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Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond

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ABSTRACT

CD40 is a TNF receptor superfamily member expressed on both immune and non-immune cells. Interactions between B cell-expressed CD40 and its binding partner, CD40L, predominantly expressed on activated CD4+ T cells, play a critical role in promoting germinal center formation and the production of class-switched antibodies. Non-hematopoietic cells expressing CD40 can also engage CD40L and trigger a pro-inflammatory response. This article will highlight what is known about the biology of the CD40-CD40L axis in humans and describe the potential contribution of CD40 signaling on both hematopoietic and non-hematopoietic cells to autoimmune disease pathogenesis. Additionally, novel therapeutic approaches to target this pathway, currently being evaluated in clinical trials, are discussed.

Contents

Abbreviations: ANCA, anti-neutrophil cytoplasmic antibodies; CD, Crohn's disease; CSF-1, colony stimulating factor 1; CSR, class switch recombination; DC, dendritic cell; ESSDAI, EULAR Sjogren's syndrome disease activity index; FDC, follicular dendritic cell; FSGS, focal segmental glomerulosclerosis; GC, germinal center; GMCSF, granulocyte/macrophage colony stimulating factor; HIGM, Hyper IgM syndrome; HLA, human leukocyte antigen; IBD, inflammatory bowel disease; ICAM, intracellular adhesion molecule; IDO, Indoleamine 2,3-dioxygenase; ITP, immune thrombocytopenia; mAb, monoclonal antibody; MHC, major histocompatibility complex; MMPs, matrix metalloproteinases; NK, natural killer cell; PASI, psoriasis area and severity index; PsA, psoriatic arthritis; PSGA, physician static global assessment; pSS, primary Sjogren's syndrome; RA, Rheumatoid arthritis; sCD40L, soluble CD40 ligand; SELENA, Safety of Estrogens in Lupus National Assessment; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index; SSc, systemic sclerosis; TAM, tissue-associated macrophages; TCR, T cell receptor; TEAE, treatment emergent adverse events; Tfh, follicular T helper; TNFR, tumor necrosis factor receptor; UC, ulcerative colitis.

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1. Introduction

Robust activation of B lymphocytes requires stimulation through the antigen receptor along with costimulation [1]. CD40 is a costimulatory member of the tumor necrosis factor receptor (TNFR) superfamily [2]. Along with its ligand (CD40L or CD154), CD40 is a transmembrane protein and, like other members of the TNFR superfamily, forms a trimer, with high order clustering of the protein required for optimal signaling [3,4]. The first functional characterization of CD40 was performed on human B lymphocytes, where it is constitutively expressed and signaling through CD40 promotes B cell activation and proliferation [5]. CD40L was initially identified on the surface of activated CD4+ T cells [6–8], and CD40-CD40L interactions between B and T cells were shown to be critical for germinal center (GC) responses and isotype class switching in response to T-dependent antigens [9].

Further studies identified a broader expression pattern beyond B and T lymphocytes for CD40 and its ligand. CD40 is known to be expressed on additional hematopoietic cells, such as monocytes and dendritic cells (DCs) where it promotes cell survival and cytokine production [10] and can also signal to induce activation, proliferation and cytokine production from CD40L expressing CD4+ T cells [11]. CD40 signaling influences cellular responses on non-hematopoietic cells as well, including, endothelial cells, epithelial cells, fibroblasts and neuronal cells [12–15]. Additionally, CD40L has been shown to be expressed on a wide variety of cells including, mast cells, basophils, B cells, natural killer (NK) cells, macrophages, megakaryocytes and platelets, highlighting a broad role for the CD40 pathway in cellular biology [16,17]. Although CD40 is considered the major binding partner for CD40L, CD40L is able to bind other receptors in the integrin family, namely αIIbβ3, α5β1 (VLA-5), αMβ2 (Mac-1) [18].

