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Autoimmune therapies targeting costimulation and emerging trends in multivalent therapeutics

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Abstract

Proteins participating in immunological signaling have emerged as important targets for controlling the immune response. A multitude of receptor–ligand pairs that regulate signaling pathways of the immune response have been identified. In the complex milieu of immune signaling, therapeutic agents targeting mediators of cellular signaling often either activate an inflammatory immune response or induce tolerance. This review is primarily focused on therapeutics that inhibit the inflammatory immune response by targeting membrane-bound proteins regulating costimulation or mediating immune-cell adhesion. Many of these signals participate in larger, organized structures such as the immunological synapse. Receptor clustering and arrangement into organized structures is also reviewed and emerging trends implicating a potential role for multivalent therapeutics is posited.

Autoimmune disease

Autoimmunity results from the breakdown of mechanisms controlling immune tolerance and the failure of the host immune system to distinguish self from non-self **antigens**. Autoimmunity induces an attack by autoantibodies and/or autoreactive **T cells**. Central tolerance in primary lymphoid organs (thymus and bone marrow) provides the mechanism by which T cells and B cells are typically removed if they carry receptors that recognize self antigens. For self-reactive T cells or B cells that escape from the thymus or bone marrow, there is a backup mechanism of ‘peripheral tolerance’ that renders these cells inactive or anergic by several processes such as apoptosis or by the function of regulatory T cells [1,2]. Despite tight regulation, inappropriate humoral and cellular-mediated immune responses against self antigens occasionally occur and can lead to disease.

Immune reactions propagate a number of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, Graves’ disease, Crohn’s disease, lupus erythematosus and insulin-dependent diabetes mellitus. Autoimmune diseases are chronic inflammatory diseases assumed to arise from a combination of genetic traits, environmental factors and/or the

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breakdown of regulatory mechanisms [1-5]. Both humoral (autoantibodies) and cellular (autoreactive lymphocytes) responses mediate autoimmune diseases. Some autoimmune diseases are mediated by cellular damage. Autoimmune diseases that are mediated by cellular damage occur when lymphocytes or antibodies bind to cell-membrane antigens and stimulate cellular lysis and inflammatory response. For example, insulin-dependent diabetes mellitus is caused by the attack of autoreactive T cells against insulin-producing cells (β cells) in the pancreas. Multiple sclerosis patients produce autoreactive T cells that infiltrate brain tissue and cause inflammatory lesions along the myelin sheath of nerve fibers [2]. Some autoimmune diseases are mediated by stimulation or suppression of autoantibody production. Two examples are Graves' disease and Myasthenia gravis, respectively. In Graves' disease, autoantibodies are produced and bind to the thyroid stimulating hormone (TSH) receptor without regulation, which leads to overstimulation of the thyroid and excess production of thyroid hormone. Myasthenia gravis is an autoimmune disease mediated by autoantibodies that block acetylcholine receptors on the motor end plate of muscles and inhibit the binding of acetylcholine to this receptor, hence weakening skeletal muscle.

Current therapies for autoimmune diseases only reduce symptoms to provide an acceptable quality of life; in many cases, these therapies include non-specific suppression of the immune system. A problem of using non-selective immunosuppressants, cytotoxic drugs or corticosteroids is that these drugs threaten the overall immune response, leaving the patient susceptible to opportunistic infections and other side effects [6]. Therefore, new therapeutic approaches that modulate the immune response in a specific manner without suppressing the general immune response are desperately needed.

Chronic inflammation is one target for autoimmune disease intervention [5]. The inflammatory pathway involves several steps including leukocyte transmigration and **cytokine** production. Discoveries of pathogenetic mechanisms of autoimmune diseases have contributed to the introduction of biologics as new therapies for autoimmune diseases. Inhibitors of the inflammatory cytokine TNF- α (infliximab, etanercept and adalimumab) have been used for treatment of rheumatoid arthritis, psoriasis and Crohn's disease. There are several other cytokines that modulate inflammatory responses such as interleukins (IL-1, -6, -10, -17 and -23), and transforming growth factor- β (TGF- β). The rationale behind attempts to block the biological effects of cytokines is that an imbalance of proinflammatory cytokines and anti-inflammatory cytokines may be responsible for the induction of autoimmunity [5].

Besides inhibition of cytokine production or signaling, blockade of cell trafficking, B-cell depletion and blocking of T-cell activation are used as therapeutic intervention strategies. Tysabri® (natalizumab), an $\alpha_4\beta_1$ **integrin** blocker, acts as an inhibitor of leukocyte extravasation and was approved for the treatment of multiple sclerosis. The drug was withdrawn later due to cases of opportunistic infections in the brain, but was later reintroduced to treat relapsing forms of multiple sclerosis. Rituximab, a **monoclonal antibody** (mAb) against CD20 antigen presented on the surface of B cells, kills B cells via complement-mediated cytotoxicity, antibody-dependent cell-mediated cytotoxicity and apoptosis [5]. In the USA, it has been approved for use in combination with methotrexate (MTX) for reducing symptoms in patients with rheumatoid arthritis who have had an inadequate response to one or more anti-TNF- α therapies.

T-cell activation & costimulatory molecules

In autoimmune diseases, T-cell activation is initiated by presentation of self-antigen to T cells. **Antigen-presenting cells** (APCs) capture self-antigen and load it onto major histocompatibility complex (MHC) class II. The antigen-primed APC then presents the self

antigen to T-cell receptor (TCR) on T cells. T-cell activation requires two signals; the interaction of the TCR on T cells and antigenic peptide–MHC complex on APCs (signal 1) and a costimulatory signal (signal 2), to promote T-cell proliferation, cytokine secretion, and effector function [7,8]. Signal 1, which imparts specificity to the immune response, must occur through the antigen-specific receptor of T cells. Signal 2, the costimulatory signal, is composed of several receptor–ligand interactions including the binding of CD28 on T cells and B7 on professional APCs. The binding of leukocyte function-associated antigen-1 (LFA-1) primarily on T cells and intercellular adhesion molecule-1 (ICAM-1) primarily on APCs prolongs the interaction of the cells [2,9]. Activated T cells then either transmigrate to cells or tissue where antigen is located (i.e., β cells in Type 1 diabetes or the central nervous system [CNS] in multiple sclerosis) or T cells proceed to activate B cells to secrete antibodies (i.e., myasthenia gravis or systemic lupus erythematosus). TCR recognition of the antigen–MHC complex in the absence of the costimulatory signal can lead to T-cell anergy and interrupt the immune response. Abatacept, a CTLA-4-Ig fusion protein, blocks T-cell costimulation by blocking interaction of CTLA-4, a receptor expressed on CD4⁺ T cells, with its ligand. Abatacept has been approved to treat rheumatoid arthritis and is now in clinical trials for other autoimmune diseases [10].

