells play a crucial role in the pathogenesis of RA. Synovial tissue T cells are enriched with CD4⁺ T cells that do not express the Leu 8 antigen, now known to be the human equivalent of the murine homing receptor.^{171–173} Loss of this molecule is a characteristic of activated T cells.

Synovial fluid also contains augmented expression of the activated antigens HLA-DR, CD9, CD38, CD49a/CD29 (VLA-1), and adhesion molecule CD54 (ICAM-1).¹⁷⁴⁻¹⁷⁷

In addition to CD4⁺ T cells, a number of other inflammatory mediators produced in the rheumatoid synovium, including arachidonic acid metabolites, vasoactive amines, platelet-activating factor, and complement cleavage products, contribute to the inflammation. Recently, IL-17 produced primarily by the T_H17 cells has also been implicated in human autoimmune inflammation. A potential key role in diseases such as RA has only recently been attributed to IL-17, although it was discovered decades ago. Elevated levels of IL-17 in blood and synovium of RA patients have been correlated with synovial levels and joint damage.¹⁷⁸⁻¹⁸⁰ Recent advancement in research toward new and better-tolerated therapies to attenuate the inflammation and pain associated with RA as well as halt the progression of erosive joint damage has led to the development of strategies for modulating stimulation of immune cells or blocking cytokines as potential therapeutic strategies.

Injections of soluble antigens have been shown to induce antigen-specific immune tolerance. It is thought that tolerance could be achieved through anergy/deletion of CD4⁺ T cells or the induction of CD4⁺ T_{reg} cells that produce IL-10 or TGF- β .¹⁸¹ Oral, and more recently nasal, tolerance resulting from administration of antigens has attracted attention as a potential treatment of autoimmune diseases. Oral administration of type II collagen (CII) has been shown to suppress arthritis in mice¹⁸² and rats.¹⁸³ Oral administration of chicken CII to patients with RA provided significantly less progression of the disease compared with placebo-treated patients.¹⁸⁴

In another study, peripheral blood mononuclear cells of RA patients responded well in vitro to CII₂₅₆₋₂₇₁ epitope and its overlapping variants.¹⁸⁵ Similarly, oral administration of this peptide has been shown to suppress arthritis in a CIA mouse model.¹⁸⁵ Specific T-cell activity, proliferation, and secretion of IFN- γ in spleen cells were actively suppressed in CII₂₅₀₋₂₇₀-fed mice, and the serum anti-CII, anti-CII₂₅₀₋₂₇₀ antibody activities and frequency of specific antibody-forming spleen cells were significantly lower in CII₂₅₀₋₂₇₀-fed mice than in controls. Moreover, IL-4-producing cells (T_H2 cells) were upregulated when CIA mice were treated with type II collagen (CII₂₅₀₋₂₇₀), suggesting that oral administration of CII₂₅₀₋₂₇₀ can suppress the cellular and humoral immune response in collagen-induced arthritis. Although the majority of animal studies have yielded positive results with the oral tolerance regimen, under some circumstances, mucosal application of antigen may instead exacerbate the disease process.¹⁸⁶

Therapeutic effects of murine MHC class II (I-A^q)-derived recombinant T-cell receptor ligands (RTL), consisting of the α 1 and β 1 domains of MHC class II molecules that are covalently linked to the CII immunodominant peptide (CII₂₅₇₋₂₇₀), were also found to reduce the incidence and clinical and histological signs of CIA.¹⁸⁷ Treatment with RTL resulted in increased CII-specific IgG1 and a reduction in IgG2a isotype that is normally associated with a pathogenic response. The mechanism of RTL in CIA appears to be, due to an increased expression of the FoxP3 gene, an upregulation in anti-inflammatory cytokines (IL-10 and IL-13) and a downregulation in proinflammatory cytokines (IL-17 and IFN- γ).¹⁸⁷

Abatacept, also known as CTLA4-Ig, was approved in 2005 by the FDA for the treatment of patients with moderate-to-severe active RA who have had an inadequate response to disease-modifying antirheumatic drugs (DMARDs), including methotrexate (MTX) and TNF antagonists. It is a fusion protein composed of the extracellular domain of the CTLA4 molecule complexed to the Fc domain of human IgG1. The IgG1 solubilizes the fusion protein; thus, unlike the membrane-bound CTLA4 molecule, its binding to CD80/CD86 does not induce a negative signal to T cells. Its binding blocks the engagement of the CD28 costimulatory signal required for T-cell activation, resulting in T-cell anergy.¹⁸⁸ Patients receiving abatacept responded better than those receiving placebo. Quality of life was also improved with abatacept and benefits were sustained at 12 months, these included significant reduction in disease activity as well as improved physical function.¹⁸⁹