Given the wide impact of the CD40 axis, tight regulation of CD40/CD40L expression is necessary to maintain appropriate immune responses. Importantly, expression of CD40 pathway components have been shown to be dysregulated in a variety of disease settings. Compared to healthy controls, CD40L expression is elevated on various cell subsets from patients with systemic lupus erythematosus (SLE), including B cells, T cells and monocytes [19–21] as well as on circulating T cells from patients with Rheumatoid arthritis (RA) and psoriatic arthritis (PsA) [22]. Overexpression of CD40L on RA T cells correlates with poor clinical outcomes, including higher disease activity and fewer remissions [23]. Moreover, soluble CD40L (sCD40L) has also been found to be increased in several autoimmune conditions [17]. Similarly, a SNP in the sequence of CD40 leads to increased CD40 protein levels in the thyroid and associates with disease in a subset of patients with Graves' disease [24]. Together, these results support a role for the CD40 pathway in autoimmune disease and suggest that aberrant CD40 signaling could contribute to the initiation or maintenance of pathogenic autoimmune responses.

While early work focused on the essential role of the CD40-CD40L pathway in the generation of humoral immune responses to T-dependent antigens, an abundance of studies has demonstrated that the action of the CD40 axis extends well beyond T-cell/B-cell interactions. This review will highlight what we have learned about the biology of the CD40 pathway in humans with an emphasis on its well documented role in promoting GC responses as well as its contribution to non-humoral aspects of immunity. The CD40-CD40L axis has been implicated in a variety of clinical settings including cardiovascular disease and transplantation [25–27], however, this review will specifically focus on its contribution to autoimmune disease pathogenesis and will detail the past and current approaches used to target this pathway clinically.

2. Role of CD40-CD40L in human humoral immune responses

The role of CD40 in B cells has been extensively characterized and has been shown to be essential for productive primary and secondary humoral immune responses to T dependent antigens. This section will describe the impact of CD40 signaling on a variety of aspects of human B cell biology.

2.1. Impact of CD40 signals on human B cells – In vitro observations

In vitro studies established a critical role for CD40 as a costimulatory signal that, when delivered in combination with cytokines or other stimuli, promotes B cell activation and proliferation. CD40 stimulation results in upregulation of CD80 and CD86 on human peripheral B cells as well as naïve, memory and GC B cells from human tonsil [28,29]. CD40 signals also induce upregulation of CD95/Fas and major histocompatibility complex class II (MHCII) on human B cells [30–32]. In addition to early activation events, CD40 signals promote progression through the cell cycle and B cell expansion. In combination with anti-CD20, IL-4 or IL-21, CD40 stimulates robust proliferation of circulating and tissue resident B cells [5,33–35].

The cytokine milieu plays a critical role in shaping the overall immune response and CD40 signaling can influence cytokine production by human B cells. CD40 stimulation induces IL-12 production from naïve and memory tonsillar B cells [36]. Studies suggest that CD40 can signal with IL-4 to induce NFκB activation and IL-6 production by human B cells [37]. In concert with BCR signals, CD40 co-stimulates production of several pro-inflammatory cytokines including IL-6, TNFα and lymphotoxin-alpha [30]. In contrast, CD40 stimulation alone has been shown to induce production of IL-10, a cytokine with suppressive capacity [30]. Pending the context of the additional stimuli, CD40 signals, therefore, can promote a cytokine milieu that supports cell activation and GC formation or a more suppressive environment, highlighting CD40 as a key rheostat for immune activation.

CD40 signals also support the differentiation of B cells into Ig-secreting plasma cells, and in combination with additional signals, drive switching to various antibody isotypes. In the context of IL-4 signaling, CD40 promotes secretion of IgM, IgG and IgE antibodies [35,38–42] while CD40 stimulation in concert with IL-10 and TGFβ can induce IgA secretion [33]. In combination with IL-21 and BCR signals, CD40 promotes the differentiation of both naïve and memory human B cells into $CD38^{hi}$ plasma cells and induces class switch recombination (CSR) to IgG1 and IgG3 from naïve B cells [34]. These data support a central role for CD40 in the generation of humoral immune responses and highlight the context dependent nature of CD40 signals in the directional output of the response.