The costimulatory signal is important in directing T-cell activation since the TCR has low affinity for the antigen–peptide complex, has a small size, hindering engagement, and is only present in small quantities [6]. In the absence of the costimulatory signal, antigen-specific T cells fail to respond effectively and become anergic or die. Several **costimulatory molecules** have been identified and classified in families. Among these molecules, there are both stimulatory and inhibitory signals. T-cell costimulation plays an important role in the development of effective immune response including T-cell proliferation, differentiation and survival (Table 1) [6,8,11–86,201]. Here, the function of these signals is summarized and current therapeutic efforts are reviewed. Particular emphasis is placed on inhibition of signals to treat autoimmune diseases. Finally, emerging trends in multivalent therapeutics that aim to interrupt costimulation and/or cell adhesion within larger, organized structures such as the immunological synapse (IS) are discussed.

Costimulatory molecules of the immunoglobulin superfamily

CD28–B7 costimulatory pathway

The interaction of CD28 on T cells and B7 family on APCs provides a crucial positive costimulatory signal for T-cell activation. CD28 is a disulfide-linked homodimeric-transmembrane member of the immunoglobulin superfamily and is constitutively expressed on CD4⁺ T cells and CD8⁺ T cells. The ligands for CD28, B7-1 (CD80) and B7-2 (CD86), are constitutively expressed on dendritic cells (DCs) and induced on activated macrophages and activated B cells. CD80 and CD86 are upregulated during maturation of DCs [87]. Binding of CD28 and their ligands promotes proliferation of activated T cells and differentiation of naive T cells into T helper cells and enhances the production of antibodies by B cells [8,88].

CTLA-4–B7 costimulatory pathway

Cytotoxic T-lymphocyte antigen 4 (CTLA-4 or CD152) is structurally homologous to CD28. CTLA-4 is expressed on T-cell membranes and binds to the same two ligands as CD28 (B7-1 and B7-2) with higher affinity. CTLA-4 is upregulated and activated after binding of CD28 and B7 ligands and T-cell activation. CTLA-4 is undetectable on resting T cells, whereas CD28 is expressed on both resting and activated cells [2,20]. CTLA-4 has similar structure to CD28, but their functions are different. The CD28–B7 interaction delivers a positive costimulatory signal to T cells, whereas the CTLA-4–B7 interaction

delivers an inhibitory signal to T cells resulting in the attenuation of T-cell activation and proliferation. Due to the higher binding affinity to B7, the upregulated CTLA-4 competes for the B7-1 or B7-2 ligands to turn off T-cell activation and hence regulate lymphocyte homeostasis [88].

Therapeutics targeting CD28-mediated costimulation

Abatacept is a fusion protein composed of the extracellular binding domain of CTLA-4 fused to an Fc domain of human IgG. It was developed to block the binding of CD28 to B7 [88]. Abatacept was approved for the treatment of moderate to severe rheumatoid arthritis (RA) in the case of inadequate response to anti-TNF α therapy. A recent report, however, has shown that RA patients respond poorly to abatacept [89]. Furthermore, abatacept was shown to lack efficacy in organ transplantation in non-human primates, which was suggested to be due to the lower affinity of abatacept to CD86 compared with CD80 [8]. For this reason, belatacept was developed. Belatacept is a second-generation CTLA-4-Ig, differing from abatacept only by two amino acids leading to a superior binding to CD80 (twofold increase) and CD86 (fourfold increase) compared with abatacept [89]. Belatacept provided more of the potent immunosuppressive properties required for transplantation [20]. Belatacept is intended to provide extended graft survival and is currently continuing through clinical trials.

PD-1–PDL costimulatory pathway

Programmed death-1 (PD-1 or CD279) is an inhibitory costimulatory molecule [90]. It is a member of the immunoglobulin superfamily expressed on activated T cells, B cells, monocytes, and macrophages [91]. PD-1 binds PDL-1 (B7-H1) and PDL-2 (B7-DC). PDL-1 is expressed on B cells, DCs, and T cells including Treg. PDL-2 expression is limited to DCs and macrophages. Ligation of PD-1 on lymphocytes delivers a negative costimulatory signal to T cells and plays a critical role in the regulation of peripheral tolerance [92-95]. Engagement of PD-1 by its ligands, PDL-1 or PDL-2, blocks TCR signaling and transduces a signal that inhibits T- and B-cell proliferation, cytokine production and cytotoxicity of CD8⁺ T cells [91,95].

Therapeutic targeting of PD-1–PDL interactions

An *in vitro* study has shown that PDL-1-Ig stimulated PD-1, thus inhibiting CD4⁺ and CD8⁺ T-cell proliferation [8]. PDL-1-Ig has been shown to ameliorate collagen-induced arthritis (CIA) in the CIA mouse model as evaluated by clinical score, histology and reduced production of inflammatory cytokines. These results suggested a beneficial effect of PDL-1-Ig in this arthritis model via an anti-inflammatory action and inhibition of cell proliferation [23]. PDL-1-Ig prolonged corneal allograft survival in mice by augmented ligation of the PD-1 negative costimulatory molecule [25].

ICOS–ICOSL costimulatory pathway

Inducible costimulator (ICOS) is also a member of the immunoglobulin superfamily of costimulatory molecules. ICOS is induced after TCR engagement upon T-cell activation. ICOSL (B7H) is constitutively expressed on DCs, monocytes, macrophages, B cells, endothelial cells, fibroblasts, and renal epithelial cells [32]. ICOS has a similar sequence to CD28 and CTLA-4. Unlike other costimulatory molecules such as CTLA-4, ICOS can be upregulated in the absence of a CD28–B7 interaction [6]. ICOS promotes T-cell differentiation and cytokine production [8,96]. ICOS can stimulate both Th1 and Th2 effector cytokine production, especially IL-4 and -10 [8,96]. Since ICOS signaling does not sustain IL-2 production, it does not stimulate T-cell clonal expansion [97,98].