Infliximab is a chimeric monoclonal antibody that binds to circulating and transmembrane TNF- α and thereby neutralizes its biological effect. Circulating TNF- α is a form of TNF- α generated by cleavage from the transmembrane precursor. Both forms of TNF- α have been shown to sustain the inflammation process. Transgenic mice overexpressing TNF- α were observed to develop a destructive arthritis resembling RA. Moreover, high levels of TNF- α detected in synovial fluid and in the tissues of patients with RA¹⁹⁰ suggest that TNF- α plays a pivotal role in physiologic and pathogenic response.¹⁹¹ In addition to infliximab, a number of agents that block TNF- α have been developed for clinical use, including etanercept, adalimumab, and certolizumab. Etanercept is a conjugate between a human TNF- α receptor and the Fc-portion of the human IgG1. Adalimumab is a recombinant human IgG1 monoclonal antibody specific for TNF- α ; it not only inhibits binding of TNF- α to its receptors but also lyses cells expressing membrane-bound TNF- α . Certolizumab, on the other hand, is a polyethylene glycol (PEG)-conjugated humanized anti-TNF Fab fragment. Although anti-TNF- α drugs have been shown to be effective and relatively safe for use in treating RA, blockade of this cytokine may have effects beyond the suppression of synovial inflammation, which can influence mortality of these patients. Mycobacterial infections have been reported among patients undergoing therapy with infliximab.^{192,193} Therefore, tuberculin skin tests and a chest radiograph are usually recommended before initiation of anti-TNF treatment. Also, avoidance of anti-TNF therapy is recommended for patients with severe chronic heart failure.¹⁹⁴ Aside from TNF- α , cytokines appear to be of particular importance in the development of RA and, therefore, have been investigated as therapeutic targets. Kineret (Anakinra) is a recombinant, non-glycosylated form of the IL-1 receptor antagonist (IL-1Ra), a naturally occurring receptor antagonist known to counteract the proinflammatory effects of interleukins. Patients taking background MTX with anakinra had significant clinical improvement as compared with the placebo group.^{195,196} Simultaneous blockade of TNF- α and IL-1 receptor had very promising results in experimental murine arthritis;¹⁹⁷ however, this did not translate well to clinical outcome. Moreover, the risk of mycobacterial infections under combination therapy increased significantly; it is therefore not recommended for RA treatment.¹⁹⁸ Tocilizumab (MRA) is a humanized monoclonal antibody against IL-6 receptor (IL-6R). Randomized phase II studies showed that patients receiving tocilizumab had reduced disease activity in a dose-dependent manner.

B lymphocytes play several critical roles in the pathogenesis of RA. They are the source of rheumatoid factors and anticitrullinated protein antibodies, which contribute to the immune

complex and complement of T-cell activation in the joints. It is therefore assumed that the depletion of B lymphocytes provides a potential therapeutic approach to controlling disease activity and inducing remission. This hypothesis has been proven in an open-label study of rituximab, a chimeric monoclonal antibody against the protein CD20. CD20 is widely expressed on B cells from early pre-B-cells to later stages in differentiation, but it is absent on terminally differentiated plasma cells. Rituximab was significantly superior to placebo when added to MTX.¹⁹⁹ Safety and tolerability of rituximab were found to be comparable to that of the placebo. Moreover, there have been no higher infection rates under B-cell depletion therapy.¹⁹⁹

3. BLOCKING SIGNAL-2

Blocking Signal-2 formation has been shown to alter the immune response via induction or energy of a certain T-cell phenotype (i.e., T_{H1} or T_{H2}). Inhibition of Signal-2 has been used for the treatment of autoimmune diseases. Monoclonal antibodies and peptides (Table II) that inhibit Signal-2 have been shown to suppress allograft rejection,^{200,201} type-1-diabetes,^{202,203} RA,^{204,205} and psoriasis.^{206,207} Despite the great promise of mAb, these drugs are costly to produce and difficult to formulate or deliver. Peptides and peptidomimetics are a better alternative to mAbs. Despite progress in designing small molecule inhibitors, a limited number of small molecules have been designed to target Signal-2, but none has yet reached the clinical setting.

A. LFA-1/ICAM-1 Interaction

Studies performed in vivo and in vitro have demonstrated that T-cell activation requires interaction of adhesion receptors. Initially, when T cell, encounter APC, adhesion receptors physically anchor and provide feedback to the T cells about the surface milieu of the APC. This may indeed be followed by TCR signaling and tightening of the distance between the two membranes through translocation of the receptors.^{8,208} Adhesion receptors such as ICAM-1 are considered to be involved in intracellular signaling, leading to the accumulation of the immune receptors along with MHC molecules at the contact area for the efficient presentation of the MHC-p complex to the T cells.²⁰⁹ Thus, adhesion is considered a costimulatory signal required to generate TCR signaling and, further, immune response. There are several adhesion receptors on T cells that can deliver a costimulatory signal including LFA-1. LFA-1 interacts with ICAM-1, -2, and -3.^{209,210} The expression of ICAM-1 on DC is necessary for longstanding interaction between CD8⁺ T cells and DC for activating effector CD8⁺ T cells and their survival.²¹¹ Knocking out ICAM-1 expression resulted in elimination of the long-lasting T-cell interaction with DC for memory induction and lowering of the production of IFN- γ cytokine.²¹¹ This result is attributed to the inability to form the IS in the absence of ICAM-1.²¹¹ There is a prolonged polarized synapse upon DC-T cell interactions before asymmetry T-cell division during mitosis to produce two different cells with distinctive functions.²¹²

Efforts made toward understanding the structure, function and mechanisms of interaction between LFA-1 and ICAM-1 have produced possibilities of new therapies. Currently, several strategies have been developed using LFA-1- and ICAM-1-targeted therapeutics, which include antibodies, peptides, peptidomimetics, small molecules, and antisense oligo-

nucleotides to suppress LFA-1/ICAM-1 costimulatory signal for the treatment of inflammation,²¹³ autoimmune diseases,²¹⁴ allograft rejection,^{200,215–219} and cancer.²²⁰ Other than blocking ICAM-1/LFA-1 interaction, the mechanisms of action of these antagonists could involve the downregulation of surface expression of these receptors as well as the prevention of the formation of active conformation of these molecules.