2.2. Human deficiency in CD40-CD40L

We have learned a tremendous amount about the role of the CD40 axis in humoral immunity from humans who have defects in this pathway. Individuals with CD40 or CD40L deficiency develop Hyper IgM syndrome (HIGM), a heterogeneous group of disorders with defects in CSR characterized by normal to high concentrations of circulating IgM and abnormally low levels of IgG, IgA and IgE [43–46]. Patients with HIGM do not respond well to immunization and have an increased susceptibility to opportunistic infections including respiratory, gastrointestinal and CNS infections [47–50] which is thought to be at least partially related to defective humoral responses as treatment with IVIG results in a marked reduction in infection rates [51].

Analysis of tissue from patients with HIGM demonstrate an indispensable role for the CD40 pathway in GC formation. The lymph nodes of patients with CD40L deficiency have normal primary follicles containing Bcl-2+ B cells but are largely devoid of GCs [52]. Additionally, follicular dendritic cells (FDCs) are both quantitatively and qualitatively altered in HIGM patients. This includes profound reductions in FDC number as well as phenotypic abnormalities such as markedly reduced expression of CD21 and CD23 [52]. The differentiation of FDCs is known to be dependent on factors derived from both T and B cells [53] and therefore abortive B cell activation in patients with defects in CD40 signaling could contribute to FDC abnormalities. Cumulatively, these data demonstrate a critical role for CD40-CD40L interactions in the formation and maintenance of the GC response.

Defective GC responses in humans with CD40 mutations can also contribute to deficient formation of B cell memory. In patients with X-linked HIGM, there is a significant reduction in the memory B population, including a near absence of circulating IgD-CD27+ class-switched memory B cells [54].

Cumulatively, these studies describe a critical role for the CD40-CD40L pathway in the selection, activation and differentiation of human B cells as well as in the formation of GCs and CSR. In addition to a pivotal role in humoral immunity, the importance of this pathway is evident in cell-mediated immunity with several molecules in the clinic aimed at modulating this pathway. The non-humoral contributions of CD40 axis to the immune response will be described next.

3. CD40/CD40L pathway in cell-mediated immunity

3.1. T cells

Expression of CD40L is dynamically regulated in human $CD4^+$ T cells. Within a few hours after activation, $CD4^+$ T cells transiently express CD40L. During this early phase, interaction of CD40L with membrane bound CD40 from other cells results in proteolytic cleavage, endocytosis and lysosomal degradation of CD40L, as well as release of soluble CD40L as a cytokine. Additionally, CD40L mRNA is downregulated in T cells [17]. Late CD40L expression (48 h after activation) by CD4 T cells is CD28-dependent and is positively and directly regulated by IL-2 in humans. The CD28-dependent upregulation of CD40L on human T cells can be inhibited by daclizumab (anti-CD25/IL-2RA) further demonstrating that this process is IL-2 mediated [55]. Moreover, addition of IL-4 has no effect on CD40L expression, while IL-12 can partially increase ligand expression on human T cells [56]. This biphasic expression of CD40L could support different biological roles: early CD40L was shown to result in B cell activation, Ig secretion, isotype switching and memory formation, while sustained CD40L expression has been reported to inhibit B cell terminal differentiation of a B cell lymphoma cell line [57,58]. Interestingly, it was also demonstrated that a splice variant of CD28 named CD28i acted as an adaptor protein and formed a complex with CD40L in human T cells. This association is thought to regulate the threshold for T cell activation driven JNK, PAK and Akt pathways [59].

CD40-CD40L interactions influence the differentiation of $CD4^+$ T cells into effector subsets. Binding of CD40L on T cells with CD40 on DCs induces IL-12 production by DCs, which in turn results in skewing of Th1 responses [60,61]. While the role of CD40L-mediated licensing of DCs by CD4 T cells is well accepted, the contribution of this pathway to CD8 T cell activation is less established. Antigen-specific CD8 T cells in humans have been reported to express CD40L and provide help to DCs [62]. Furthermore, IL-12 and subsequent STAT4 signaling can promote CD40L expression on human CD8 T cells, which enables self-priming in the absence of CD4 T cell help [63]. In this case, CD8 T cells are still able to perform cytotoxic functions, but optimal CD8 T cell priming is achieved when CD4 T cells provide help with CD40L expression, cytokine production and properly stimulated APCs. Unlike mouse CD8 T cells, activated human CD8 T cells have been reported to not express CD40 [64]. It has also been demonstrated that sCD40L inhibits Fas-induced cell-death in a T cell leukemia cell line where it binds α5β1 integrin and activates pro-survival signals such as p38, ERK1/2, PI3K and Akt [65].

