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Bioconjugate Strategies for the Induction of Antigen-Specific Tolerance in Autoimmune Diseases

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Abstract

Antigen-specific immunotherapy (ASI) holds great promise for the treatment of autoimmune diseases. In mice, administration of major histocompatibility complex (MHC) binding synthetic peptides which modulate T cell receptor (TCR) signaling under sub-immunogenic conditions induces selective tolerance without suppressing the global immune responses. However, clinical translation has yielded limited success. It has become apparent that the TCR signaling pathway via synthetic peptide antigen alone is inadequate to induce an effective tolerogenic immunity in autoimmune diseases. Bioconjugate strategies combining additional immunomodulatory functions with TCR signaling can amplify the antigen-specific immune tolerance and possibly lead to the development of new treatments in autoimmune diseases. In this review, we provide a summary of recent advances in the development of bioconjugates to achieve antigen-specific immune tolerance *in vivo*, with the discussion focused on the underlying design principles and challenges that must be overcome to target these therapies to patients suffering from autoimmune diseases.

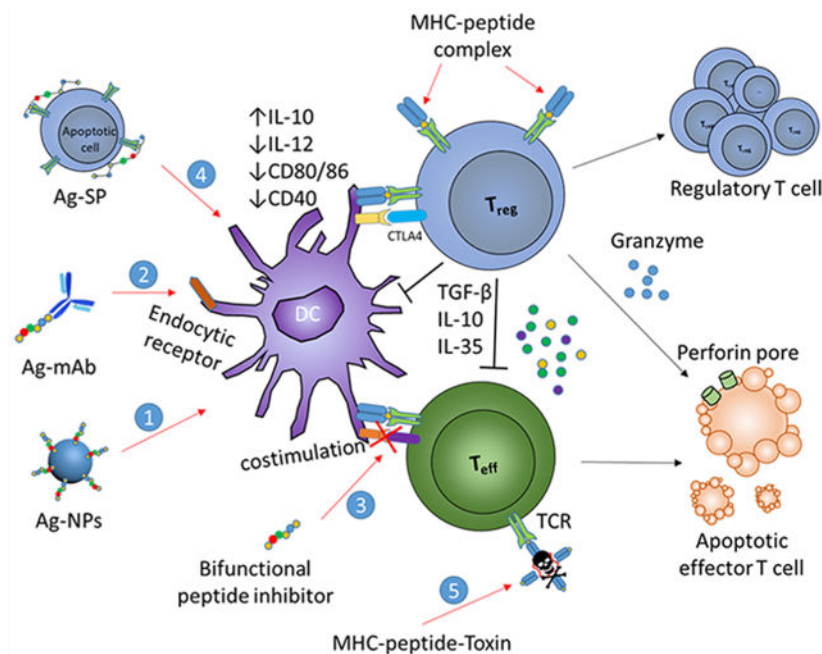
Graphical Abstract

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INTRODUCTION

Autoimmune diseases are characterized by aberrant activation of T- and B-cells *in vivo* which results in the immune destruction of the host tissues or organs.^{1,2} These diseases represent a heterogeneous group of disorders with a combination of genetic factors and environmental triggers (virus, bacteria, and other infectious pathogens).¹⁻⁴ According to the National Institute of Allergy and Infectious Diseases (NIAID), there are more than 80 autoimmune diseases affecting nearly 20 million people in the US alone and the prevalence is rising each year.⁵ Some of the most common autoimmune diseases include type 1 diabetes (T1D), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD), and multiple sclerosis (MS).⁵ The precise causes of many autoimmune diseases remain unknown, and there is no effective screening to detect individuals at risk for most autoimmune diseases, making disease prevention almost impossible.^{1,2} Thus, most patients have significant disease progression and tissue destruction before clinical diagnosis and initiation of appropriate treatments.

Currently available treatments for autoimmune diseases include physical therapy, immunosuppressants, corticosteroids, anti-inflammatory drugs, and cell or tissue transplantation, among others.^{6,7} These treatments alleviate the symptoms but do not alter the overall chronic course of diseases. For example, immunosuppressants (i.e., cyclosporine A) inhibit the activity of the immune system by reducing the proliferation and function of cells associated with immune reactions and show partial efficacy in many autoimmune diseases.⁷ However, their therapeutic effects are dependent on chronic drug administration that can lead to systemic immune suppression, with the potential risk of development of cancer and opportunistic infections.⁷ Antigen-specific immunotherapy (ASI) is the treatment able to modify the outcome of the diseases by restoring self-tolerance toward autoantigens.

^{8–10} Under sub-immunogenic conditions (i.e., in the presence of low levels of stimulatory molecules), ASI introduces autoantigens to antigen presenting cells (APCs) in order to correct the immune responses, acting directly through T cell receptor (TCR) on effector T cells (via clonal deletion or anergy) and/or via regulatory T cells (Tregs) that secrete anti-inflammatory cytokines.^{8–10} One of the main implications of this intervention is its specificity: compared with other immune therapies, ASI selectively targets disease-relevant T cells, while leaving the normal immune system intact. Initial experimental approaches have included oral antigen administration,¹¹ particulate autoantigen delivery,¹² altered peptide ligands,¹³ and dose escalating immunotherapy,¹⁴ among many others. Although these approaches have shown efficacy in preclinical models, results from human clinical trials have not shown the same level of efficacy as in mice.^{9,10} In spite of intensive research, to date, no FDA approved ASI is available for treating patients with autoimmune diseases. Induction of antigen-specific tolerance to dominant immune responses driving autoimmunity remains an unmet challenge.

Though our current understanding of the enormously complex human immune system is far from complete, it is clear that modulation of the TCR signaling pathway via administration of autoantigen alone is insufficient and that alternative strategies which can amplify the antigen-specific immune tolerance in animal models are needed to increase the validity and predictive power in the development of new treatment in humans.¹³ In the past two decades, many technologies have been developed in the creation of a new generation of ASI in order to attenuate the inflammation in autoimmune diseases. Efforts have been devoted to designing delivery vehicles which target key antigen-presenting cells,¹⁵ or combination immunomodulation which codelivers immune-modulators with autoantigens.¹⁶ These strategies target TCR signaling pathways, cosignaling molecules and cytokines, or inhibit the formation of the immunological synapse, enabling the augmentation of TCR-mediated tolerance. Among these different approaches, one of the most attractive strategies is to incorporate additional immunomodulatory functions into autoantigens through bioconjugation (Figure 1). In fact, modification of autoantigen through functional conjugation to polymers, nanoparticles (NPs), antibodies, or small molecules have been extensively used in vaccines.^{16–18} In this review, we highlight the recent advances in experimental mouse models using various bioconjugate strategies aimed at the induction of autoantigen-specific tolerance for the potential treatment of autoimmune diseases. We focus our discussion on the design principles for each of the strategies, along with their limitations and potential for clinical translation. Some common autoimmune diseases and corresponding bioconjugate strategies reviewed in this paper are summarized in Table 1.

IMMUNOLOGICAL BASIS OF SELF-TOLERANCE AND TARGETING DC–T CELL INTERACTIONS FOR THE PROMOTION OF ANTIGEN-SPECIFIC TOLERANCE

The central role of the immune system is to discriminate between self-and non-self-antigens and respond specifically to remove non-self-pathogens invading the body while leaving the self-organs intact. The immune response is a complex process involving multiple coordinated actions of diverse immune cells and molecular signals (Figure 2). Specialized

sentinel cells known as APCs phagocytose antigens and present fragments of them to T cells via major histocompatibility complex (MHC) molecules. In the presence of appropriate inflammatory cues, CD8⁺ and CD4⁺ T cells with matching receptors are activated by interacting with APCs presenting an antigen, and proliferate and differentiate into cytotoxic “killer” cells (CD8⁺ T-cells) or “helper” cells (CD4⁺ T-cells). The protective functions of T-cells are collectively known as cellular response. In addition, B-cells acquire and process antigen, and with the “help” from CD4⁺ T cells, differentiate into antibody-producing plasma cells. Immune protection mediated by secreted antibodies is referred to as humoral response.

In addition to immune activation, the immune system is equipped with mechanisms to suppress immune responses. In healthy individuals, the immune system maintains unresponsiveness to antigens recognized as “self”, and thus does not react to antigens expressed in endogenous tissues. When such self-tolerance is lost, underlying self-recognition can result in tissue destruction mediated by humoral or cellular mechanisms, or both, leading to autoimmune diseases.