1. Antibodies—Antibodies have been used to determine the functions of ICAM-1 and LFA-1 in disease pathologies, and these antibodies have therapeutic activities in preventing organ transplant rejection^{221–224} and suppressing autoimmune diseases, including arthritis,²²⁵ insulin-dependent diabetes mellitus,^{202,203,226} multiples sclerosis,^{227–231} and lupus.²³² The first LFA-1/ICAM-1-targeted monoclonal antibody tested clinically was anti-LFA-1 antibody Odulimomab (antibody 25.3) in bone marrow^{216,219} and kidney transplant,^{217,218} while anti-ICAM-1 antibody Enlimomab (antibody R6.5 or BIRR1) has been investigated in kidney transplant²³³ and RA.²³⁴ Anti-ICAM-1 antibody is progressing through phase I and II trials for the treatment of RA.²⁰⁵ Blocking ICAM-1/LFA-1 interaction using Enlimomab failed to show any benefit in a randomized renal transplantation study.²³⁵ Efalizumab, a recombinant humanized monoclonal antibody that binds to CD11a (the α -subunit of LFA-1), has been successfully used in the treatment of psoriasis.^{207,214,236–242} Recently, a crystal structure of the efalizumab Fab in complex with the LFA-1 I-domain reveals that the antibody binds to the I-domain distinct from the ICAM-1 binding site and blocks the binding of LFA-1 to ICAM-1 via steric hindrance.²⁴³ Efalizumab, under the trade name Raptiva[®] has been approved by the FDA in 2003 for the treatment of moderate-to-severe plaque psoriasis; it has been withdrawn from the market because of increased patient susceptibility to PML.²⁴⁴ Although monoclonal antibodies for adhesion molecules have successfully demonstrated effectiveness in suppressing autoimmune diseases in patients, the risk of secondary infections and immunogenic response has been shown to increase. Therefore, peptides and small molecules are being investigated as immunotherapeutics, and these molecules offer distinct advantages in terms of development and treatment costs.

2. Peptides—The Siahaan group has discovered several cell adhesion peptides that block LFA-1/ICAM-1 interaction (Signal-2). These peptides were derived either from domain-1 of ICAM-1 or from α - and β -subunits of LFA-1 (Table II).^{159,245–252} Studies carried out using in vitro cellular models, such as homotypic or heterotypic T cell adhesion or mixed lymphocyte reaction, have clearly demonstrated the ability of these peptides to inhibit the binding of T cells to APC by blocking the binding of adhesion receptors to their natural ligands.^{245,247–252} It should be noted that although the suppression of autoimmune diseases can be accomplished by delivering Signal-2 blockers, inhibiting Signal-2 may also suppress the general immune response to fight infections.

3. Antisense oligonucleotides—In addition to expression on immune cells, ICAM-1 is also constitutively expressed on endothelial and epithelial cells, and during inflammation ICAM-1 expression is upregulated. Antisense oligonucleotide (Alicaforsen, ISIS 2302) binds to RNA and blocks the translation of ICAM-1 protein within the cell; this molecule was investigated in phase II and III clinical trials for the treatment of Crohn's disease and

ulcerative colitis.^{253,254} Although the initial trials with a small group of subjects was promising, later larger trials in both diseases failed to show efficacies.^{255–261} The utility of ISIS 2302 was also evaluated for treating RA, and this drug was not efficacious in the clinical trials.²⁶²

B. CD28/B7 Interaction

Interactions between CD28 and B7-1 (or CD80) or B7-2 (or CD86) have a role in T-cell differentiation and proliferation. Inhibition of CD28 signal prevents recruitment and activation of naïve T cells as well as interfering with epitope spreading. This inhibition is being investigated for development of treatments for autoimmune diseases and organ transplantations.^{93,263–269} CD28-deficient mice have impaired immune responses to viral antigens or autoantigens,^{270,271} suggesting that T-cell responses to autoantigens or alloantigens are dependent on costimulation through CD28.²⁷² Antibody-induced modulation of a costimulatory signal via the CD28 pathway has been shown to prolong allograft survival in rats.²⁷³

Administration of anti-CD28 Fab fragments efficiently led to suppression as well as reversal in the induction of EAE²⁶⁸ and uveoretinitis²⁷⁴ in mice. The activity of anti-CD28 Fab suppresses EAE due to ablation of TNF- α production and prevention of T-cell infiltration into the CNS without stimulating CD28.^{268,269,275,276} Unfortunately, the Fab fragment is difficult to develop as a drug because it is rapidly cleared from the body.²⁷⁷ To solve the clearance problems, the Fab fragment has conjugated to polyethylene glycol or fused with serum albumin, and these conjugates have been shown to have longer half-lives in the body.²⁷⁷ Although CD28 blockade using mAb attenuated EAE and prevented subsequent relapses, it did not completely eliminate the encephalitogenic response, suggesting that the activation of encephalitogenic T cells independent of CD28 costimulation may not have been completely abolished. This observation is consistent with findings in CD28-deficient mice. CD28 $^{-/-}$ mice developed autoimmune heart disease, although it was less severe than that observed in heterozygous littermates.²⁷⁰ Similarly, breeding NOD mice with CD28 $^{-/-}$ was found to exacerbate autoimmunity.²⁷⁸

C. CTLA-4/B7 Interaction

Blocking the CTLA-4/B7 negative signal has been proposed to improve the response of cytotoxic T cells to antigen presentation by APC, thus enhancing their activation and proliferation.²⁷⁹ The signal transmitted through CTLA-4 leads to dephosphorylation of the second messengers in the CD3 complex and subsequently leads to the control of the production of various cytokines produced by T_H1 and T_H2 cells.^{280,281} It has been suggested that CTLA-4 is involved in the development of T_{reg} cells in organ transplantation models,²⁸² and CTLA-4-deficient animals have been shown to be resistant to immunotolerance.²⁸³ A single injection of anti-CTLA-4 2 days after immunization in mice resulted in a mild increase in severity and incidence of EAE.²⁷⁹ When anti-CTLA-4 mAb was administered on the second day after clinical signs of EAE were observed followed by its administration every other day for the following 6 days, a marked increase in the disease score was observed and there was a considerable increase in the production of IL-2, TNF- α , and IFN- γ .²⁸⁴ Histological evaluation of mice treated with anti-CTLA-4 mAb showed more

inflammation in the brain and the spinal cord compared with those treated with control antibody, suggesting that inhibition of CTLA-4 signal enhances the activation and proliferation of antigen-reactive T cells.²⁸⁵ Blocking the CTLA-4 signal is not a good approach for developing therapeutic agents for autoimmune diseases. In contrast, inhibition of CTLA-4 signal can activate T cells for fighting tumors.²⁸⁶ Several clinical trials have been conducted using the anti-CTLA-4 antibody to treat melanoma and other types of malignancies (Table II). Two of these antibodies, ipilimumab and tremelimumab, have reached phase III clinical trials.²⁸⁷