3.2. Dendritic cells and macrophages

As a bridge between innate and adaptive immune systems, professional antigen presenting cells such as DCs present antigen to T cells via Major Histocompatibility Complex/ T cell receptor (MHC/TCR) interaction resulting in effector T cell functions [66]. An effective T cell response also requires expression of co-stimulatory molecules such as CD80 and CD86 on DCs, and only then the T cells are properly activated [67]. Engagement of CD40 on DCs by CD40L has been shown to upregulate both MHC and co-stimulatory molecule expression. This results in DC maturation, proper licensing of DCs and effective priming of T cells [68].

Binding of CD40L to CD40 results in the recruitment of TNF Receptor associated factor (TRAFs) family of proteins to the intracellular domain of CD40. The downstream signaling pathway that gets activated depends on the specific TRAF recruited, and TRAF6 is known as the main player in DC biology [69]. TRAF6 recruitment to CD40 results in p-38 dependent MAPK pathway and MyD88-dependent NFKb pathway activation. Activation of these pathways result in survival and maturation of DCs, along with cytokine secretion [70]. Several studies also reported that the non-canonical NFKb pathway could be activated in a CD40-dependent manner via the serine/threonine kinase NIK (NFKb-inducing kinase) in DCs, which can cross-prime CD8 T cells [71,72].

As mentioned earlier, CD40L can also be secreted and act as a cytokine. Circulating soluble CD40L (sCD40L) has been reported in different disease settings [17]. One report demonstrates that sCD40L is present in monomeric and multimeric forms in the plasma of HIV patients that are on antiretroviral therapy. They further demonstrate that while the multimeric sCD40L is immune-stimulatory, monomeric form does not result in true stimulation. Additionally, in the presence of TLR ligands, stimulation with both monomeric and multimeric sCD40L results in the dysfunction of DCs and a defect in IL-12 secretion [73]. In a different study in HIV patients, sCD40L was shown to be immunosuppressive via indolamine-pyrrole 2,3-dioxygenase (IDO)-induced Tryptophan metabolism in DCs and subsequent generation of T regulatory cells [74].

In a cancer setting, trimeric sCD40L was fused to a cancer-specific marker (EpCAM) and was reported to have a beneficial role in that it induces potent and specific DC maturation, and cancer cell apoptosis [75]. It is interesting to note the context-dependent activity of sCD40L, and further studies are required to fine tune the efficacy of current therapies.

CD40/CD40L pathway also plays an important role in the initiation of immune responses by and effector function of macrophages [76]. A recent study demonstrated that macrophages from CD40L-deficient patients lack fungicidal activity with decreased oxidative burst in vitro. Additionally, macrophages have reduced cytokine production, which could be reversed with addition of exogenous recombinant IFN-gamma. Transcriptome analysis revealed differential regulation of genes in macrophages from CD40L-deficient patients, with 48 downregulated and 61 upregulated genes compared to macrophages from healthy controls [77]. In general, CD40 signaling in macrophages result in a pro-inflammatory milieu which could be beneficial in host defense yet detrimental in autoimmune diseases or inflammatory disease. For example, a recent study demonstrated that blocking TRAF6 signaling in human macrophages, while preserving CD40-TRAF2/3/5 signaling was effective in reducing atherosclerosis while still keeping CD40/CD40L intact for proper immune responses [78].