Therapeutic targeting of ICOS–ICOSL interactions

The ICOS–ICOSL costimulatory pathway is crucial for T-cell activation, differentiation and effector function. Anti-ICOS mAb treatment resulted in significant inhibition of graft-versus-host disease (GVHD) and reduced the number of Ag-specific T cells that reject the bone marrow graft [36]. The blockade of ICOS–ICOSL was effective in prolonging cardiac and liver allograft survival [8]. ICOS blockade also prevented development of spontaneous disease in prediabetic non-obese diabetic (NOD) mice [99]. Transient blockade of ICOS/ICOSL profoundly reduced the severity of glucose-6 phosphate isomerase (G6PI)-induced arthritis [33]. These observations suggested that ICOS targeting may be a beneficial therapeutic scheme for allograft transplantation and autoimmune diseases [96].

BTLA–HVEM costimulatory pathway

B- and T-lymphocyte attenuator (BTLA or CD272) is an immunoglobulin-like molecule belonging to the immunoglobulin superfamily. BTLA is not expressed by naive T cells, but it is induced during activation and remains expressed on Th1 but not on Th2 cells [39]. It is also constitutively expressed at high levels on B cells. BTLA was identified as an inhibitory receptor with structural and functional similarities to CTLA-4 and PD-1 [100]. The engagement of BTLA and herpes virus entry mediator (HVEM), which is a member of the TNF receptor (TNFR) superfamily, delivers an inhibitory signal. BTLA interaction with HVEM impairs T-cell activation resulting in decreased T-cell proliferation and cytokine production.

Therapeutic targeting of BTLA–HVEM interactions

Blockade of BTLA–HVEM via anti-BTLA mAb in combination with CTLA-4-Ig can prolong cardiac allograft survival [41]. In a different study, a combination therapy of anti-BTLA mAb and CTLA-4-Ig induced donor-specific tolerance in a murine model of islet allograft [42].

Costimulatory molecules of the TNF–TNFR family

CD40–CD40L costimulatory pathway

CD40 is constitutively expressed on B cells, DCs, monocytes, platelets, and macrophages, and can be induced on non-hematopoietic cells such as endothelial cells and fibroblasts in the presence of inflammation [43]. Ligation of CD40 and its subsequent signaling leads to activation of B cells, as well as activation and maturation of DCs, priming of helper and cytotoxic T cells, and propagation of a variety of inflammatory reactions [44]. The ligand for CD40 (CD40L or CD154), a member of the TNF family, is expressed on activated T cells, after stimulation by TCR–MHC complex engagement and signaling from CD28–B7 [8]. Blockade of the CD40–CD40L pathway alone or together with the CD28–B7 pathway can lead to long-term allograft survival in animal transplantation models and can inhibit the development of autoimmune diseases [44].

Therapeutic targeting of CD40–CD40L interactions

Blocking CD40–CD40L was examined by using an anti-CD40L mAb, which has shown promise in both rodent and non-human primate models [9]. Anti-CD40L mAb can prevent acute allograft rejection and promote allograft and xenograft survival in rodents and non-human primates [8]. Although treatment with anti-CD40L mAb promoted long-term allograft survival in experimental transplantation, clinical trials in autoimmune diseases and transplantation were halted due to unanticipated thromboembolic complications. This complication may be explained by the high expression of CD40L on platelets; however, observed stabilization of arterial thrombi were thought not to involve CD40 molecules [9].

An anti-CD40 mAb has also been developed and was shown to block binding of CD154 and to inhibit CD154-induced B-cell proliferation *in vitro*. Anti-CD40 mAbs promoted moderate prolongation of graft survival when used alone. When combined with belatacept, anti-CD40 mAbs synergized the effect in prolonging graft survival in non-human primate models of renal and islet transplantation [9].

OX40–OX40L costimulatory pathway

OX40 (CD134) is predominantly expressed on activated T cells, but not naive CD4⁺, CD8⁺ T cells, and memory T cells [15]. Following activation, OX40L is induced on activated B cells and mature DCs, endothelial cells and T cells. It is not expressed on resting APC. Expression of OX40 is delayed and decreased in the absence of CD28. OX40-positive T cells were found at the site of inflammation in experimental allergic encephalomyelitis (EAE), GVHD and RA [101]. Targeting the OX40–OX40L interaction with anti-OX40L mAb can delay disease progression in EAE, colitis, arthritis, asthma and diabetes in mice [52–56].

GITR–GITRL costimulatory pathway

Glucocorticoid-induced TNFR-related gene (*GITR* or *TNFRSF18*) is a transmembrane protein of the TNFR superfamily. GITR is preferentially expressed by Treg and is also upregulated in conventional effector T cells upon activation [61]. GITR ligand (GITRL) is mainly expressed on B cells, macrophages, DCs, endothelial cells, and activated T cells, but not on resting T cells [57,61]. Binding of GITRL to its receptor is involved in the development of autoimmune or inflammatory responses due to costimulation of effector T cells, inhibition of Treg cells, and activation of macrophages [58]. GITR fusion protein binding to GITRL prolonged allograft survival and showed potential anti-inflammatory properties in animal studies [61,62]. Neutralizing anti-GITRL mAb protected against the progression of diabetes in mice [63].

Molecules of the integrin family

LFA-1 and ICAM-1 belong to the integrin family and immunoglobulin superfamily, respectively. Although not traditionally considered a ‘costimulatory’ signal, they mediate strong interaction of leukocytes to cells bearing the receptor (e.g., APC), and enhance T-cell activation signaling [102]. Binding of LFA-1 and ICAM-1 stabilizes the interaction of TCR– and MHC–peptide complexes in IS.