Self-tolerance is maintained by a coordinating operation of central and peripheral mechanisms (Figure 3). In central tolerance, most T- and B-lymphocytes with receptors specific for autoantigens are eliminated at an early stage in lymphoid cell development in the thymus and bone marrow, respectively. This process is also known as negative selection and it allows most self-reactive B and T cells to be removed before they enter the peripheral. However, it is becoming clear that the negative selection is not a perfect process as the peripheral repertoire of healthy individuals contains a high frequency of diverse, self-reactive lymphocytes.¹⁹ Fortunately, a healthy immune system can effectively prevent both the self-reactive B and T cells that escaped from central deletion from initiating potentially dangerous immune responses against the body’s own tissues. This is achieved by a number of regulatory mechanisms outside of the primary lymphoid tissues collectively known as peripheral tolerance (Figure 3). These mechanisms affect the survival, differentiation, and function of T and B cells and play an important role in the induction and maintenance of self-tolerance.

Full activation of T cells requires an antigen-specific signal delivered through the TCR and appropriate costimulatory signals. However, engagement of TCR alone produces intrinsically unresponsive T cells that remain alive for an extended period, a state known as anergy.²⁰ These cells are associated with diminished proliferation and cytokine production, and compromised effector functions.²⁰ Another peripheral tolerance mechanism is activation-induced cell death (AICD) caused by the interaction between Fas receptors (FasR) and Fas ligands (FasL).²¹ AICD in T cells by FasR/FasL-mediated apoptosis is an important regulatory mechanism to down-regulate inflammation and promote peripheral tolerance as mice with defective FasR or FasL develop a number of autoimmune diseases.²¹ Unlike anergy, activation-induced cell death requires stimulation of T cells by competent APCs and it is dependent on IL-2 (interleukin 2), which enhances AICD by up-regulating FasL expression.²²

A third mechanism of peripheral tolerance which prevents autoimmune reactions is the suppression of self-reactive lymphocytes by Tregs. It is now known that various subsets of Tregs exist in the immune system. CD4⁺ Tregs are classified into thymus-derived natural Tregs, which are CD4⁺CD25⁺Foxp3⁺, and peripherally induced Tregs including type 1 Tregs (Tr1) which secrete IL-10 (interleukin 10), Th3 (T helper 3) Tregs which secrete IL-10 and TGF- β (transforming growth factor beta), and Foxp3⁺ Tregs. Besides, based on their regulatory function, other lymphocyte subsets are also recognized as Tregs: inducible CD8⁺ Tregs, double negative Tregs (CD3⁺CD4⁻CD8⁻ Tregs), CD4⁺V α 14⁺ natural killer T cell, and $\gamma\delta$ T cells.²³ These Tregs have been shown to suppress T-cell responses by either induction of effector T cell death or by elaborating soluble immunosuppressive factors such as TGF- β and IL-10.²³ The most important subset of Tregs expresses the transcription factor forkhead box P3 (Foxp3). Evidence has accumulated that Tregs, defined by the expression of CD4, CD25, and Foxp3 have an indispensable role in the maintenance of self-tolerance.²⁴ In mice, depletion of Foxp3⁺CD25⁺CD4⁺ Tregs results in the spontaneous development of a variety of autoimmune diseases, including autoimmune thyroiditis, diabetes, and IBD, and that the disease could be reversed by adoptive transfer of Tregs.^{24,25} Immune suppression mediated by Tregs is antigen-dependent. In the secondary lymphoid organs, Tregs encounter specific antigens presented on antigen presenting cells and in the presence of IL-2, undergo clonal expansion.²⁶ The immune suppression of effector T cells by Tregs is more complicated. Both antigen-specific and bystander suppression of effector cells have been reported.^{27,28} However, it is speculated that antigen-specific suppression is more effective than bystander suppression.²⁹ Tregs can exert their suppressive functions directly on the effector T cells or indirectly on the function and maturation of DCs.²⁹ It has been shown that inhibition by Tregs can be mediated by immunoregulatory cytokines such as TGF- β , IL-10, and IL-35.²⁹ These cytokines inhibit the production of inflammatory cytokines such as IL-12, causing a decrease in the T helper type 1 (Th1) response and IFN- γ (interferon gamma) production, thereby inhibiting the self-reactive immune responses.²⁹ Tregs were also found to be able to produce granzyme B, which induced apoptosis in effector T cells via cell-to-cell interaction. This mechanism decreases the number of effector T cells and promotes the induction of self-tolerance.²⁹ In addition to the direct effect of Tregs on effector T cells, Tregs can inhibit the maturation and function of DCs by promoting the tolerogenic phenotype of DCs. Although the mechanism is unknown, it is believed that Tregs achieve this by cell surface molecules or cytokines such as CTLA-4, IL-10, and TGF- β .

B cell activation and plasma cell differentiation are critically dependent on T cell help.³¹ T helper cells (Th) recognize peptide/MHC II on B cells and provide costimulation via CD40L and CD40 interaction as well as cytokines such as IL-4 and IL-21, which in turn sustain B cell activation and differentiation into antibody-secreting cells. If helper T cells specific for the autoantigens are deleted, suppressed, or rendered anergic, self-reactive B cell responses do not occur. Thus, CD4⁺ T helper cells are ultimately crucial for B cell tolerance.³¹

Antigen presenting cells especially DCs play essential roles in the initiation and maintenance of peripheral tolerance.^{15,32} Both T- and B-cell-mediated immunity are tightly linked to DCs through antigen presentation, costimulatory interaction, and cytokine factors. As outlined above, induction of antigen-specific tolerance is a complex process that involves

activation and/or inhibition of multiple immunological pathways. Therapeutic manipulation of these pathways by different strategies provides mechanisms to induce antigen-specific immune suppression without compromising the ability for global immune responses. One of the first targets in tolerance induction is the MHC/peptide/TCR interaction. T cell tolerance can be achieved by inducing a partial or altered signal through the MHC/peptide/TCR trimolecular interaction.^{8–10} Typically, TCR stimulation in the absence or low levels of costimulation leads to T cell anergy, depletion, or differentiation to Tregs.^{8,9} Targeting MHC/peptide/TCR based on administration of autoantigens is being pursued treating autoimmune diseases, such as MS, SLE, and T1D.^{8–10} Antigen dose, routes, and kinetics that promote tolerance have been explored to modulate disease progression but with limited success.^{11–14} These failures may be related to the choice of antigen, dose, frequency, route, and mode of delivery, but most probably suggest TCR signal alone is insufficient in reversing the disease-causing autoimmunity *in vivo*.

Exogenous targeting the costimulatory and/or coinhibitory pathways appears to be a logical approach for therapeutic manipulation to down-regulate the pathologic immunity.^{16,33–35} It is now clear that both costimulatory and coinhibitory molecules are essential for the initial induction and function of Tregs. Along with this line of research, a variety of costimulatory and coinhibitory pathways have been explored. CD28, B7 (CD80/CD86), CTLA-4, CD40, CD154, ICOS, OX40, and 4–1BB have been targeted with agonists or antagonists to modulate the APC–T cell interaction.^{16,36} These treatments alone or combined with autoantigen have sparked enormous research. Much work has been conducted on the B7-CD28/CTLA-4 costimulatory system which plays a critical role in effector T cells activation versus suppression. For example, Abatacept is an FDA approved B7-binding fusion protein used to treat RA.³⁷ It has also been shown to be effective in treating T1D.³⁸ Blockade of CD28 with anti-CD28 antagonists promotes the engagement of CTLA-4 with B7, delivering a negative signal into effector T cells and inhibiting their activation. Several antagonists against CD28 have been developed and inhibited disease progression in both rodents and humans.³⁹ In line with this research, similar pathways have been targeted for autoimmune modulation. Efficacy in several murine autoimmune diseases has been demonstrated where 4–1BBL/4–1BB, PD-1/PD-L1 were targeted alone or in combination with TCR stimulation.^{16,36} These studies demonstrated the potential of targeting DC/T cell interaction in tolerance induction. However, immunomodulatory effects of targeting costimulatory or coinhibitory molecules are potentially more complex. Achieving a delicate balance appears to be important for an effective tolerance induction: while costimulation through 4–1BB, OX40 promotes Tregs proliferation, it also reduces Tregs suppressive capacity.^{40,41} Similarly, in an EAE (experimental autoimmune encephalomyelitis) mouse model of MS, mice were immunized with MOG_{35–55} (myelin oligodendrocyte glycoprotein) and then treated with CTLA-4Ig (anti-B7 monoclonal antibody) to achieve B7 blockade. Surprisingly, it was revealed that CTLA-4Ig treated mice had about two times higher disease scores compared with PBS treated control mice, implying that the B7 blockade exacerbated disease. This study suggested that a certain level of B7/CD28 engagement was required for Tregs proliferation and function.⁴²