The importance of CD40 biology in macrophage function has been nicely demonstrated in the cancer setting. As a main player in the immunosuppressive tumor microenvironment, tissue-associated macrophages (TAMs) could be detrimental in dampening immune response as they secrete IL-10, IDO and TGF-beta [79]. Wiehagen et al. demonstrated that dual targeting of TAMs with a CD40 agonist and colony stimulating factor 1 (CSF-1) blockade converts TAMs into a pro-inflammatory phenotype. CSF-1 is known to induce generation of myeloid-derived suppressor cells and tolerogenic DCs; therefore, blocking this pathway while providing a positive stimulus for macrophage maturation resulted in increased CD8 T cell responses and reduced regulatory T cell activity in mice [80]. In a different study, Luheshi and colleagues showed that culturing monocyte-derived human macrophages in the presence of Th1/Th2 cytokines and CD40 agonism resulted in pro-inflammatory macrophages, while CD40L stimulation only produced suppressive macrophages. Interestingly, while Th1 cytokines generated both tumor killing macrophages and potent T cell activation, Th2 cytokines resulted only in enhancement of T cell responses due to increased IL-12 production [81].

3.3. NK cells

CD40 pathway agonists that have been used in animal models and cancer settings have T-cell independent effects via acting on other cells such as NK cells [82]. CD40L is upregulated on the surface of human and mouse NK cells when activated with IL-2 [83]. NK cells can be directly activated in an autocrine manner by their CD40L engagement with CD40 and this results in NK cell proliferation, activation and improved target killing [84–86].

Activation of CD40/CD40L pathway can also indirectly activate NK cells, mainly through IL-12 secretion by APCs. One study demonstrated that immunization with bone-marrow derived DCs resulted in long-lasting NK cell mediated anti-tumor immunity in mice. The authors show that one month after immunization, when the mice are challenged with tumors, endogenous DCs upregulate CXCL-10 and chemoattract NK cells into the spleen. In the spleen, CD40L expressing T effector memory cells activate DCs, resulting in IL-12 production and subsequent NK cell activation [87]. In a different study, Jackaman and colleagues demonstrate that IL-2/CD40 agonist combination therapy resulted in NK cell infiltration into mesothelioma tumors. Furthermore, they show that NK cells did not play an effector role in this system, rather helped in establishing a long-term memory response in this human cancer setting [88]. In a different study examining the interplay between human DCs and NK cells, it was demonstrated that CD40 and CD40L interaction increased cytotoxic function of NK cells but not IFN-gamma secretion [89].

3.4. Granulocytes

It is well established that granulocytes also express CD40 or CD40L in different settings. Human peripheral blood and nasal polyp eosinophils have been reported to both express CD40 that could be regulated by circulating IgA (upregulated) and IL-10 (downregulated) [90]. Functionally, ligation of CD40 with CD40L resulted in increased survival and secretion of granulocyte/macrophage colony stimulating factor (GMCSF) in human peripheral blood eosinophils [90]. In a separate study, researchers isolated fresh eosinophils from the peripheral blood of healthy and asthmatic patients, and upon culturing for 48 h, CD40 was expressed spontaneously. Engagement of CD40 resulted in delayed apoptosis and increased eosinophil survival. In the same study, they demonstrated that eosinophils from the sputum of asthmatic patients expressed CD40 [91]. Finally, a recent study demonstrated that human eosinophils can act as antigen presenting cells and activate a multitude of T helper responses (both pro- and anti-inflammatory). This report demonstrated that culturing eosinophils with allergen antigens results in the upregulation of CD40 as well as human leukocyte antigen (HLA) and CD80/CD86 [92].

Stimulatory abilities of human mast cells and basophils in stimulating IgE synthesis by B cells have been compared. This study showed that unlike mast cells, monocyte-derived basophils produced IL-4 and IL-13 and upregulated CD40L after Fc∈RI stimulation [93]. Similar to eosinophils, it was demonstrated that basophils were able to present antigen, produce IL-6 and IL-4, and amplify humoral responses with the help of CD4 T cells in CD40-dependent manner [94].