Therapeutic targeting of ICAM-1/LFA-1 interactions

The effect of anti-ICAM-1 mAb on preventing rejection was reported in a rat transplantation model [103]. Intravenous administration of anti-ICAM-1 mAb alone could not prevent acute lung allograft rejection; however, combination with cyclosporine significantly reduced infiltration of leukocytes into the allograft [103]. Anti-ICAM-1 mAbs were studied in clinical trials for Crohn’s disease (alicaforfen) and ischemic stroke (enlimomab) [84,85]. None of these were commercialized.

Anti-LFA-1 mAb (efalizumab) has been shown to be effective in reducing the severity of chronic psoriasis without lymphocyte depletion and prevented rejection in kidney transplantation [104,105]. Recently, efalizumab was withdrawn from clinical use due to concerns about the development of progressive multifocal myeloencephalopathy (PML) in several patients receiving the drug as treatment for psoriasis [106]. In other studies, Posselt *et al.* described a novel immunosuppressive regimen using efalizumab, which permitted long-term islet allograft survival while reducing the need for corticosteroids and calcineurin inhibitors (CNI) [107].

Costimulatory mediators & orientation in the IS

The IS is a junction between immune cells in which a variety of receptors and adhesion molecules interact with their ligands on the opposing cell surface [108]. One such junction occurs between T cells and APCs. The IS includes specific binding between MHC class II with 'loaded' peptide and the opposing TCR that recognizes the presented antigen [108]. Pairs of many of the aforementioned costimulatory receptors and their ligands also accumulate at the center of the IS while clusters of LFA-1 and ICAM-1 and other receptor–ligand pairs are localized to the peripheral ring of the IS [109,110]. T-cell activation requires sustained conjugate formation between T cells and APCs because the number of peptide–MHC complexes can be very low and because the interaction between the TCR and its ligand has low affinity [111–113]. In the IS, the ligands and their receptors are arranged in an orderly manner, increasing the probability that multiple ligands will bind simultaneously with multiple receptors to enhance binding specificity, affinity and avidity and to prolong the duration of signaling [114]. Reports suggest that, T-cell activation which takes place during a 24–48 h interval [115] is mediated by the longevity of stabilized LFA-1–ICAM-1 binding [114]. Antigen presentation is amplified and sustained by translocation of TCR and MHC complexes to the center of the IS [110,112,114].

Many of the proteins participating in the IS have become the focus of therapeutic interventions (Table 1). The surface proteins located within the IS on T cells include T-cell receptors, costimulatory molecules CD2, CD28, CD40L, LFA-1, very late antigen (VLA)-4, ICAM-1, ICAM-3 and various cytokine and chemokine receptors [116]. Proteins on APCs include CD80, CD86, CD40, ICAM-1, ICAM-3 and other surface and cytoplasmic proteins [117]. The structure of the mature IS was described as a 'bull's eye' with three distinct regions [109]. The central region of the IS, called the central supramolecular activation cluster (cSMAC), contains TCR complexes interacting with peptide–MHC complexes as well as interactions of costimulatory molecules such as CD2, CD28 and CD40 [109,110,114,116,118,119]. The intermediate ring of the bull's eye, called the peripheral supramolecular activation cluster (pSMAC), contains complexes of integrin LFA-1 binding to ICAM-1 and the cytoskeletal protein talin, among others [110,118]. The outermost region is the distal supramolecular activation cluster (dSMAC), contains CD45 and CD43 [109,119].

After T-cell and APC contact, the binding of TCR to peptide–MHC complexes leads to the formation of small TCR microclusters thought to contain about 100 TCR/ MHC each, distributed in the periphery of the T cell–APC interface (dSMAC) [120,121]. The increased density of TCR and MHC by forming microclusters facilitates the engagement of this low affinity pair. Segregated microclusters of LFA are also formed in dSMAC [122]. TCR and LFA-1 microclusters then translocate to pSMAC [119]. After T cells begin to contract and have unstructured morphology, TCR microclusters migrate towards the center region of the contact area (cSMAC), while integrin microclusters accumulate in a surrounding area (pSMAC) [110,111,119,122–124]. Once at the center, TCR microclusters assemble to form larger clusters and a stable cSMAC to sustain the activation signal [125]. Similarly, MHC and ICAM-1 microclusters are also formed at the contact interface on the APC. cSMAC is surrounded by pSMAC containing adhesion molecules such as LFA-1 and ICAM-1. ICAM-1 on APCs forms oligomers that bind to LFA-1 clusters on T cells [116,126]. The interactions of integrins and their ligands play a critical role in the formation of tight and prolonged contacts between T cells and APCs. A stable IS cannot be formed in the absence of integrins [116].

In addition to the formation of TCR clusters, clustering of costimulatory molecules and their ligands is also required for T-cell activation to occur. The costimulatory molecules CD28

and CTLA-4 have been reported to accumulate in the cSMAC [112]. The accumulation of CD28–B7 pairs enhanced T-cell response by modifying T-cell signaling and by stabilizing the IS [109]. Other costimulatory receptor–ligand pairs, including PD-1–PDL-1/PDL-2, ICOS–ICOSL and CD40–CD40L were also reported to localize to the cSMAC [109]. CD28 and CTLA-4 are expressed as homodimers on the T-cell surface with monovalent and bivalent binding capacities, respectively. B7-1 is also present on the surface as a homodimer, while B7-2 is monomeric [120]. A homodimeric CTLA-4 molecule can bind two B7-1 molecules at low concentration of CTLA-4. At high concentration, CTLA-4 can form a linear array of B7-1–CTLA-4 multimers containing 7–18 receptor–ligand pairs [127]. At low concentration, one CD28 molecule can bind two molecules of B7-1. At high concentration, two molecules of CD28 can bind a dimer of B7-1 without forming a multimeric array [127]. As a result of multimerization, CTLA-4 has 10–100-fold higher binding avidity for B7 ligands than CD28 [120].

Strategies & design of multivalent, targeted therapeutics

Multivalent receptor–ligand interactions regulate many important biological responses. Pairs of receptors and ligands often orientate in arrays to elicit a cellular response [128]. For example, the IS requires sustained conjugate formation between T cells and APCs mediated by a large number of ‘clustered’ binding events. Receptor clustering and arrangement into long-range orderly arrays enhances binding and prolongs the duration of antigen recognition and simultaneously provides important signals leading to an inflammatory or regulatory response. Multivalent ligand strategies have been shown to increase avidity of ligands as expected [129]. In addition, multivalent conjugates can potentially offer novel autoimmune interventions by improving ligand-binding specificity, by providing costimulatory or regulatory signals, by triggering receptor-mediated endocytosis, or by inducing unique cell responses [130–133].