Another promising target is the CD40/CD154 interaction. Engaging CD40/CD154 delivers activating intracellular signals to ACPs and subsequently controls T-dependent B cell

response and T cell priming. Thus, disrupting CD40/CD154 interaction is likely a viable strategy in treating autoimmune diseases in which activated T and B cells are involved.^{43,44} In fact, CD154-blocking antibodies (Ab) can prevent or ameliorate autoimmunity in several disease models, including T1D,⁴⁵ RA,⁴⁶ SLE.⁴⁷ However, anti-CD154 Ab treatment led to thromboembolic complications.⁴⁸ Alternative small molecules which target CD40 are emerging in autoimmunity. For example, a CD40 blocking peptide derived from human CD154 was discovered recently.⁴⁹ Daily administration of CD40 blocking peptide successfully reversed T1D in NOD (nonobese diabetic) mice.⁴⁹

BIOCONJUGATE STRATEGIES FOR THE INDUCTION OF ANTIGEN-SPECIFIC TOLERANCE

Antibody–Antigen Conjugates.

Monoclonal antibody (mAb)-based therapies have become a critical part of the treatment for patients with autoimmunity, cancer, and infectious disease. In autoimmune diseases, antigen–antibody conjugates are mainly used to target DCs, the most efficient antigen presenting cells, to enhance the antigen uptake, processing, and presentation.^{15,32,50} A direct delivery of antigens to DCs subset in the most appropriate tissue in the absence of costimulatory stimuli not only leads to the deletion of antigen-specific T cells, but also to the induction of peripheral tolerance mediated by Tregs, as demonstrated in murine models of autoimmune arthritis, EAE, and T1D.^{51–54} One of the early examples was demonstrated by Finkelman et al. in 1996.⁵⁵ The authors showed a rat IgG2b anti-DC antibody induced rat IgG2b-specific T cell and B cell tolerance, unresponsive to the subsequent challenge. The tolerance induction appeared to be dependent on the specific DC surface receptor, as injection of a DC-specific hamster anti-CD11c mAb stimulated antibody responses rather than tolerance.⁵⁵ Since then, a variety of different antibodies which deliver autoantigens via DC surface receptors have been explored for tolerance induction. DEC-205 is one of the well-studied DC surface receptors with tolerogenic potential.^{51,54–62} Autoantigens conjugated to anti-DEC-205 in the absence of activating stimuli are selectively delivered to DEC-205⁺ DCs, inducing profound peripheral T cell tolerance.^{51,54} Antigen targeted DEC-205 is internalized by receptor-mediated endocytosis, and traffics to late endosomal compartments where high concentrations of MHC II molecules are present (Figure 4).⁵⁶ Low-dose of antigen delivered via DEC-205 exhibited 100-fold increase in antigen presentation (Figure 4),⁵⁷ and favored the conversion of naïve CD4⁺CD25[−]Foxp3[−] T cells into functional CD25⁺Foxp3⁺ Tregs.^{51,54,58} These regulatory responses were very efficient as mice administrated with appropriate autoantigens fused with anti-DEC-205 were protected from autoimmune arthritis,⁵⁹ and EAE,⁶⁰ and T1D.^{61,62} In the T1D studies, linking either CD4⁺ or CD8⁺ epitope to anti-DEC-205 led to the deletion of autoreactive T cells and protected mice from disease in two different mouse T1D models, strongly suggesting this specific endocytic receptor is a valid target for tolerance induction.^{61,62}

Antigen–antibody conjugates targeting plasmacytoid DCs (pDCs) via sialic acid binding Ig-like lectin H (Siglec-H) have been shown to inhibit Th cell-dependent autoimmunity in an antigen-specific manner in mice, even in the presence of strong immune stimulation.⁶³ Siglec-H is an efficient endocytic receptor on plasmacytoid DC precursors and Siglec-

mediated antigen delivery of myelin autoantigen induced a hyporesponsive state in CD4⁺ T cells without conversion to Foxp3⁺ Tregs, and effectively delayed the onset of EAE.⁶³

Targeting several other receptors on DCs has been shown to be effective in Tregs and tolerance induction. Antigen–antibody conjugates targeting migratory DCs promoted peripheral tolerance induction as delivering to Langerin⁺ (CD207) DCs and pDCs induced Foxp3⁺ Tregs proliferation and EAE suppression.⁶⁴ Furthermore, DCs expressing CD103 in the intestine primed Foxp3⁺ Tregs.⁶⁵ These specialized CD103⁺ DCs in the gut lymphoid tissues produced both TGF- β and retinoic acid and efficiently promoted the differentiation of Tregs.⁶⁵

Protein expression pattern differs between mice and humans. To test whether antigen targeting autoantigens to human DCs could suppress autoimmunity, the human IgE Fc domain was fused with autoantigen.⁶⁶ In human Fc ϵ RI α -transgenic mice, such antigen-Fc ϵ conjugate was efficiently delivered to DCs, enhanced antigen presentation by at least 1000-fold, and resulted in the deletion of antigen-specific T cells.⁶⁶ These results warrant consideration of DC targeting for further clinical studies.

Antibody–antigen conjugate represents a simple and effect approach for the induction of antigen-specific immunosuppression. However, like all therapeutic proteins, antibody–antigen conjugates induce antibody responses in patients.⁶⁷ Such antidrug antibodies are a common cause for the treatment failure and adverse hypersensitivity reaction. Nevertheless, management of the immunogenicity of therapeutic antibody by optimizing the antibody design⁶⁷ or by tolerogenic strategies⁶⁸ have been tested to overcome this limitation.

Albumin–Antigen Conjugate.

The effectiveness of antigen-specific immunotherapy is dependent on the accumulation of antigens in the antigen presenting cells. Subunit peptides and proteins have a short half-life *in vivo* due to rapid renal clearance, degradation, and nonspecific tissue accumulation. One effective approach is to tag antigens to albumin, a natural transporter protein with long circulating half-life.⁶⁹ Recently, Tregitopes,⁷⁰ MHC class II epitopes in the Fc fragment of IgG which were capable of specifically activating Tregs, were fused with albumin.⁷¹ Administration of Tregitope-albumin extended antigen half-life. Compared to antigen alone, mice that received a single dose of Tregitope-albumin fusion protein showed a significant reduction of antigen-specific T cell proliferation in OVA-specific T cell proliferation. Albumin fusion, administered either prophylactically or therapeutically ameliorate the disease progression in chronic inflammatory demyelinating polyneuropathy (CIDP) in a spontaneous mouse model.⁷¹ In addition to increased half-life, recent studies demonstrated that amphiphilic vaccines bind to albumin *in situ* efficiently accumulated in the antigen presenting cells in the lymph node (LN) following subcutaneous injections, leading to up to 30-fold increase over unmodified vaccines in antigen-specific CD8⁺ T cell proliferation.^{72,73} These studies provide evidence that autoantigen–albumin conjugate improves antigen presentation by prolonging antigen half-life and enhancing lymphatic accumulation.

As a natural transporter protein with long circulatory half-life, albumin has been considered as one of the most attractive carriers for delivery of therapeutic agents in

immunomodulation.^{74,75} In addition, the successes of albumin-based conjugates such as long-acting insulin (Levemir) and glucagon-like peptide (Victoza), which are attached to an albumin-binding moiety, potentially promote the development of albumin-based biologicals in clinical studies.⁷⁵ While an albumin-based conjugate platform demonstrated superior immunomodulation effects, how the modification impacts on antigen uptake, processing, and presentation have not been elucidated. In addition, it remains to be investigated whether antigen–albumin conjugates can be a general approach in other autoimmune diseases.

Soluble Peptide–MHC Complex.