4. BIFUNCTIONAL PEPTIDE INHIBITORS

Recently, we have discovered a novel and selective method to suppress autoimmune diseases using bifunctional peptide inhibitor (BPI) molecules that simultaneously target both Signal-1 and Signal-2 (Fig. 3). Results from our studies have demonstrated that two different BPI molecules (i.e., PLP-BPI and GAD-BPI) can induce immunotolerance in autoimmune diseases in two different animal models, EAE^{288,289} and NOD mice.¹⁵⁹ In addition to these two BPI molecules, we also have ongoing and promising studies toward developing similar molecules to treat collagen-induced arthritis in DBA/1J mice, a mouse model for RA. In the case of PLP-BPI, the antigenic-peptide epitope derived from the PLP (PLP₁₃₉₋₁₅₁) was conjugated to LABL peptide derived from α_L integrin (CD11a₂₃₇₋₂₄₆) to make BPI molecules.^{288,289} GAD-BPI is made from GAD₂₀₈₋₂₁₇ and LABL peptides. We hypothesize that the antigenic-peptide fragment (i.e., PLP₁₃₉₋₁₅₁ and GAD₂₀₈₋₂₁₇) binds to MHC-II and the LFA-1 peptide fragment (LABL) binds to ICAM-1 on the surface of APC. Because both peptides are conjugated via a linker, simultaneous binding of BPI to MHC-II and ICAM-1 will prevent the translocation between TCR:MHC-II-peptide (Signal-1) and ICAM-1/LFA-1 complexes (Signal-2) that forms the IS. Inhibition of IS formation selectively alters the activation of T cells from T_{H1} to T_{H2} phenotypes and/or induces the production of T_{reg} cells. Because these BPI molecules contain specific antigenic peptides, we hope that we can target only a specific subpopulation of T cells involved in the onset and progression of autoimmune diseases without affecting the general immune response.

Studies with experimental models of autoimmune disease and allergy have shown that the administration of soluble peptides based on known T-cell epitopes leads to suppression of the specific response, induction of bystander suppression, and prevention and treatment of hypersensitivity. Injections of antigenic peptides, such as PLP₁₃₉₋₁₅₁^{266,290} and GAD₂₀₈₋₂₁₇¹⁶⁷ in saline, have been shown to suppress the progression of EAE and T1D, respectively, in mice models. However, treatment with antigenic peptides is potentially dangerous and can lead to fatal anaphylactic reactions. Many researchers have modified these antigenic peptides via mutation²⁹¹ or development of altered peptide ligands (APL)²⁹² in an effort to improve their therapeutic potential in treating autoimmune diseases as well as address their safety concerns.

Our data showed that PLP-BPI has better activity in suppressing EAE than do other peptides, including VP2-BPI (a BPI with an epitope peptide derived from Theiler's encephalomyelitis virus capsid protein, VP2₇₄₋₈₆), which is known to bind to MHC-II in SJL/J mice,²⁹³ PLP-BPI_{sLABL} (PLP-BPI with a scrambled sequence of LABL), OVA-BPI

(BPI in which PLP₁₃₉₋₁₅₁ has been replaced with OVA₃₂₆₋₃₃₇ derived from ovalbumin), and the unlinked mixture of PLP₁₃₉₋₁₅₁ and LABL. PLP-BPI-treated mice had very low EAE clinical scores and minimal loss in body weight compared with other groups. In addition, some of the PLP-BPI-treated mice did not develop the disease.^{288,289} Similarly, dosing regimens designed for therapeutic treatment instead of prophylactic treatment showed that BPI was able to reverse disease severity very quickly.²⁸⁹ Some of our unpublished work shows that PLP-BPI_{sPLP} (PLP-BPI with a scrambled sequence of PLP₁₃₉₋₁₅₁) has no EAE-suppressing activity, suggesting the significance of a unique structure in PLP-BPI such as the need for both PLP₁₃₉₋₁₅₁ and LABL peptides and covalent linking of these two peptides in the same molecule. Also, more importantly, we found out that these molecules are highly antigen-specific. We observed that splenocytes isolated from PLP₁₃₉₋₁₅₁-immunized mice responded to *in vitro* re-stimulation with PLP₁₃₉₋₁₅₁ but not MBP₈₇₋₈₉ and vice versa.²⁸⁹ It was also interesting to find that splenocytes isolated from Ac-PLP-BPI-NH₂ (a modified form of PLP-BPI) showed significantly less proliferation in re-call to PLP₁₃₉₋₁₅₁, suggesting that injection of Ac-PLP-BPI-NH₂ reduces and/or suppresses the number of PLP₁₃₉₋₁₅₁-responsive populations. In a parallel study, GAD-BPI had the capacity to suppress the progression of T1D in NOD mice as demonstrated by significantly less insulinitis and lower blood glucose levels in GAD-BPI-treated mice compared with control.¹⁵⁹