Neutrophils also express CD40L, and the interplay between CD40 expressing platelets has been studied. CD40L expressed on activated neutrophils along with reactive oxygen species further activate platelets and result in secretion of soluble CD40L from the platelets [95]. Additionally, CD40L expressed by platelets is capable of binding CD40 expressed by endothelial cells triggering chemokine secretion, resulting in chemotaxis of leukocytes into the site of injury [96]. One cell type that is recruited to sites of inflammation is the neutrophil. Binding of soluble CD40L on the surface of neutrophils also enhances adhesion via CD40/CD40L interaction itself and Mac-1 (Cd11b) upregulation [97]. All these mechanisms have the potential to play crucial roles in inflammation and induction of vascular disease.

4. CD40/CD40L in autoimmune disease pathogenesis

4.1. In the germinal center

The CD40 pathway plays an important role in autoimmune disease that are thought to be largely driven by autoantibodies. CD40L expressed on T follicular helper (Tfh) cells in both in lymphoid tissue GCs as well as in GCs that form in tissue ectopic follicles is believed to play a major role in the generation of plasma cells with autoimmune specificities. In systemic rheumatic diseases where autoantibodies are believed to play an important role in progression of disease, such as SLE, Lupus Nephritis, Sjögren's syndrome, Myositis, Systemic sclerosis, ANCA (anti-neutrophil cytoplasmic antibodies) Vasculitis, RA, IgG4 Related disease and Pemphigus, CD40L is believed to be central to the production of pathogenic autoantibodies (Fig. 1). CD40 signaling is also believed to be required for the generation of autoantibody producing plasma blasts and plasma cells in non-rheumatic conditions that have a large autoantibody component, such as Myasthenia gravis, Neuromyelitis Optica, NMDA receptor encephalitis, thyroid Hashimoto's or Grave's disease, Idiopathic thrombocytopenic purpura, hemolytic anemia or IgA Nephropathy.

The anti-CD20-depletion drug Rituxan has been in clinical trial for several autoimmune and non-autoimmune indications, and is approved