In the immunological system, multimerization of ligands and receptors plays an important role in orchestrating the localization and function of immune cells. Binding of cell-surface proteins with their ligands on leukocytes is necessary for signal transduction inducing immunological function and regulation. The binding of antigen to TCR can either activate or inhibit T cells depending on antigen array size, ligand valency and concentration [134]. For example, multivalent MHC complexes (MHC–Ig dimers) demonstrated stable binding to antigen-specific T cells with high avidity for cognate T-cell receptors [135]. In addition, a scaffold of multivalent single-chain T-cell receptor fusion protein complexes exhibited enhanced antigen binding and biological activity due to an increase in avidity between ligand and polymeric carrier [136].

In addition to T-cell receptor signaling, multivalent interaction of costimulatory molecules also regulate binding avidity and specificity to their receptors and T-cell and B-cell responses [137]. Monomeric costimulatory molecules such as CD80/86 on APCs and CD28 or CTLA-4 on T cells bind their ligands/receptors with low affinity and dissociate rapidly. Costimulatory molecules, therefore, require high avidity binding by clustering or ‘oligomerization’ of ligands and receptors. It has been reported that CTLA-4 requires dimerization for high avidity binding and oligomerization was required for CD80/86 to bind CTLA-4 with high avidity [138]. Conjugation of CTLA-4 Ig to liposomes improved the efficiency of the targeting and blocking of B7 costimulatory molecules by increasing the binding avidity through a multivalent ligand effect and by increasing the concentration of CTLA-4-Ig at the target site [139].

Other examples of multivalency involving cell-adhesion molecules are also prevalent in the literature. The interaction of monomeric LFA-3 and CD2 was reported to have low binding

affinity, whereas multimeric LFA-3 bound CD2 with much higher avidity [140]. Another report showed that multimerization of RGD led to an increase in binding affinity for the $\alpha_v\beta_3$ integrin receptor and substantially enhanced specificity for $\alpha_v\beta_3$ integrin *in vivo* when compared to the more promiscuous monomeric RGD [141]. In a different study, a scaffold of RGD was created using dendrimers to target $\alpha_v\beta_3$ integrin, which also showed higher receptor selectivity compared with monovalent RGD [141]. Compared with monomeric CD200-Fc fusion proteins, a pentamer of CD200 with cartilage matrix protein yielded a higher avidity and more specific binding to CD200 receptor on myeloid cells such as macrophages [142].

High-affinity binding and selectivity are important properties of targeted therapeutics [101,143,144]. Targeted therapeutics often use specific types of membrane-bound protein including surface antigens or cell-surface receptors as targets. Tools for targeting specific cellular populations have been widely explored by using various targeting ligands including antibodies, aptamers, peptides and small molecules [145-148]. This strategy can result in an increased therapeutic efficacy and reduced side effects and toxicity [149]. The ideal targeted therapeutic should have low affinity for the targeted receptor expressed in normal cells, but high affinity to receptors on diseased or activated cells [101]. Therapeutics with a high affinity may actually show poor specificity if the target receptors are expressed in both diseased and healthy cells, even if the differential receptor expression (diseased vs healthy) is high [101].

As an example, multivalent therapeutics and drug-delivery systems have been reported to differentially target integrins in cancer models, which may provide insight into approaches in autoimmune diseases. One active targeting method for cancer treatment focuses on $\alpha_v\beta_3$ integrin, which mediates cell-cell attachment and cellular adhesion to extracellular matrix. An example of a more selective targeting strategy was the use of an $\alpha_v\beta_3$ integrin ligand conjugated to an anti-galactose antibody. The antibody portion interacted weakly with monovalent oligosaccharide but bound with higher affinity to multivalent galactose epitopes. Binding of the $\alpha_v\beta_3$ integrin ligand to integrin on cancer cell surface resulted in triggering complement-mediated cell lysis. Only cancer cells with high levels of $\alpha_v\beta_3$ integrin were killed. This study implicated multivalent interactions to distinguish cells with different levels of $\alpha_v\beta_3$ integrin to induce differential killing of cancer cells [150].

In many examples of multivalent therapeutics, the specificity of targeting was improved by transitioning from the low affinity of a single, free ligand to multivalent ligand presentation on a single construct (higher avidity). Multivalent ligands may be attained synthetically or by attaching to a carrier such as dendrimers, quantum dots, polymers, microparticles or nanoparticles. The resulting array should allow receptor access to the ligand (i.e., proper flexibility and spacing) to facilitate binding to multiple receptors simultaneously. When properly designed, multimeric ligands usually exhibit substantially stronger binding compared with their corresponding monomers [151]. Studies have shown enhanced binding avidities of multivalent therapeutics on 1 to 9 orders of magnitude when compared with the ligand alone [143]. Several investigators have also designed multivalent therapeutics displaying different types of ligands to achieve even more selective targeting [151]. These strategies are now emerging in the form of multivalent therapeutics targeting costimulation or adhesion of immune cells.

The importance of multivalency: a case study of LFA-1 & ICAM-1

LFA-1 mediates multiple events in the immune system, such as leukocyte trafficking, transmigration and formation of the IS. As mentioned, binding of LFA-1 to ICAM-1 is needed for the initiation and stabilization of T cell-APC conjugation. As discussed earlier,

the affinity of integrins are often regulated by the alteration of integrin conformation, whereas avidity regulation is mediated by changes in cell-surface receptor diffusivity or density that alter the number of adhesive bonds [152]. The ligands of LFA-1 are members of the immunoglobulin superfamily known as ICAMs expressed on APCs, endothelial cells and epithelial cells, and are inducible by inflammatory cytokines.