Soluble peptide–MHC II complexes (pMHC II) have long been recognized for their ability to blunt autoimmune responses.^{76–79} This method directly targets cognate T cells without the need of APCs for antigen presentation. Early studies using pMHC monomers have shown promise in the preclinical study in EAE but with limited clinical beneficial effects in Phase I trial in MS patients.^{80,81} Similarly, a Phase I/II clinical trial for RA using an epitope of human cartilage glycoprotein (HCgp39) loaded pMHC II monomer yielded only short-term effects.⁸² Clinical responses were seen in patients treated with 7 infusions of 150 $\mu\text{g}/\text{kg}$ peptide/MHC over 6 weeks. One possible limitation for monomeric pMHC is that, in general, pMHC/TCR interaction is very weak and classically lasts for no longer than a few seconds, especially for autoantigens.⁸³ To improve the direct stimulation of antigen-specific T cells, multivalent pMHC complexes have been designed. Unlike the monovalent pMHC, the multivalent interaction between pMHC and T cells greatly enhances both the TCR binding affinity and stability.⁸³ Soluble peptide–MHC multimers, in addition to being a powerful tool in the monitoring of T cell response, can mediate downstream signaling after T cell receptor engagement.^{84,85} In the absence of costimulation, peptide–MHC multimer leads to anergy, deletion of cognate T cells, or induction of Tregs.⁸⁵ To avoid the transfer of peptides from multimers to cell surface MHC molecules which could possibly exacerbate autoimmune responses, peptide antigens are often covalently linked to MHC. Such pMHC class II multimer can inhibit autoreactive CD4⁺ T cells in T1D^{85–88} or MS.^{77,89} Masteller et al. developed soluble peptide/I–Ag7 dimer with a linked peptide specific for islet-reactive BDC2.5 transgenic CD4⁺ T cells in NOD mice.⁸⁷ These dimers were shown to specifically bind BDC2.5 T cells. Treatment with BDC2.5 peptide I–Ag7 dimer protected mice from diabetes mediated by adoptive transfer of diabetogenic BDC2.5 CD4⁺ T cells.⁸⁷ In a similar approach, Casares et al. demonstrated soluble dimeric peptide–MHC II chimera not only prevented the onset of T1D disease but also restored normoglycemia in diabetic animals.⁸⁶ In a spontaneous T1D model, administration of soluble IAg7 dimers covalently linked to β -cell autoantigen-derived peptide GAD65 blocked the progression of insulinitis and the development of diabetes.⁸⁸ In addition to pMHC class II dimers, pMHC class I complexes have been reported to inhibit alloreactive CD8⁺ CTL (cytotoxic T lymphocyte) *in vivo*,^{90,91} and covalent linkage of MHC class I with long rigid linkers efficiently inhibited CTL target cells by interfering with TCR-mediated activation of lymphocyte function-associated antigen 1 (LFA-1).⁹² Another intriguing idea is to deplete the autoreactive T cells with a pMHC multimer immunotoxin.^{93–96} In this approach, cytotoxic drugs such as ²²⁵Actinium, saporin, and doxorubicin were conjugated to pMHC multimer, resulting in a rapid antigen-specific T cell deletion. Together, though long-term efficacy and toxicity data are lacking, these results

suggested that multimeric peptide-MHC might be useful in the development of immunospecific therapies for autoimmune diseases.

Antigen–NPs Conjugates.

NPs delivery has recently been investigated to promote tolerance induction. The use of NPs for tolerogenic vaccine delivery improves the antigen stability, targets key immune cells, and enhances antigen presentation. For these purposes, autoantigens and/or suppressive signals are often encapsulated or conjugated to nanosized vehicles. Here we limit the scope of the discussion to techniques and approaches that can modulate the immune system via molecular conjugates.

Antigen-Linked NPs as APC Targeted Delivery.

The efficacy of NPs in immune modulation largely depends on their ability to target and alter the functions of antigen presentation cells *in vivo*.^{15,32,52} NP's physical and chemical characteristics such as size, shape, geometry, and surface chemistry affect their biodistribution, uptake, and intracellular trafficking.⁹⁷ Because their sizes are comparable to those of microorganisms, a particulate system is more efficient in antigen delivery than soluble antigens.^{97,98} In the absence of danger signals, the presentation of autoantigens on APCs can induce antigen-specific tolerance. Getts et al. showed that antigen-decorated 500 nm polystyrene NPs induced long-term T cell tolerance in mice with EAE.⁹⁹ Intravenous injection of disease-relevant peptide such as PLP_{139–151} (proteolipid protein) conjugated polystyrene NPs enhanced the antigen uptake in splenic phagocytes via Macrophage Receptor with Collagenous Structure (MARCO), a scavenger receptor, and prevented the onset of EAE, a model of human MS.⁹⁹ These beneficial effects are associated with the vaccine-elicited Tregs and inhibition of effector T cell activation.⁹⁹ A more biocompatible poly(lactic-*co*-glycolic acid) (PLG) NPs have also been shown to function as a safe, cost-effective, and efficient carrier for the induction of antigen-specific T cell tolerance.¹⁰⁰ Myelin antigen-coupled PLG NPs were able to ameliorate the ongoing diseases in an EAE model and, importantly, minimize epitope spreading when administered systemically.¹⁰⁰ Autoantigen–polymer conjugate PLGA NPs with favorable release kinetics were developed in a recent study. It was found the Tregs induction was dependent on antigen density as well as nanoparticle concentration.¹⁰¹

Using NPs to codeliver antigen and immunomodulators is an emerging trend in antigen-specific immunotherapy. Yeste et al. used small gold NPs (60 nm in diameter) for delivery of EAE-relevant peptide antigen MOG_{35–55} and 2-(1Hindole-3-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), a small molecular agonist activating aryl hydrocarbon receptor transcription factor (AhR) (Figure 5A).¹⁰² The addition of ITE promoted the differentiation of CD4⁺ CD25⁺ Foxp3⁺ Tregs. Using gold nanoparticle incorporated with β -cell antigen proinsulin, the same group reported the induction of a tolerogenic phenotype in DCs and Tregs generation *in vivo*, which in turn suppressed autoimmune diabetes in a mouse model.¹⁰³ Similarly, poly(lactic-*co*-glycolic) NPs combined autoantigen and rapamycin were shown to efficiently induce tolerogenic DCs, and suppress both T-and B-cell-mediated immunity in mice.¹⁰⁴

Targeting antigen to APCs in the lymph nodes is emerging as an efficient strategy for tolerance induction.^{105,106} NPs with optimized parameters such as size, charge, and shape were able to drain to the lymph nodes and accumulated in the LN-resident APCs.⁹⁷ Immunological tolerance can thus be elicited by conjugating autoantigens to size-optimized NPs (5–100 nm) such as quantum dots. Hess et al. in a recent study showed quantum dots (QDs) with uniform size (<20 nm) efficiently concentrated in the draining lymph nodes following subcutaneous injection, colocalizing with macrophages expressing the scavenger receptor MACRO.¹⁰⁷ Treatment of autoantigen loaded QDs markedly reduced disease incidence when compared with soluble antigen group in an EAE mouse model of MS. Importantly, the antigen density has been correlated with the efficacy of tolerance induction, with low-density antigen displayed on high numbers of tolerogenic particles driving the most efficient tolerance.¹⁰⁷ Though the underlying mechanism is still not clear, this finding suggests, in addition to efficient antigen delivery to APCs in the LN, an antigen display in which the spatial organization of the antigens on the surface of NPs regulates autoimmunity.

NPs Coated with Autoimmune Disease Relevant Peptide/MHC.

To improve the therapeutic outcomes of soluble peptide-MHC multimers in autoimmune diseases, pMHC conjugated NPs has been proposed.^{108–111} pMHC coated NPs can provide protection of the pMHC molecules from degradation, which would prolong the circulating half-life. In addition, the particle size, pMHC valency, spacing, and density can be fine-tuned to optimize the regulation of T cell responsiveness.^{109,112,113} Tsai et al. coated iron oxide NPs with T1D-relevant pMHC complexes and found such pMHC class I-coated NPs elicited TCR clustering and expanded memory-like autoregulatory T cells, which subsequently inhibited the activation of other autoantigen-specific autoreactive CD8⁺ T cell populations in the pancreas.¹⁰⁸ Treatment of pMHC-NPs not only blunted T1D progression in prediabetic mice but also restored normoglycemia in newly diagnosed diabetic NOD mice.¹⁰⁸ Similarly, NPs coated with autoimmune-disease relevant peptides bound to MHC II (Figure 5B) profoundly induced antigen-specific T cells with regulatory function, and led to the suppression of disease in mouse models of RA, T1D, and MS, without compromising the global immunity.¹¹⁰ These pMHC-NPs provide a “plug and play” vaccine platform to reprogram the immune responses, with the possibility to codeliver other tolerogenic signals, such as costimulatory inhibitors or suppressive cytokines.