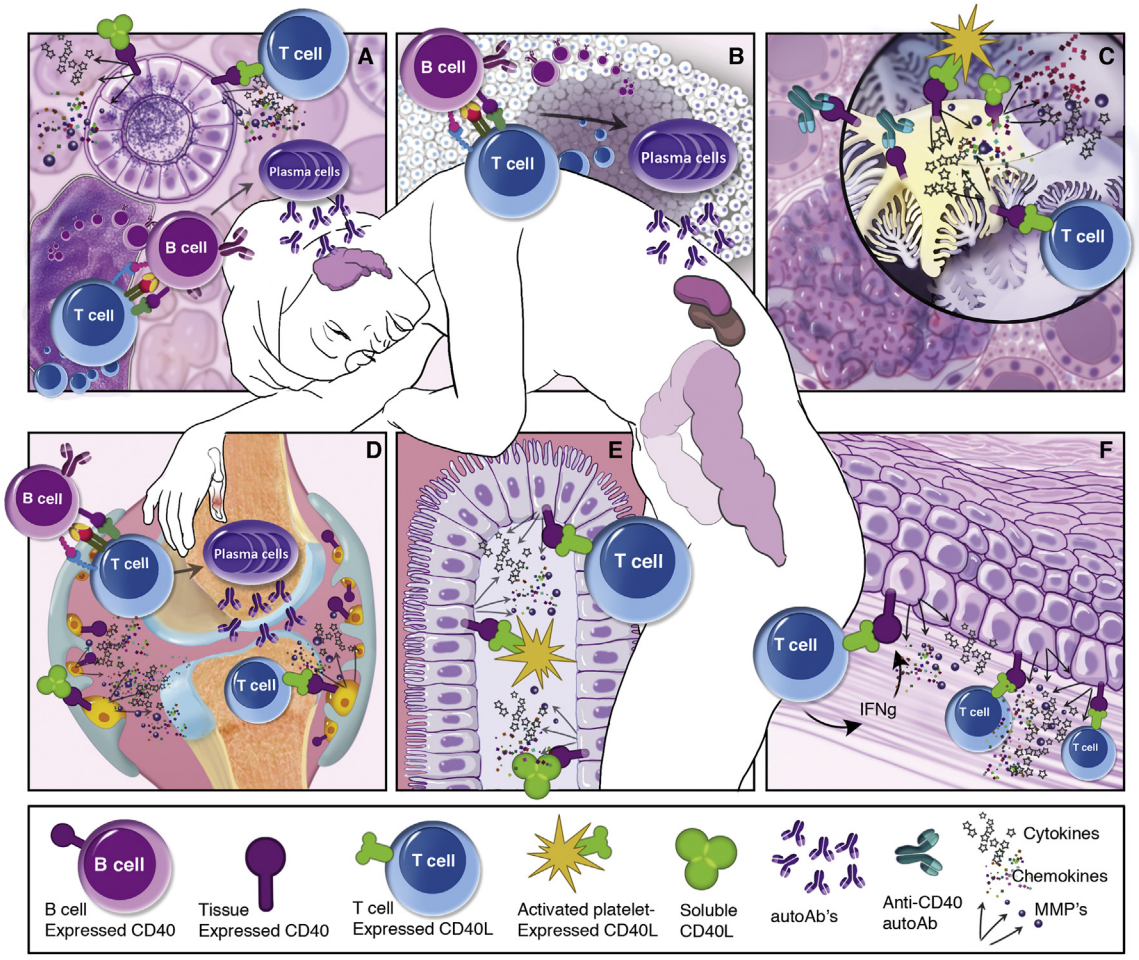

Fig. 1. CD40L ability to drive inflammation in non-lymphoid tissues. (A) Salivary Gland: CD40 expressed directly by salivary gland ductal epithelial cells can be engaged by CD40L-expressing T cells or soluble CD40L released by activated platelets to drive local inflammation. Moreover, CD40 expressed by B cells in ectopic lymphoid follicles can be driven to differentiate into autoreactive plasma cells by CD40L-expressing T cells. (B) Spleen: CD40L expressed by GC T cells drives class switch recombination and plasma cell differentiation of B cells in lymphoid tissues. (C) Kidney: CD40 expressed on podocytes in the Bowman's capsule *can be triggered by CD40L-expressing T cells, soluble CD40L, CD40L expressed by activated platelets, or agonist anti-CD40 autoantibodies that develop in* Focal segmental glomerulosclerosis that may contribute to disease pathogenesis by altering glomerular permeability. (D) Joint: CD40 expressed on synoviocytes can contribute to joint destruction by trigging production of proinflammatory mediators following interaction with T cell-expressed or soluble CD40L. Furthermore, autoantibodies may be produced locally by infiltrating B cells that are activated to differentiate by CD40L-expressing T cells. (E) Gut: *Intestinal* epithelial cells of inflamed mucosa express CD40 and can contribute to inflammatory bowel diseases through activation of release of MMPs and chemokines that allow for chemotaxis of lymphocytes and neutrophils resulting in inflammation. (F) Skin: CD40 is expressed by *keratinocytes in diseases such as* psoriasis and cutaneous lupus that can be further upregulated by IFNγ resulting in cytokine production that may be linked to cutaneous fibrosis.

in RA as well as ANCA Vasculitis, Pemphigus vulgaris and several oncology indications. Even though antibody-producing plasmablasts and plasma cells do not express CD20, Rituxan has been reported to reduce some autoantibodies [98]. These results suggest that short-lived autoreactive CD20- plasma blasts can be continuously produced in the GC from CD20+ B cells where CD40L has a known role, and in these diseases, targeting the CD40L pathway may also be expected to reduce the pathogenic autoantibodies.

4.2. Beyond the germinal center

As discussed above, the CD40 pathway is intricately involved in humoral immune processes. However, CD40 can be expressed on a host of non-hematopoietic cells that is often upregulated with inflammation and can be triggered by tissue infiltrating T cells that express CD40L, sCD40L, or activated platelets that express CD40L. Binding of CD40L by endothelial cells and other non-hematopoietic cells can induce these cells to produce pro-inflammatory molecules including cytokines, matrix metalloproteinases (MMPs), chemokines and other immunologically relevant proteins [25,99,100]. Below several diseases are discussed where CD40 expression on stromal cells may play a direct role tissue damage.