LFA-1 on resting leukocytes circulating in the blood is normally inactive (i.e., a non-ligand-binding conformation). The conformational change of LFA-1 from a low- to high-affinity state can be activated by the binding of other receptors on leukocytes, such as chemokine receptors, antigen-specific TCRs or B-cell receptors (BCRs) [153]. The high-affinity state of LFA-1 mediates its ability to bind ICAMs. There are three different LFA-1 conformations that occur at different stages of activation [153]. The low affinity conformation of LFA-1 is a bent form where the LFA-1 I domain is bent and close to the cell membrane. The extended form with a closed binding domain has intermediate affinity and the extended form in which the ligand-binding I domain is opened to bind domain 1 of ICAM-1 with the highest affinity [82,154]. The interaction of intermediate affinity LFA-1 with ICAM-1 induces the high-affinity conformation that stabilizes adhesion.

The dynamic adhesion of LFA-1 is also controlled by its avidity resulting from clustering of LFA-1 [153]. Microclusters of LFA-1 on T cells are formed after initial multivalent binding of LFA-1 to ICAM-1 [72]. ICAM-1 is the primary ligand of LFA-1, expressed on both T cells and APCs. Some have suggested that ICAM-1 on APC may act as a costimulatory receptor that facilitates the immune response [126]. Oligomerization and clustering of ICAM-1 increases ligand binding avidity by allowing the 'rebinding' of receptors and ligands after dissociation, since monomeric adhesion molecules tend to have relatively fast dissociation rates [126]. ICAM-1 molecules organize into non-covalently linked homodimers via interactions between domain 4 of ICAM-1 (D4-D4) on the cell surface. Two homodimers bind via D1, resulting in W-shaped tetramers (Figure 1). These tetramers can form linear arrays between the T cell and APC. This linear structure may bend and close to form a peripheral ring in the IS facilitating the adhesion between APC and T cells. The adhesion ring may be composed of thousands of LFA-1/ICAM-1 pairs [126].

LFA-1 and ICAM-1 possess characteristics that can be leveraged for multivalent therapeutics. As mentioned, LFA-1 and ICAM-1 on resting leukocytes, APC, endothelial and epithelial cells are normally inactive (i.e., a non-ligand binding conformation). Once triggered (e.g., by binding of TCR to MHC class II complex), molecules like LFA-1 change conformation to enhance binding to ICAM-1 [126]. Second, LFA-1 and ICAM-1 are upregulated on activated T cells and on endothelial cells in inflammatory diseases or autoimmune diseases. Third, LFA-1 and ICAM-1 ligand binding is controlled by their avidities resulting from clustering [126,153]. This last effect has been directly observed in a study to prevent human rhinovirus infection. Trivalent and tetravalent anti-ICAM-1 mAbs decreased the dissociation rate constant of these multivalent antibodies compared with the monomeric form and dramatically improved efficacy to protect cells from rhinovirus infection *via* an increase in binding avidity to ICAM-1, which is a major human rhinovirus receptor [155].

Multivalent ligands conjugated to nanoparticles have been found to offer several features that were distinct from monomeric ligands in solution. First, this type of multivalent targeting strategy has been proven effective when targeting a variety of different types of receptors that cluster [156]. The binding of LFA-1 and ICAM-1 is also regulated by binding avidity resulting from receptor oligomerization [157,158]. This LFA-1 and ICAM-1 clustering during activation was expected to enhance interaction with multivalent ligands.

Indeed, nanoparticles displaying ligands (antibodies or peptides) were found to differentially target LFA-1 or ICAM-1 on activated T cells or APC populations [159-161].

Besides enhancing binding avidity and selectivity, multivalent therapeutics may also facilitate internalization of the construct via receptor-mediated endocytosis. For example, targeting LFA-1 and ICAM-1 internalization was triggered by the multivalent engagement of ligands [162]. The conjugation of multiple LFA-1 and ICAM-1 ligands on nanoparticles mediated intracellular delivery. This may offer more potent or more sustained inhibition of receptor signaling by internalization of surface receptors. Also, intracellular drug delivery enables targeted delivery of immune therapeutics to cells expressing these receptors. As one may expect, the valency of ligand can also be optimized to adjust binding, internalization, or cellular response [156,163].

Since the oligomeric states of LFA-1 and ICAM-1 molecules contribute to their ability to regulate T-cell responses, it was hypothesized that these nanoparticles may also interfere with signaling at the IS between DCs and T cells. *In vitro* studies of co-cultures of DCs with T cells showed that nanoparticles targeting ICAM-1 or LFA-1 were significantly better inhibitors of synapse formation compared with similar concentrations of free ligands [164]. Furthermore, the targeted nanoparticles dramatically shifted the response of these cells as evidenced by a significant shift in cytokine expression profiles and T-cell proliferation. Multivalent therapeutics, therefore, provide a unique, emerging opportunity to develop new interventions in immune signaling and to shape the immune response.

Future perspective

In autoimmune diseases and immune therapy in general, current interventions often lead to unwanted side effects such as general suppression of the immune system or, at the other extreme, inflammatory cytokine storms. Drugs targeting costimulatory molecules play an important role in directing T-cell activation. The roles of these proteins and the latest therapeutic approaches applied to autoimmune diseases have been reviewed in this article. In addition, the contributions of these proteins as mediators of costimulation or adhesion in the IS were reviewed. Multivalent therapeutic agents were proposed as a logical strategy to intervene in orchestrated signaling events between cells. Evidence from the literature suggests that multivalent ligands can contribute to an increase in ligand-targeting specificity and that these constructs may offer unique strategies towards novel autoimmune therapies.

There are a great number of membrane-bound proteins on immune cells that participate in multivalent signaling events. Many research groups have successfully designed and synthesized multivalent therapeutics using small molecules, peptides, mAbs or aptamers to increase binding affinity, avidity and selectivity to receptors. In addition, drug-delivery systems such as nanoparticles, polymers, dendrimers, and the like, are being explored as multivalent constructs and carriers of drugs. Such systems serve as a platform with readily adjustable binding specificity, stability and, perhaps, efficacy. Finally, multivalent presentation of ligands can induce unique cellular responses not observed from the monomer, such as triggered, receptor-mediated endocytosis.

Multimeric ligands seem to provide a great opportunity as a promising option for a specific treatment of autoimmune diseases. Further development of a multivalent ligand delivery system would have a great impact on autoimmune disease therapy. Future studies may focus more on discovery and targeting costimulatory/adhesion molecules which are proteins, oligosaccharides and carbohydrates that require a clustered state of the ligands. In addition to the therapeutic agents, diagnostic agents may be developed to be used in diagnostic applications of autoimmune diseases. A variety of chemistry methods for conjugation of

multimeric ligands may be more widely investigated to provide options for coupling ligand to the multimeric scaffold.