However, the major barrier for pMHC-NPs approach lies in the complexity of the production of pMHC-NPs that match human leukocyte antigen of individuals.¹¹⁴ Although NPs-based approaches are promising in the treatment of autoimmune diseases, the clinical translation of these strategies remains in its infancy due to the concerns underlying immunogenicity and toxicity associated with synthetic NPs.

Small Molecule–Antigen Conjugates.

Compared with protein drugs, small molecular compounds have the advantages in manufacturability, cost, stability, and safety. Small molecular ligands that specifically block the engagement of costimulatory molecules or inhibit the formation of the immunological synapse are thus conjugated to autoantigens to amplify the tolerance induction. Such bispecific heterodimers are designed to selectively inhibit the maturation of T cells specific

for autoantigens. Siahaan and colleagues have designed a bifunctional peptide inhibitor (BPI, Figure 6), which consists of an antigenic peptide epitope conjugated to an inhibitory peptide blocking DC-T cell interaction.^{115–119} Conjugating peptide autoantigens to LFA- α -blocker left (LABL) peptide inhibited the formation of the immunological synapse by binding to both MHC II and intercellular adhesion molecular-1 (ICAM-1), and skewed the differentiation of T cells from stimulatory to regulatory cells.¹⁰⁷ Similarly, BPI composed of an antigenic peptide and a B7 binding peptide derived from CD28 receptor was designed to simultaneously target MHC II and B7 on the surface of antigen presenting cells.¹¹⁹ Administration of low concentrations of BPI consisting of appropriate disease-specific antigens was highly potent in T1D, and RA.^{115–119} To overcome the short *in vivo* half-lives of peptide, White et al. in a recent study covalently conjugated the bifunctional peptide to the fragment crystallizable (Fc) region of the human IgG1 antibody via a sortase-ligation strategy.¹²⁰ The initial tests of the conjugate showed significantly reduced EAE symptoms.¹²⁰

The LEAPS (Ligand Epitope Antigen Presentation System) platform represents another technology to the development of antigen-specific immunomodulating peptide vaccines for autoimmune diseases.¹²¹ In this technology, disease-specific peptide epitopes were conjugated to an Immune Cell Binding Ligand (ICBL), a short peptide which activates precursors to initiate and redirect appropriate T cell responses. Depending upon the nature of the attached ICBL, the LEAPs technology can direct the antigen-specific T cell responses toward Th1, Th2 or Tregs.¹²¹ Conceived in the late 1980s, LEAPS technology has since been demonstrated to be able to block the progression of several autoimmune diseases, including RA in the collagen-induced arthritis model¹²² and experimental autoimmune myocarditis in a mouse model.¹²¹

Small molecule immunomodulators, including vitamin D3,^{123,124} mycophenolic acid, dexamethasone,¹²³ D-man-nose,¹²⁵ and rapamycin^{104,123} have been shown to be effective in augmenting the tolerance induction through a variety of mechanisms which include the induction of Tregs, or by altering the profile of pathogenic immunity. However, the combination of most of these “tolerogenic” adjuvants with immunogens is achieved by coadministration or encapsulation in particulate carriers. Chemical conjugation of antigen and immunomodulators ensures delivery to the same antigen presenting cells and are widely used in the generation of immune responses.¹²⁶ Due to the lack of direct head to head studies, it remains to be determined whether covalent linkage can be more effective in immune suppression. Nevertheless, small molecules have been conjugated to autoantigen for immune suppression. In a recent study, Perdicchio et al. conjugated sialic acid to autoantigens for the induction of Tregs. Sialic acids bind to Ig-like lectins (Siglecs), which are inhibitory receptors expressed on DCs.¹²⁷ The authors suggested that modification of antigens with sialic acids targeted DCs via Siglecs and altered the immunogenicity of antigens.¹²⁷ Administration of sialylated antigens led to inhibition of the proliferation and functions of effector T cells and induction of Tregs, even under inflammatory conditions.¹²⁷ Modulation of T cell responses via myelin antigen sialylation was effective in mice with EAE, providing evidence that sialic acid–antigen conjugates could induce antigen-specific immune tolerance.¹²⁷

Although cross-linking antigen to immunomodulatory is a straightforward design for immune suppression, one of the potential complications with using chemical conjugation is that it offers limited protection to the immunogen, resulting in premature degradation once exposed to the harsh physiological environments.

Cell–Peptide Conjugates.

Although the precise mechanisms have not been determined, physiological cell death (apoptosis) is intrinsically tolerogenic.¹²⁸ Unlike pathological cell death (necrosis), under homeostatic conditions, apoptotic cells are engulfed and processed by antigen presenting cells and are presented to induce tolerance to autoantigens.¹²⁸ Exploiting this intrinsic peripheral tolerance process, several groups have tried to target autoantigens to different types of cells.^{129–139} One of the most studied approaches to induce tolerance is intravenous treatment with antigen-coupled, ethylene carbodiimide (ECDI)-fixed splenocytes.^{129–135} In this approach, splenocytes were isolated from the donor mice and were chemically coupled with antigen via a small molecular linker ECDI. ECDI also induces apoptosis of splenocytes, creating a cell-based autoantigen processed by tolerogenic pathways.¹²⁸ It has been shown that ECDI-coupled splenocytes induce tolerance via both direct and indirect mechanisms.¹³⁰ In the direct pathway, peptide-coupled cells directly present auto-antigens to antigen-specific T cells via T cell receptor, leading to anergy through TCR stimulation in the absence of costimulation. In an indirect pathway, intravenous infusion of antigen-coupled cells accumulates in the marginal zone of the spleen where they are phagocytosed and presented on the surface of macrophages, which produce IL-10, upregulating the expression of the immunomodulatory costimulatory molecules PD-L1.¹³¹ Antigen-coupled cell infusion also induces Tregs that are essential for long-term tolerance maintenance.¹³¹ Notably, antigens chemically conjugated *ex vivo* to splenocytes have been shown to induce antigen-specific tolerance in T1D^{132,136} and EAE.¹³³ Recently, autologous peripheral blood mononuclear cells chemically coupled with an array of myelin peptides were tested in MS patients.¹³⁴ This first-in-man, phase 1 clinical trial demonstrated that the treatment was safe, and a high dose of treatment reduced antigen-specific T cell responses.¹³⁴ This study established the safety and feasibility of using antigen-coupled cells in MS patients, providing evidence to justify further clinical investigations on the treatment of autoimmune diseases.

Immunological tolerance can also be induced by antigen coupled red blood cells (RBCs).^{137–139} Transfusion of RBCs covalently modified with disease-relevant autoantigen payload has been shown to blunt the contribution to immunity from B cells, CD4⁺, and CD8⁺ T cells in an antigen-specific manner.¹³⁸ Mice treated with autoantigen-coupled RBCs demonstrated prophylactic and therapeutic efficacy in EAE, and this strategy also protected the majority of NOD mice from T1D.¹³⁸

The strategies just described rely on the isolation and *ex vivo* manipulation of autologous cells. Such *ex vivo* cell approaches have in general been tedious, complex, and inefficient. Kontos and co-workers reported an innovative strategy by which an antigen was coupled to circulating RBCs via *in situ* binding.¹³⁹ Autoantigens were fused to an erythrocyte binding domain and following intravenous infusion, bound to RBCs avidly, deleting both CD8⁺ and

CD4⁺ T cells in an antigen-specific manner. Using mimotope peptides, the authors reported complete prevention of hyperglycemia was achieved by injection of RBC-targeted peptide conjugate in an adoptive T cell transfer-induced T1D model.¹³⁹ As this strategy uses molecularly defined peptide conjugates for *in situ* erythrocyte targeting and avoids the *ex vivo* cell manipulation, it could be, at least in principle, readily translated to the clinic for human studies.

Polymeric Peptide Antigens.