Another important issue we looked at is the safety of these BPI molecules. As with any other peptide-based therapy, there is an associated risk involved in the use of BPI such as the possibility of an anaphylactic response, a life-threatening immediate hypersensitivity reaction caused by injections of the antigen-related peptides. Owing to hypersensitivity reactions in patients during phase II clinical trials, development of an MS-targeted peptide drug was suspended.^{294,295} In our studies, *i.v.* injection of PLP-BPI at 4–5 weeks after immunization caused anaphylactic reaction to a much lower number of mice than does PLP₁₃₉₋₁₅₁. Additionally, a modified form of PLP-BPI (*i.e.*, Ac-PLP-BPI-NH₂-2 with a longer linker and its N- and C-terminal acetylated and amidated, respectively) was found to be even less aggressive in inducing anaphylactic reactions.²⁸⁹ We speculate that the lower incidence of anaphylaxis in BPI is due to the presence of LABL peptide that inhibits LFA-1/ICAM-1 interactions at the site of antigen recognition or the addition of another moiety at the N- or C-terminal to the parental allergic peptide PLP₁₃₉₋₁₅₁. Involvement of LFA-1/ICAM-1-mediated heterotypic aggregation of activated T cells to mast cells has been implicated in augmenting mast cell degranulation and histamine release.²⁹⁶

5. PEPTIDE SAFETY

A potential problem that can arise when treating diseases with multiple injections of antigen-related peptides is the possibility of anaphylactic shock.^{291,297} As a result of the fact that peptides are smaller in size than proteins, it is thought that they are safer and less likely to induce an anaphylactic response in the immune system. Despite the numerous advantages of peptides over proteins, anaphylactic reactions during treatments involving peptides have been widely described in the literature.^{288,291,297}

In general, the mechanism of anaphylactic response is thought to occur when an antigen crosslinks with IgE bound to FcεRI on mast cells, which leads to degranulation and the release of histamine and cytokines, both of which are inflammatory mediators.²⁹⁶ The administration of anti-IgE antibodies along with myelin-specific peptides during the treatment of EAE has been shown to inhibit anaphylaxis, which suggests that IgE plays a large role in anaphylaxis when treating with peptides.²⁹⁸

APL have been shown to suppress anaphylaxis in EAE without affecting its activity toward myelin-reactive T cells.²⁹² Short linear peptide sequences generally lack the ability to crosslink adjacent IgE molecules on mast cells and basophils.²⁹⁹ Because a nonimmunogenic peptide sequence can become immunogenic when only one of its amino acid residues is altered, care must be exercised in designing an APL so that it retains its activity toward its target and does not become more antigenic.

The avoidance of IgE activation is necessary to circumvent allergic response. It is optimal to use peptides shorter than 20 amino acids, as longer peptides have been associated with more adverse events compared with their shorter counterparts.²⁹⁹ Five-to-six amino acids have been shown to be sufficient minimal antigenic determinants.³⁰⁰ Alternatively, APL that can bypass the body's ability to create an anaphylactic response against a peptide without affecting its activity toward its target have been shown to work and must be utilized in creating effective therapies.

6. MECHANISM OF PEPTIDES THAT TREAT AUTOIMMUNE DISEASES

It has been described before that the immune system functions as a balance between pathogenic effector cells and regulatory cells.²⁹⁹ The suppression and activation of these two will lead to either an immunogenic response or an immunotolerant response (Fig. 4). Autoimmune diseases typically arise due to an imbalance between these two responses. Therefore, to combat autoimmune diseases, we must try to restore balance in the immune system. One way to restore the balance is by either inducing the suppressive cells or activating the regulatory cells.

DC are considered to be the most prominent APC in inducing an autoimmune disease; Yamazaki et al.³⁰¹ and Steinman et al.³⁰² provided excellent reviews on the function of DC in tolerance and immune responses. They are believed to send a “danger” signal to induce an immunogenic response, but that is not always the case.³⁰³ The function of the DC depends on whether it is in a mature (activated) or immature state.³⁰⁴

The maturation of DC into a T-cell stimulatory mode is activated by an inflammatory stimulus that is initiated by the uptake of an insoluble antigen (Fig. 4, left side).^{299,304} Once the DC is in the mature state, its phenotype changes and there is an upregulation of costimulatory and adhesion molecule expression on its cell surface. Presentation of antigen in this way leads to the differentiation of proinflammatory cellular responses.²⁹⁹ Such an inflammatory cellular response triggered by a mature DC (mDC) includes the induction of T_H1 cells that produce IFN-γ and IL-2.³⁰⁴ Other proinflammatory responses include the induction of T_H17 and the release of IL-17.¹²⁸ These inflammatory responses triggered toward self tissues usually lead to an autoimmune disease such as MS.

On the other hand, an immature DC (iDC) is one that did not take up and process any antigens. It has been shown, using antibodies specific for peptide-free MHC-II molecules, that iDC express empty MHC-II molecules on their surface and can bind antigenic peptides in solution. These bound antigens can be presented to T cells without internalization and processing.³⁰⁵ Soluble peptides that are presented by iDC lead to a differentiation of T_{reg} cells (Fig. 4, right side).²⁹⁹ Activation of regulatory T cells leads to the production of regulatory cytokines such as IL-10 and TGF- β . Such cytokines have been shown to have an important role in ameliorating autoimmune diseases in animal experimental models.^{306–308} In the case of some autoimmune diseases, expanding the “regulatory pool” leads to a downregulation of the effector response (T_{H1}) and enhancement of the immunosuppressive response (T_{H2}).²⁹⁹