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Key Terms

Antigen: A substance that is recognized by an antibody.

T cell: A type of white blood cell that plays a role in cell-mediated immunity. Some T cells (e.g., cytotoxic) protect the body from infection by identifying and destroying infected cells. Other T cells (e.g., regulatory) can dampen an inflammatory immune response.

Cytokine: A peptide, protein, or glycoprotein that acts as a signaling molecule, regulates the extent and duration of immune response and mediates intercellular communication.

Integrin: A heterodimeric transmembrane glycoprotein that mediates cellular attachment to other cells or extracellular matrix. Integrin also plays a role in cell signaling.

Monoclonal antibody: A class of antibodies generated by a single clone of immune cells. Monoclonal antibody binds specifically to a particular epitope.

Antigen-presenting cell: A cell that presents antigen to T cells and sends out signals to induce a response to the antigen.

Costimulatory molecules: Membrane-bound or secreted protein that is required for signal transduction leading to T-cell activation.

Executive summary

- Autoimmune disease results from the breakdown of immune tolerance and failure of the immune system to distinguish between self and non-self antigens, hence producing autoreactive antibodies or autoreactive T cells.
- T-cell activation requires two signals, the interaction of the T-cell receptor (TCR) on T cells and antigenic peptide–MHC complex on antigen-presenting cells (APCs; signal 1) and a costimulatory signal (signal 2), to promote T-cell proliferation, cytokine secretion and effector function.
- Monoclonal antibodies and fusion proteins have been developed as agonists or antagonists against costimulatory molecules for the treatment of autoimmune diseases.
- T-cell activation requires sustained conjugate formation between T cells and APCs and communication through the TCR and peptide–MHC complexes.
- Multivalent interaction of costimulatory molecules regulates binding avidity and specificity to their receptors. The multivalent interaction modulates T - cell and B-cell responses.
- In the immunological synapse, the ligands and their receptors are arranged in an orderly manner, increasing the probability that multiple ligands will bind simultaneously with multiple receptors to enhance binding specificity, affinity and avidity and to prolong the duration of signaling.
- The potential of multivalent therapeutics targeting large, organized interfaces (e.g., the immunological synapse) has been reviewed.

Figure 1. Multivalency in immune cell signaling

(A) The linear array of LFA-1 when triggered with PMA or divalent cations. (B) ICAM-1 forms a tetramer when binding to a cluster of LFA-1. (C) Non-ligand binding conformation of LFA-1 on the surface of resting T cells and the dimeric form of ICAM-1 on APC. (D) Simultaneous binding of multimeric LFA-1 to an array of ICAM-1. LFA-1 conformation has been changed into an active form to bind to ICAM-1. APC: Antigen-presenting cell; PMA: Phorbol 12-myristate 13-acetate; TCR: T-cell receptor.

Table 1

Summary of costimulatory molecules

Molecule	Expression	Ligand(s)	Expression	Function	Drugs/mAb
CD28	T cells, thymocytes, NK eosinophils and melanoma cells [6,8,11,12]	CD80 (B7-1) CD86 (B7-2)	Upregulated on activated DCs [13], B cells and macrophages [6] Upregulated on activated monocytes, DCs and B cells, T cells and APCs [6]	Enhances T-cell proliferation and cytokine production through induction of IL-2 expression [6] Promotes T-cell differentiation into IL-4-producing T-helper 2 cells [6,14] Stimulates B cells for antibody production [8] Augments the kinetics and level of expression of OX40, 4-1BB and CD30 [6,15] Inhibition of CD28/B7 reduces specific Ab production, prolongs the survival of organ transplants and inhibits autoimmune diabetes and lupus	Abatacept (CTLA-4-Ig) targeting CD80/86 has been approved by FDA to use in RA. It is also in Phase II clinical trials in psoriasis [16-19] Belatacept (CTLA-4-Ig) has clinical indication in renal transplantation [20,21] Galliximab is an anti-CD80 mAb. It has a clinical indications in non-Hodgkin's B-cell lymphoma [22] and psoriasis [16]. It is now in Phase III clinical trials [201]
PD-1	B cells, monocytes/macrophages and activated T cells [23]	PDL-1 (B7-H1) PDL-2 (B7-DC)	Found on nonhematopoietic cells [23] T cells, B cells, dendritic cells and macrophages [23,24]	Transmits signals that inhibit T- and B-cell activation [23]	PDL-1-Ig fusion protein reduced clinical score in collagen induced arthritis mouse model and prolonged graft survival in mice [23,25] Anti-PD-1 antibody antagonists (MDX-1106, CT-011) are currently in Phase I clinical trials in melanoma [26,27,201]
CTLA-4 (CD152)	Activated T cells [14]	B7-1 (CD80) and B7-2 (CD86)	T cells, B cells, macrophages, DCs, endothelial cells, epithelial cells and fibroblasts [14]	Transmits an inhibitory signal to T cells and inhibits T-cell activation [14] Depletes the expression of IL-2 and IL-2R [8] CTLA-4 binds B7 with a much higher affinity than CD28	Anti-CTLA-4 mAb (Ipilimumab and tremelimumab) have clinical indications in melanoma and other tumors. They have been studied in Phase II/III clinical trials [28-31,201]
ICOS (CD278)	Activated T cells [32]	ICOSL (B7RP-1 or CD275)	Constitutively expressed on B cells, dendritic cells, monocytes, endothelial cells, fibroblasts and epithelial cells [32]	Enhances T-cell differentiation, cytokine production and survival [32]	Anti-ICOSL mAb antagonist has been shown to reduce the severity of G6PI-induced arthritis [33], reduce lethality and symptoms in GVHD in mice [34] Anti-ICOS mAb prolongs cardiac and bone marrow graft survival in mice [35-37]
BTLA (CD272)	B cells and inducible during activation of T cells [38,39]	HVEM	Naive or resting T cells, NK cells, B cells and DCs, myeloid cells [38,39]	Delivers the inhibitory signal of T-cell activation [38,39] Decreases IL-2 production [38,39] Balances activation and inhibition during an immune response [40]	Anti-BTLA in a combination with CTLA-4-Ig has shown efficacy in allograft rejection [41,42]