The physicochemical properties, and thus the immunological fate of an antigen, can be tuned by conjugating to different types of polymers. For example, while antigen conjugated to oxidized mannan has been shown to induce cellular and humoral immune responses,¹⁴⁰ reduced mannan conjugates skewed the predominant T-helper 1 responses to T-helper 2 responses.¹⁴¹ Protein antigen conjugated with polyethylene glycol (PEG), a non-immunogenic synthetic polymer, induces immune tolerance of antigen-specific Th cells.¹⁴² Interestingly, size, structure, and linker chemistry have been found to play an important role in the levels of tolerance induction in a peptide-polyglycerol conjugate.¹⁴³ Polyglycerol with an ester linkage was the most tolerogenic, while the same polymer with an amide linkage induced strong effector T cell proliferation.¹⁴³

An autoantigen delivery platform based on Soluble Antigen Arrays (SAGAs) has been developed recently.^{144–146} This delivery system has a polymeric backbone with grafted autoantigens and inhibitory signals. One of the advantages with this system is that it is highly tunable: by varying the polymer backbone and the type, density of peptide attachment, a wide variety of design parameters such as molecular size, solubility, flexibility, antigen valency, spacing, and binding avidity can be controlled.¹⁴⁵ These parameters have been correlated with therapeutic efficacy in murine models of autoimmune diseases.¹⁴⁵

CONCLUSION AND FUTURE PERSPECTIVE

The continued development of ASI depends highly upon our understanding of human immunology, as well as discovery and delivery of tolerogenic adjuvants which can effectively amplify the immune suppression across a wide range of autoimmune disease types. As outlined above, bioconjugates hold great promise in immunomodulation of autoimmune diseases, bridging synthetic molecular functions with immunological features. Bioconjugates can be tailored and functionalized to target DCs, to codeliver autoantigens and immunomodulators, or to target specific immunological pathways. Among all the strategies we reviewed here, myelin peptide-coupled blood mononuclear cells represent the most clinically advanced strategy to date, showing good tolerance and safety in MS patients. Despite the progress, it has become clear that current strategies for ASI are not sufficiently active for many diseases. There is clearly a need to improve therapeutic efficacy in humans. As the field of immunomodulation continues to evolve, new bioconjugate strategies will emerge as innovative treatment modalities. For example, bioconjugate strategies might be developed to control the localization, dose, and kinetics of tolerogenic vaccines. Bioconjugates might also be engineered to program immune cell differentiation and thus control immune cell fates. Future efforts will explore the biological functions of

biomaterials, and combination therapy by which disease-modifying biologics and autoantigens are conjugated should provide a framework to improve efficacy and reduce side effects of antigen-specific immunotherapy. In the long term, these efforts will continue guiding the rational design of ASI to improve current treatment and ultimately lead us toward a cure.

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ABBREVIATIONS

ASI	Antigen-specific immunotherapy
MHC	Major histocompatibility complex
TCR	T cell receptor
NIAID	National Institute of Allergy and Infectious Diseases
T1D	Type 1 diabetes
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
EAE	Experimental autoimmune encephalomyelitis
IBD	Inflammatory bowel disease
MS	Multiple sclerosis
APCs	Antigen presenting cells
Tregs	regulatory T cells
Th	T helper cell
MMR	Macrophage mannose receptor
NPs	nanoparticles
AICD	Activation-induced cell death
FasR	Fas receptors
FasL	Fas ligand
DCs	Dendritic cells
Foxp3	Forkhead box P3
CTLA-4	Cytotoxic T-lymphocytes-associated protein 4

ICOS	Inducible T-cell costimulatory
PD-1	Programmed cell death protein 1
PD-L1	Programmed death ligand 1
NOD	Nonobese diabetic
mAb	Monoclonal antibody
pDCs	Plasmacytoid dendritic cells
Siglec-H	Sialic acid binding Ig-like lectin H
HSA	Human serum albumin
CIDP	Chronic inflammatory demyelinating polyneuropathy
LN	Lymph node
HCgp39	Human cartilage glycoprotein 39
CTL	Cytotoxic T lymphocyte
LFA-1	Lymphocyte function-associated antigen 1
PLGA	Poly(lactic-co-glycolic acid)
MARCO	Macrophage receptor with collagenous structure
AhR	Aryl hydrocarbon receptor
QDs	Quantum dots
PLP	Proteolipid protein
MOG	Myelin oligodendrocyte glycoprotein
ITE	2-(1Hindole-3-carbonyl)-thiazole-4-carboxylic acid methyl ester
BPI	Bifunctional peptide inhibitor
LABL	LFA-alpha blocker left
ICAM-1	Intercellular adhesion molecular-1
Fc	Fragment crystallizable
LEAPS	Ligand epitope antigen presentation system
ICBL	Immune cell binding ligand
ECDI	Ethylene carbodiimide
RBCs	Red blood cells
PEG	Polyethylene glycol

SAgAs	Soluble Antigen Arrays
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-21	Interleukin-21
IL-35	Interleukin-35
IFN-γ	Interferon- γ
TGF-β	Transforming growth factor beta

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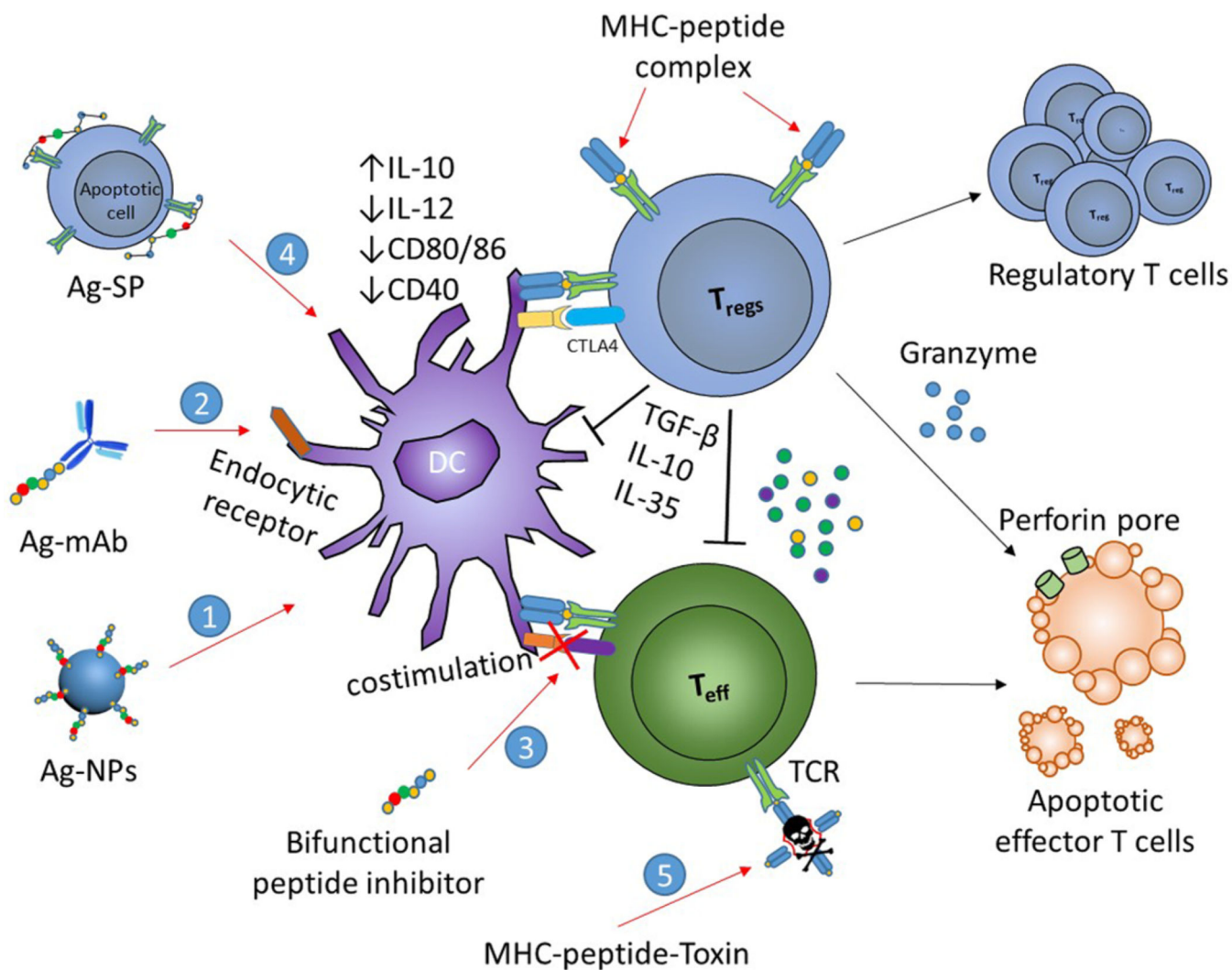


Figure 1. Bioconjugate-based strategies for the induction of antigen-specific tolerance in autoimmune diseases. Bioconjugates have been engineered to target autoantigens and tolerogenic molecules to dendritic cells (DCs) (1); to facilitate antigen-processing via endocytic receptors (2); to inhibit costimulation (3); to link to apoptotic cells for tolerogenic presentation (4); and to deliver toxin to autoantigen-specific T cells (5). These strategies lead to peripheral tolerance as results of anergy and deletion of cognate T cells, or induction of Tregs.