7. PROPOSED BPI MECHANISMS

There are several possible mechanisms that could explain how BPI molecules suppress the activation of T cells and result in suppression of autoimmune diseases. The first of these is that only the antigenic fragment of BPI molecule binds to MHC-II on the iDC (steady-state DC) and is presented to naïve T cells for differentiation to produce T_{reg} cells that generate IL-10 (Fig. 5, right side). This proposed mechanism, which is similar to the mechanism of action of soluble antigenic peptide as a therapeutic vaccine,²⁹⁹ ignores the role of cell adhesion peptide (e.g., cIBR or LABL) on the BPI molecule. It has been shown that injection of the BPI molecules generates IL-10;²⁸⁸ however, the kinetics and the amount of IL-10 production upon BPI and PLP peptide injections have not been compared. Because the BPI molecule has better efficacy than the parent antigenic peptide (PLP),²⁸⁸ it is predicted that the amount of IL-10 produced by injecting BPI molecules will be higher than that produced by injecting PLP peptide. The second possible mechanism is that the BPI molecule binds not only to MHC-II but also to adhesion molecules (e.g., LFA-1 or ICAM-1) on iDC. Thus, during the interaction between BPI-loaded iDC and naïve T cells, the BPI molecule blocks the IS formation to induce differentiation of naïve T cells to T_{reg} cells. The presence of cell adhesion peptide on BPI makes it a more efficient modulator for naïve T cell differentiation than the antigenic peptide alone. The third possible mechanism is that the BPI molecule simultaneously binds to MHC-II and LFA-1 to block the IS formation during the interaction between naïve T cells and mDC (Fig. 5, left side) to suppress the differentiation of naïve T cells to T_{H1} and T_{H17} cells. There is some indication that the BPI molecule induces the generation of IL-4-producing T_{H2} cells, which may tip the balance to lower the production of T_{H17} and T_{H1} cells. The increase in IL-4 production was observed after BPI injection into the EAE mouse model.²⁸⁸ The mechanism of action of BPI molecules may also be a combination of these three proposed mechanisms.

8. CONCLUSIONS

Immunomodulating compounds have been explored as potential treatments for autoimmune diseases, such as MS, T1D, and RA among others. However, most of the agents developed thus far aim at broad modulation of the immune response and, therefore, may present some undesirable side effects. Targeted drug delivery is a more attractive strategy to improve the efficacy and reduce the side effects of these immunomodulating drugs. In our studies, we

hope to design BPIs that target only a subpopulation of T cells responsible for the progression of the disease without affecting the general immune response. Although it has not been clearly elucidated, BPI-based therapies work either by inhibiting the formation of the IS by blocking both Signal-1 and Signal-2 or by shifting the T-cell subpopulation into T_{regs} and/or T_H2-like phenotype. The detailed mechanisms of action of BPI-type molecules, including the antigen-specific immunosuppressive activity as well as its safety, are being investigated in depth in our current studies. Additionally, we are looking at ways of improving the structure and sequence of BPI to provide a more efficient and well-tolerated immunotherapy.

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Biographies

Prakash Manikwar is a postdoctoral researcher working for Dr. David B. Volkin in the Department of Pharmaceutical Chemistry at The University of Kansas, Lawrence. He received his B. Pharmacy degree from Rajiv Gandhi University of Health Sciences in India, M.S. in Biological Sciences from South Dakota State University, and M.S. and Ph.D. degrees in Pharmaceutical Chemistry from The University of Kansas. He is currently working on analytical characterization, stabilization, and formulation of biopharmaceutical.

Paul Kiptoo is a postdoctoral researcher working for Dr. Teruna J. Siahaan in the Department of Pharmaceutical Chemistry at the University of Kansas. He received his Ph.D. in Pharmaceutical Sciences from the University of Kentucky. He is currently working on enhancing the absorption of anticancer drugs into the brain using E-cadherin peptides that inhibit E-cadherin interactions at the intercellular tight junctions and also targeting antigenic peptides to leukocytes in order to modulate the formation of the immunological synapse for the suppression of autoimmune diseases such as MS and RA.

Ahmed H. Badawi received his Bachelor of Science degree from the University of Missouri –Kansas City in Pharmaceutical Science and Chemistry. Ahmed then moved to the University of Kansas where he earned his Master of Science degree in Pharmaceutical Chemistry with honors. Currently, Ahmed is a doctoral student working for Dr. Teruna J. Siahaan in the Department of Pharmaceutical Chemistry at The University of Kansas. His project entails developing peptides for the treatment of autoimmune diseases.

Barlas Büyüktimkin is a Ph.D. candidate working for Dr. Teruna J. Siahaan in the Pharmaceutical Chemistry Department at The University of Kansas, Lawrence. He received his Doctor of Pharmacy degree from the University of Kansas, and M.S. in Pharmaceutical Chemistry from The University of Kansas. He is currently working on peptide and protein-mediated drug delivery.

Teruna J. Siahaan is a Professor and the Associate Chair of the Department of Pharmaceutical Chemistry at The University of Kansas. His research has explored the utilization and modulation of cell adhesion molecules on the cell surface for targeted drug delivery to a specific cell type, and for enhancing drug permeation through the intestinal mucosa and blood– brain barrier (BBB). His research group is very well known in the study of peptide-mediated drug delivery. He has more than 20 years of experience in both academia and industry and is a recipient of numerous awards. He has given many invited presentations and has published more than 120 research and review articles. He serves in the Editorial board of several journals and in grant review panels for National Institutes of Health (NIH), Department of Defense (DOD), and various Research Foundations. He has received several grants from NIH, National MS Society, Alzheimer’s Association, Arthritis Foundation, and American Heart Association.