Molecule	Expression	Ligand(s)	Expression	Function	Drugs/mAb
CD40	B cells, monocytes, macrophages, DCs, platelets and inducibly expressed on non-hematopoietic cells such as epithelial and endothelial cells and fibroblasts [43]	CD40L (CD154)	Activated T cells, activated B cells and platelets [43]	Promotes B-cell activation and DC maturation [8] Induces memory B cells maturation [43,44] CD40 ligation leads to the upregulation of B7-1 and B7-2 on APCs and enhances the ability of APCs to activate naive T cells [43,44]	Dactuzumab (anti-CD40 mAb) has been shown that it has an immunosuppressive effect in multiple myeloma, non-Hodgkin's lymphoma (Phase I) [45,46], diffuse large B-cell lymphoma [47] and chronic lymphocytic leukemia (Phase I) [48] Anti-CD40L mAb significantly suppressed T-cell-mediated alloimmune responses and prolonged allograft survival in monkeys [44] and has been studied in clinical trials for lupus nephritis and kidney transplantation (Phase II) [201]
OX40 (CD134)	Activated T cells [15]	OX40L (CD252)	Professional APCs, can be induced on activated B cells, mature DCs, plasmacytoid DCs and macrophages [15]	Promotes T-cell proliferation, differentiation, survival, cytokine production and effector cell function of T cells [15] OX40-OX40L interactions additionally modulate the differentiation and activity of regulatory T cells (both suppress and promote proliferation of these cells) [15]	Anti-OX40 and OX40L mAb agonists have shown benefits in cancer. Anti-OX40 mAb was studied in clinical trials (Phase I/II) [49-51,201]. There is an ongoing clinical study of human mAb OX40L for treatment of asthma (Phase II) [201] Neutralizing anti-OX40L ameliorated the severity of disease in experimental autoimmune encephalomyelitis [52], colitis [53], arthritis [54], asthma [55] and diabetes [56] <i>in vivo</i>
GITR	Naive T cells and Treg It is upregulated on effector T cells [57] and on tissue infiltrating macrophages	GITRL	B cells, macrophages, DCs, endothelial cells thymic precursors and activated T cells [57]	Suppresses function of Treg [58] Activates effector T cells and NK cells [58] Induces inflammatory activation of macrophage [57]	Anti-GITR mAb agonist induced antitumor immunity [59,60] Phase I clinical study is being performed for treatment of malignant melanoma [201] GITR fusion protein binding to GITRL prolonged allograft survival and showed potential anti-inflammatory properties in animal studies [61,62] Neutralizing anti-GITRL mAb protected the progression of diabetes [63]
CD27	Naive T cells, B cells, NK cells, memory and antigen-exposed T cells [64,65]	CD70	Activated T cells, B cells, macrophages, and activated DCs [64,65]	Stimulates T-cell activation, expansion and survival [66] Promotes B-cell activation and production of antibodies [64]	Anti-CD70 mAb agonist exhibited antitumor immunity in <i>in vivo</i> studies [67,68] Neutralizing anti-CD70 mAb improved disease severity in inflammatory and autoimmune disease [69,70]
LIGHT (TNFSF14)	Activated T cells and immature DCs [65,71]	HVEM (this is the receptor)	T cells, B cells, DCs, macrophages and NK cells [65,72]	Promotes T-cell proliferation, cytokine production, and activation of NF- κ B [8]	LT β R-Ig fusion protein has been shown to ameliorate trinitrobenzene sulfonic acid-induced colitis and prolonged survival of GVHD mice [73] Anti-HVEM mAb profoundly ameliorated GVHD in mice [74] HVEM-Ig fusion protein plus cyclosporine A prolonged allograft survival in mice [75]
4-1BB (CD137)	Activated T cells and NK cells [76-78]	4-1BBL	Activated DCs, B cells and macrophages [76-78]	Provides costimulation of T-cell activation and promotes T-cell survival [76-78]	Anti-4-1BB mAb agonist has shown to have anti-tumor immunity <i>in vivo</i> [79]. It is now being studied in Phase II clinical trials [201] Neutralizing anti-4-1BBL mAb attenuated acute rejection in rat liver transplantation [80]

Molecule	Expression	Ligand(s)	Expression	Function	Drugs/mAb
VLA-4	T cells, B cells, monocytes, NK cells, eosinophils, neutrophils and hematopoietic cells [78]	VCAM-1 (CD106) and fibronectin	Endothelial cells [78]	Regulates adult hematopoiesis [78] Promotes the adhesion of lymphocytes, monocytes, eosinophils and basophils [8]	Natalizumab (anti-VLA-4 antagonist) was approved by FDA for the treatment of relapsing forms of multiple sclerosis [81]
LFA-1	Leukocytes [82]	ICAM-1 (CD54) and ICAM-2 (CD102)	Vascular endothelium, macrophages and lymphocytes [8]	Mediates adhesive interaction between T cells and APCs in the immunological synapse and mediates leukocyte trafficking during inflammation [82]	Efalizumab (anti-LFA-1 mAb) was approved to use in psoriasis but withdrawn due to an increased risk of PML [83] Anti-ICAM-1 mAb was studied in Phase III clinical trials for Crohn's disease (alicaforfen) [84,201] and ischemic stroke (enlimomab) [85] None of them were continued in the post-marketing phase
Mac-1	Leukocytes [82]	ICAM-1 (CD54)	Vascular endothelium, macrophages, and lymphocytes [8]	Mediates leukocyte trafficking during inflammation and contributes to leukocyte activation in immune responses [82]	Anti-Mac-1 showed no effect in graft survival [86]

APC: Antigen-presenting cell; BTLA: B- and T-lymphocyte attenuator; DC: Dendritic cell; GVHD: Graft-versus-host disease; HVEM: Herpes virus entry mediator; ICOS: Inducible costimulator; LFA: Leukocyte function-associated antigen; mAb: Monoclonal antibody; PD: Programmed death; PML: Progressive multifocal myeloencephalopathy; RA: Rheumatoid arthritis.