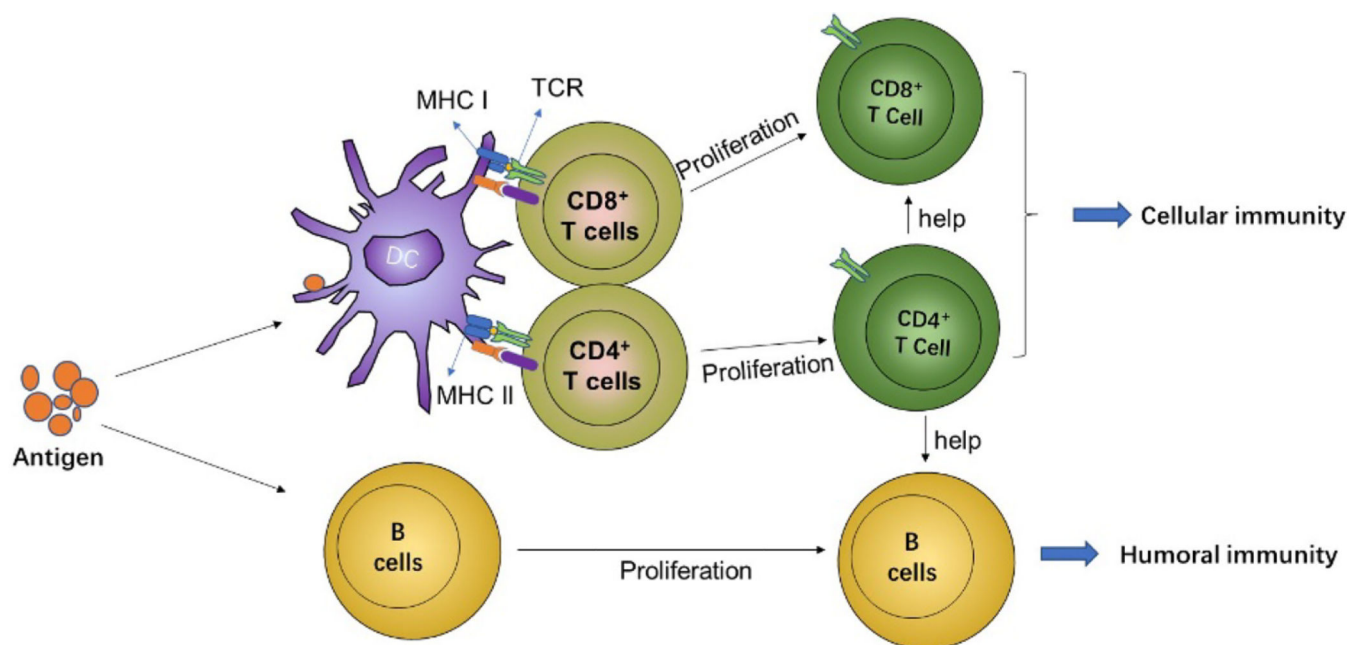


Figure 2. Induction of adaptive immune responses. DCs and B cells acquire antigens and in the presence of inflammatory cues (costimulatory signals), activate T- and B-cells. Activated $CD4^+$ T cells differentiate into T helper cell subtypes and $CD8^+$ T cells differentiate into cytotoxic T effector cells. In parallel, activated B cells differentiate into antibody-producing plasma cells.

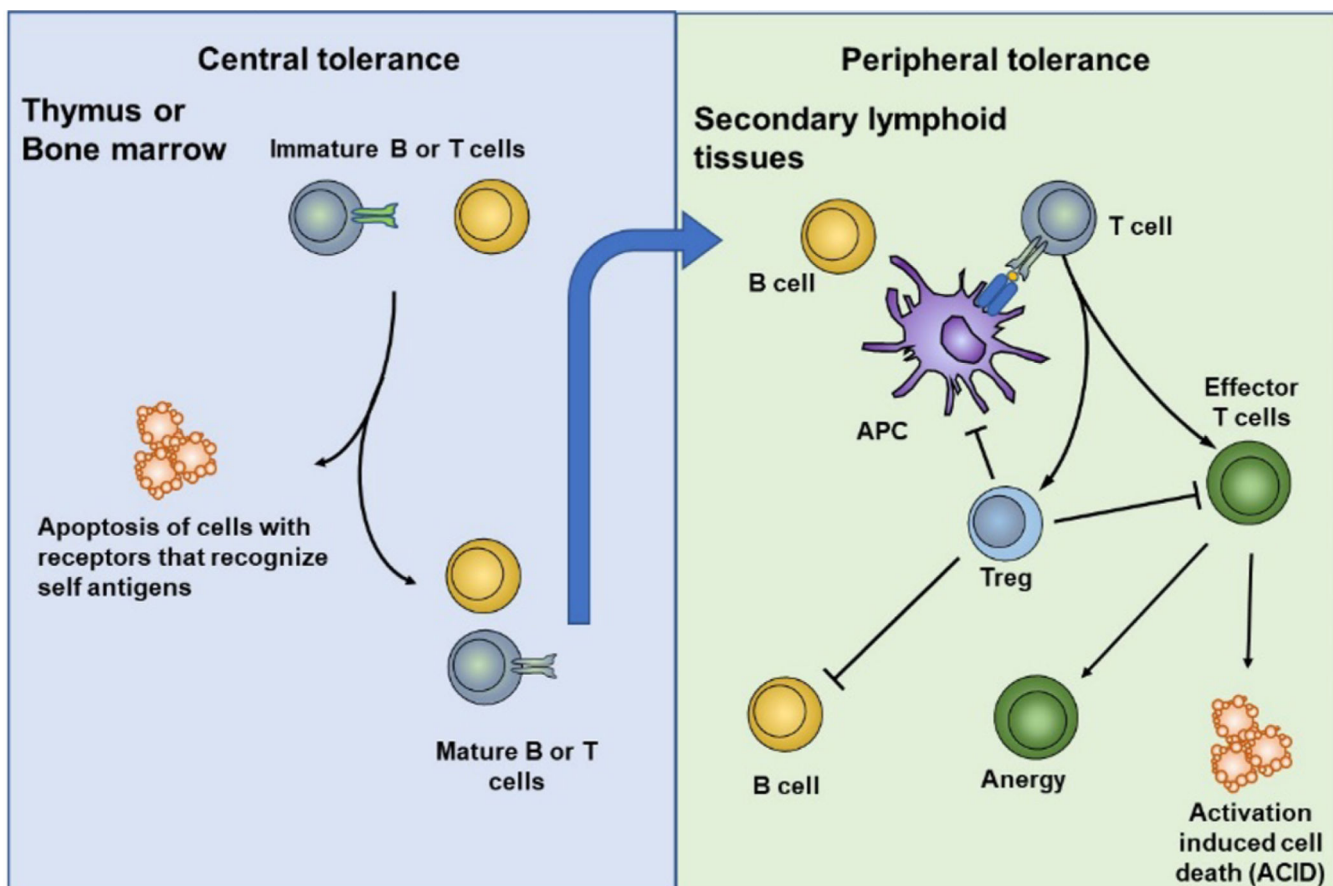


Figure 3. Central and peripheral tolerance mechanisms in T cells and B cells. Central tolerance occurs in the primary lymphoid organs where high-affinity self-reactive T cells and B cells are deleted in the thymus and bone marrow (negative selection), respectively. Peripheral tolerance occurs as mature self-reactive T cells and B cells escape central tolerance and enter the periphery, where they are inactivated (anergy), deleted (activation induced cell death, ACID), or suppressed.