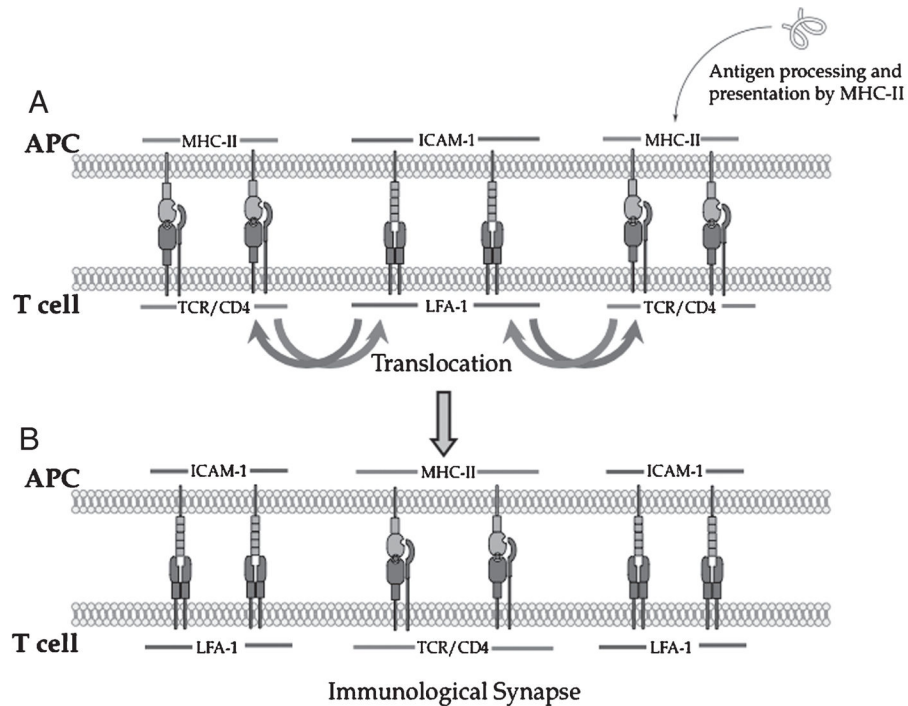


Figure 1. Mechanism of immunological synapse formation during T cell and APC interaction. **(A)** Initial contact between Signal-1 (TCR/MHC-II-peptide complex) and Signal-2 (LFA-1/ICAM-1 complex). **(B)** Translocation of Signal-1 and Signal-2 to form c-SMAC and p-SMAC of the immunological synapse. APC, antigen-presenting cell; SMAC, supramolecular activation clusters; ICAM-1, intercellular adhesion molecule-1; MHC, major histocompatibility complex; TCR, T-cell receptors; LFA, leukocyte function-associated antigen; p-SMAC, peripheral SMAC.

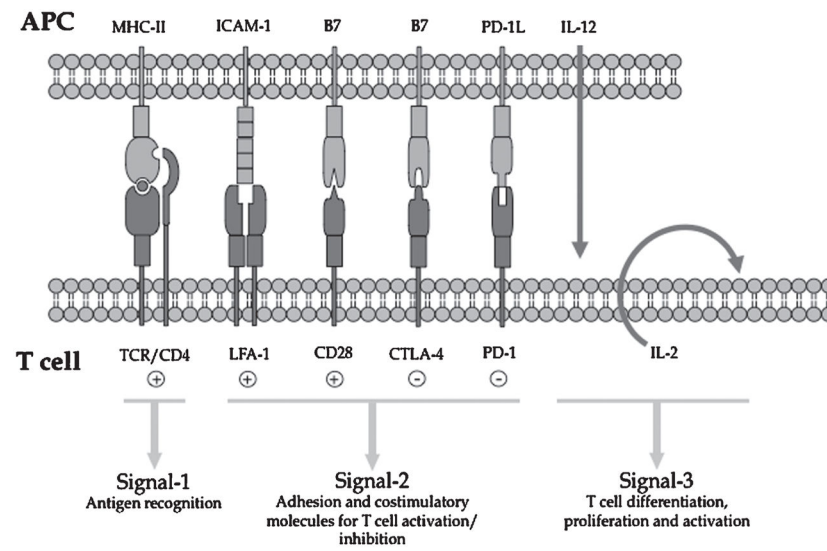


Figure 2. Signaling molecules involved in the interface of T cell and APC interaction. The interaction between T cell and APC involves several pairs of receptors (Signal-1 and -2) and is associated with the release of cytokines (Signal-3). APC, antigen-presenting cell.

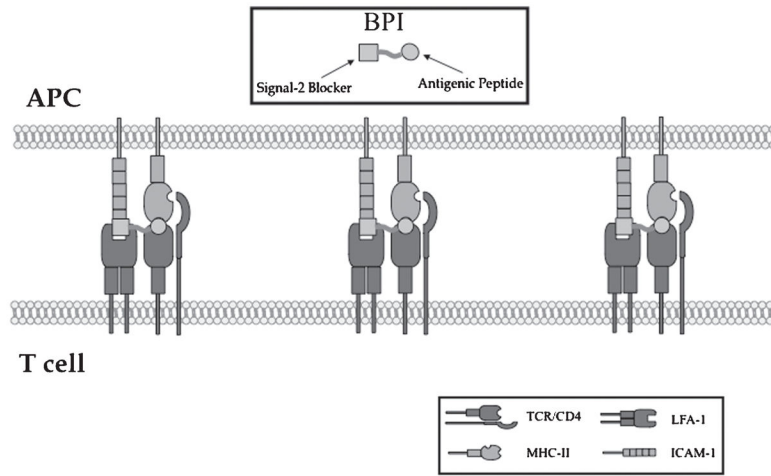


Figure 3. The proposed binding of the BPI molecule to MHC-II and ICAM-1, which inhibits the translocation of Signal-1 and Signal-2. ICAM-1, intercellular adhesion molecule-1; MHC, major histocompatibility complex; BPI, bifunctional peptide inhibitor.

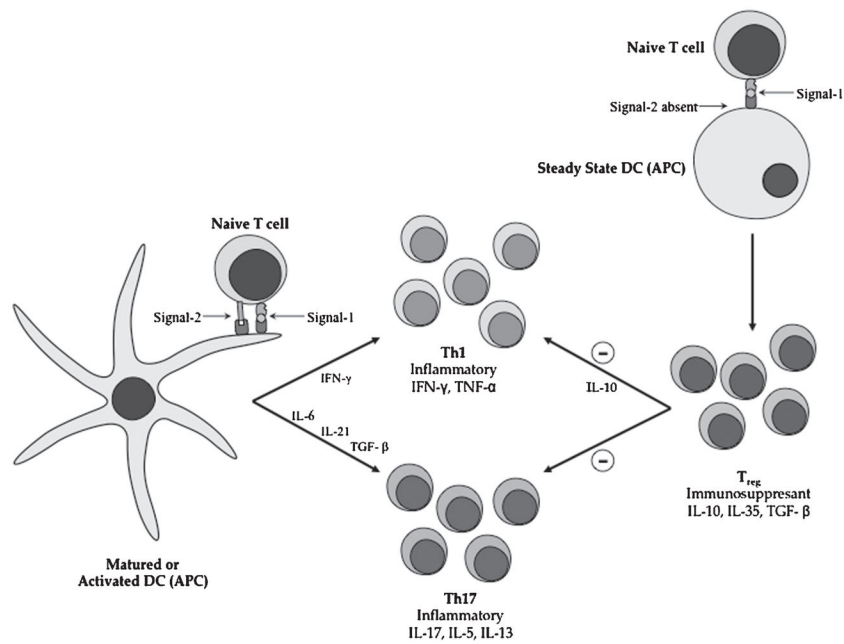


Figure 4.

The proposed mechanism of activation and suppression of immune response during autoimmune diseases. On the left, interaction between mature/activated DC and naïve T cells is mediated by MHC-II antigen/TCR complex (Signal-1) in the presence of the costimulatory signal (Signal-2) that leads to differentiation into $\text{T}_{\text{H}1}$ and $\text{T}_{\text{H}17}$ cells. On the right, interaction between immature/steady-state DC and naïve T cells is mediated by MHC-II antigen/TCR complex (Signal-1) in the absence of the costimulatory signal (Signal-2) that leads to differentiation into T_{reg} cells. T_{reg} cells produce IL-10 that suppresses the proliferation of $\text{T}_{\text{H}1}$ and $\text{T}_{\text{H}17}$. MHC, major histocompatibility complex; TCR, T-cell receptors; DC, dendritic cells.

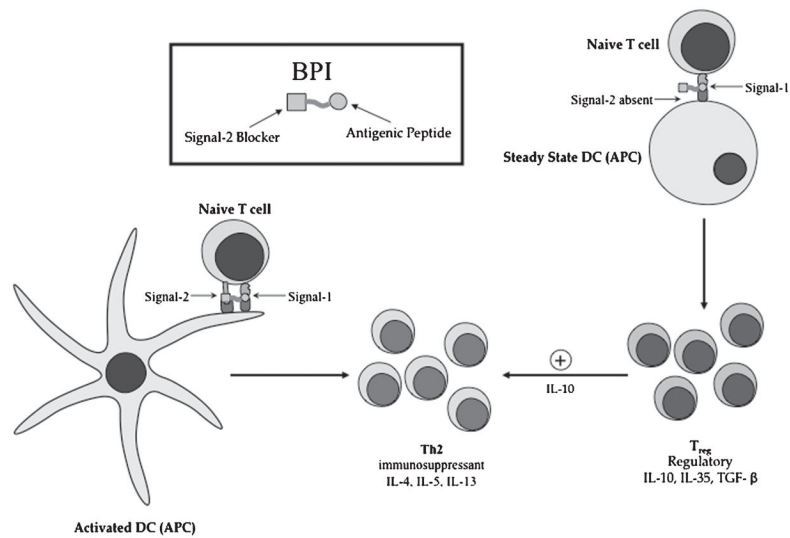


Figure 5. Potential mechanism of BPI molecules in altering the differentiation of naïve T cells upon interaction with immature and mature DC. On the right, interaction of steady-state/immature DC with BPI molecule produces differentiation of naïve T cells to IL-10-producing T_{reg} cells. IL-10 induces the proliferation of immunosuppressant T_{H2} cells. On the left, interaction of BPI molecule with mature/activated DC induces immunosuppressant T_{H2} cell differentiation. MHC, major histocompatibility complex; TCR, T-cell receptors; DC, dendritic cells; BPI, bifunctional peptide inhibitor.

Table I

List of Candidate Autoantigens in MS, T1D, and RA

Disease	Antigen	References
Multiple sclerosis	Proteolipid protein (PLP) Myelin basic protein (MBP) Myelin oligodendrocyte glycoprotein (MOG) Astrocyte-protein S100 β	309
Type 1 diabetes	Glutamic acid decarboxylase (GAD) Insulin zinc transporter (ZNT8) Insulinoma antigen (IA-2) Insulin	159-161
Rheumatoid arthritis	Human cartilage gp-39 Cartilage proteins melanoma inhibitory activity (MIA) Type II collagen Citrullinated proteins (e.g., fibrin)	310-316

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Table II

Antibodies and Peptides That Have Been Investigated for Blocking Signal-2 in Autoimmune Diseases, Organ Transplantation, and Cancer

Signal-2 Receptor	Peptide/protein ligand	Target disease	References
LFA-1/ICAM-1	Anti-LFA-1 mAb (Odalimumab, Efalizumab)	Psoriasis, Type 1 diabetes, and transplantation	203,214,216-219,237
LFA-1/ICAM-1	Anti-ICAM-1 mAb (Enlimomab)	Rheumatoid arthritis, Type 1 diabetes, and transplantation	226,233,234
LFA-1/ICAM-1	Cyclo(1,12)-PenPRGGSVLVTGC (cIBR)	Rheumatoid arthritis, inflammation, and immunosuppression	246,250
LFA-1/ICAM-1	Cyclo(1,12)-PenITDGEATDSGC (cLABEL)	Rheumatoid arthritis, inflammation, and immunosuppression	250,251,317,318
LFA-1/ICAM-1	Cyclo(1,9)-CLLRMRSIC	Inflammation	319
B7/CD28	Anti-CD28 mAb	Transplantation	320
B7/CTLA-4	Anti-CTLA4 mAb (Ipilimumab and Tremelimumab)	Melanoma and other types of malignancies	287
B7/CTLA-4	Abatacept (CTLA4-Ig)	Rheumatoid arthritis	321

CTLA, cytotoxic T-lymphocyte antigen; mAb, monoclonal antibody; ICAM, intercellular adhesion molecule.