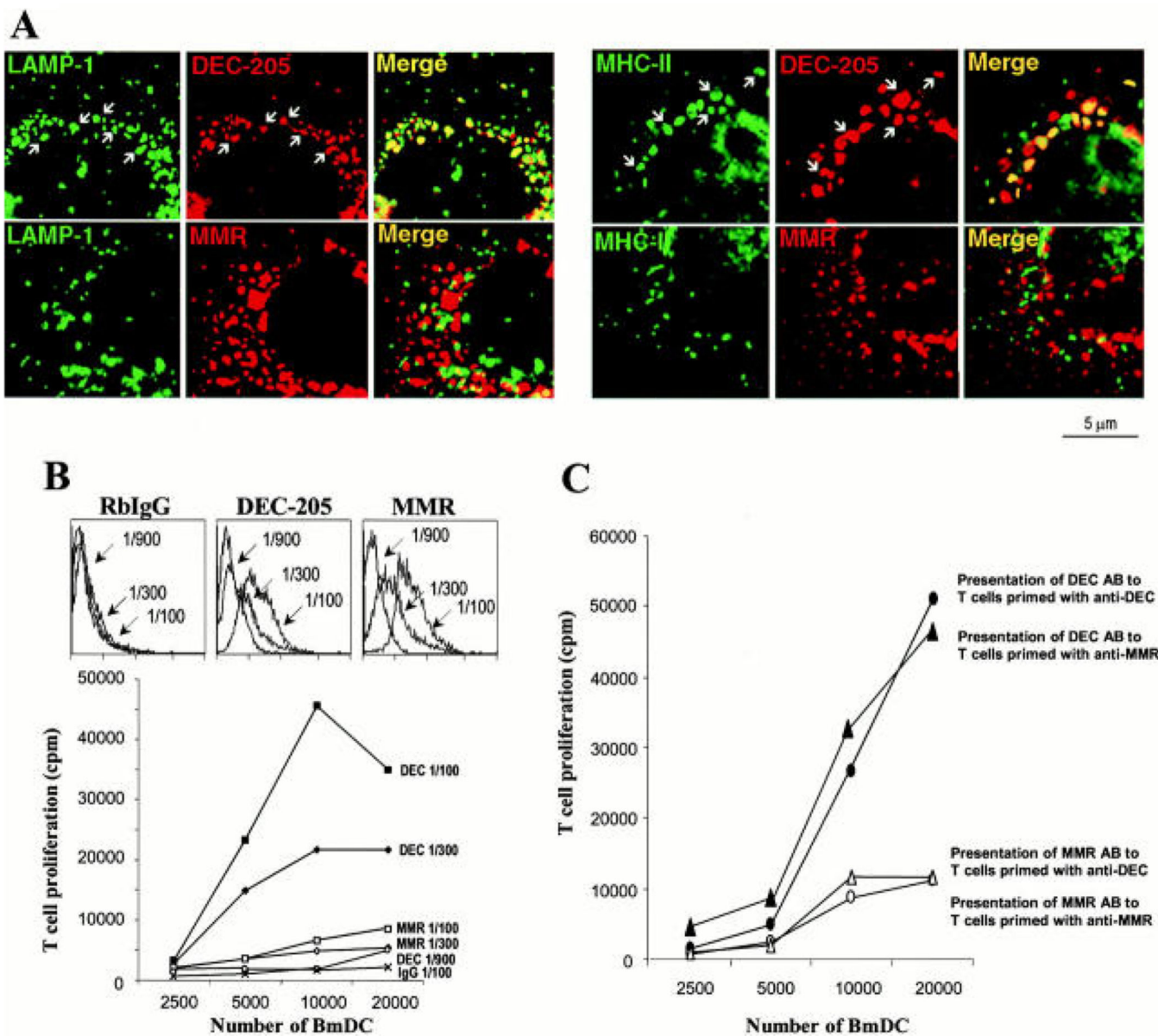


Figure 4. Antigen delivered via DEC-205 enhances antigen presentation. (A) Anti-DEC-205, but not anti-MMR (Macrophage Mannose Receptor), is internalized by receptor-mediated endocytosis, and traffics to late endosomal compartments where high concentrations of MHC II molecules are present. (B and C) Antigen delivered via anti-DEC-205 greatly enhances its presentation to T cells. Reproduced with permission from ref 57.

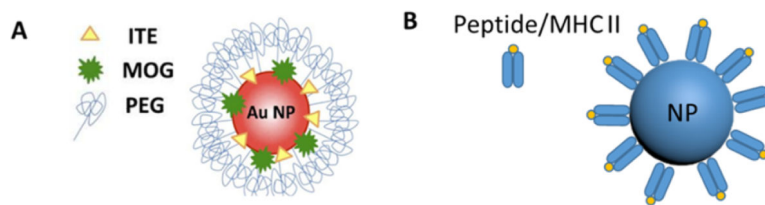


Figure 5. Tolerogenic NPs for antigen-specific immunotherapy. Schematic representation of antigen (MOG₃₅₋₅₅) and AhR ligand (ITE) conjugated gold NPs (A) and NPs coated with peptides bound to MHC class II (B). Reproduced with permission from ref 102 (A).

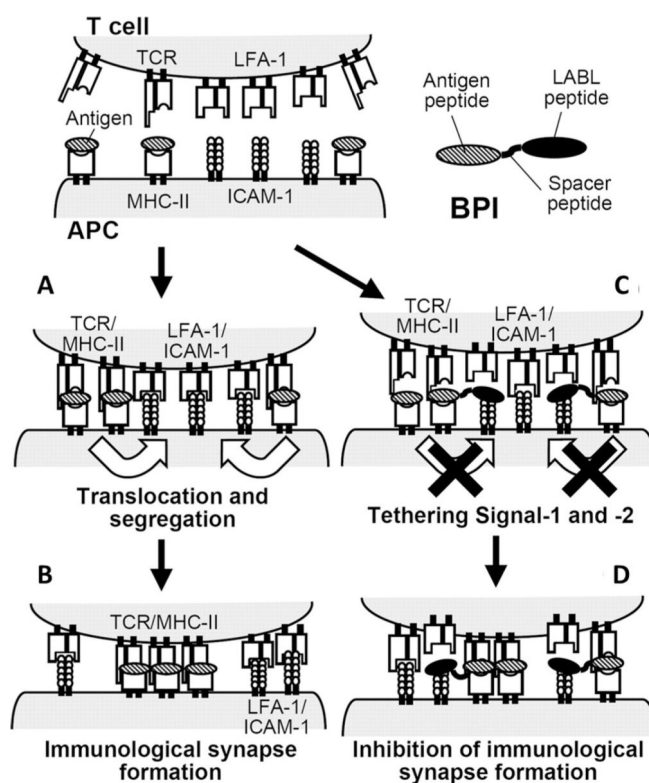


Figure 6.

Proposed mechanism of action of bifunctional peptide inhibitor (BPI). BPI prevents the formation of immunological synapse by binding to MHC-II and ICAM-1 on the surface of APC, which leads to inhibition of T cell activation. (A) Around the central zone, LFA-1/ICAM-1 and TCR/MHC-antigen complexes are first formed at the early stage of T cell activation; (B) both pairs are translocated to form complete immunological synapse. (C) In the presence of BPI, BPI binds to MHC-II and ICAM-1 on the surface of APC; (D) it inhibits their migration, which leads to inhibition of the immunological synapse formation. Reproduced with permission from ref 115.

Table 1.

Some Common Autoimmune Diseases Studied by Different Bioconjugate Strategies

common autoimmune diseases	affected cells or tissues	disease mechanism	proposed causes	bioconjugate strategies
Type 1 diabetes	Pancreatic β cells	CD4 ⁺ , CD8 ⁺ T cell and B cell	Genetics, chemicals, environmental factors	Antibody-antigen; Cell-peptide; Soluble peptide-MHC II complex
Rheumatoid arthritis	Joints, skin	CD4 ⁺ , CD8 ⁺ T cell and B cell	Genetics, environmental factors	Antigen-NPs; Small molecule-antigen; Soluble peptide-MHC II complex
Systemic lupus erythematosus	Mainly on skin	CD4 ⁺ , CD8 ⁺ T cell and B cell	Genetics and drugs	Antibody-antigen; Soluble peptide-MHC II complex
Multiple sclerosis	Nerve cells	CD4 ⁺ , CD8 ⁺ T cell	Genetics and infectious agents	Antigen-NPs; Cell-peptide; Soluble peptide-MHC II complex
Chronic inflammatory demyelinating polyneuropathy	Peripheral nervous system	CD4 ⁺ T cell	Dysfunction of immune cells	Albumin-antigen