4.2.1. Rheumatoid arthritis (Joint)

RA is the largest of the Rheumatologic diseases that targets joints, although other major organs can be involved. Rheumatoid factor autoantibodies directed to the Fc region of antibodies result in immune complex deposition of joints, resulting in joint swelling, pain and bone erosion. In a clinical trial, Rheumatoid factor has been reported to be modulated by drugs that target the immune system, suggesting that Rheumatoid factor is produced by short lived plasmablasts that have recently emerged from CD40L engagement in the GC. However, the CD40 pathway is not only linked to production of autoantibodies in RA, but has also been described to be expressed directly by synovial fibroblasts, where engagement with CD40L results in production of inflammatory mediators such as TNF, IL-1b and MMPs, contributing to further inflammation and joint destruction [101].

4.2.2. Sjögren's syndrome (Salivary gland)

Sjögren's syndrome is the second most common systemic Rheumatologic disease, that primarily involves the salivary glands (dry mouth)

and lacrimal glands (dry eyes), but can involve other major organs, and if so, can result in severe disease. Lymphoid follicles containing active T cells, B cells and other cell types develop in these tissues and are believed to be involved in tissue destruction. In addition to infiltrating immune cells, CD40 is constitutively expressed on salivary gland ductal epithelial cells and endothelial cells that also express other immunologic relevant molecules such as HLA, CD95, CD80/86, and thus have the ability to present antigen, and be immune-activating [102–104]. Furthermore, interaction with CD40L, expressed on infiltrating salivary gland T cells, or sCD40L, that has been reported to be increased in Sjögren's syndrome [105], can upregulate adhesion molecules such as intercellular adhesion molecule-1 ((ICAM1)/CD54) [106]. Recently, we have described that in a mouse model of Sjögren's syndrome, a single injection of anti-CD40L at 4 weeks of age prevented the development of Sjögren's manifestations for over 6 months, suggesting that blockade of this pathway had long lasting protective effects in a murine model of autoimmunity [107].

4.2.3. Autoimmune nephritis (Kidney)

Several diseases can result in autoimmune nephritis, including antitubular basement membrane disease, Kawasaki disease, Sjögren's syndrome, SLE, Wegener granulomatosis or Focal segmental glomerulosclerosis (FSGS). FSGS is the leading glomerular cause of end-stage renal disease resulting in renal transplantation, where recurrent FSGS often occurs after transplant [108]. The cause of the disease is unknown, however, podocyte function and survival is impacted. Podocytes, also referred to as visceral epithelial cells, wrap around glomeruli and are involved in kidney blood filtration. It has been shown that circulating factors in the blood can directly damage these cells. CD40 is not normally expressed in healthy kidneys but is upregulated by podocytes of glomeruli in individuals with FSGS and can be further induced by CD40L [109].

There are several autoantibodies that have been identified in FSGS including agonist autoantibodies to CD40 that appear to bind a cryptic peptide that may be antigenic [110]. Presence of anti-CD40 autoantibodies prior to renal transplant correlates with recurrence of FSGS, suggesting involvement with disease [111]. In vitro, anti-CD40 isolated from FSGS patients modifies human podocytes actin cytoskeleton leading to increased permeability, and recombinant CD40L upregulates proinflammatory mediators such as MMPs on podocytes [109,112]. In vivo, FSGS-derived anti-CD40 induces proteinuria in wildtype, but not CD40-deficient mice [110]. CD40 can also be triggered by sCD40L released by activated platelets. In FSGS sCD40L is increased and biologically active on podocytes, suggesting other means by which CD40 can be triggered on epithelial cells leading to tissue damage [113].

Blocking the CD40 pathway in FSGS following transplant could have several possible benefits: by induction of immunological tolerance of transplanted kidney; through inhibition of plasma cell differentiation and ensuing autoantibody production; and via blockade of CD40L-mediated activation of podocytes.

4.2.4. Inflammatory bowel disease (Gut)

Inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory diseases of the gut, whose etiopathology is unknown. These diseases are characterized by infiltrating T cells, B cells and macrophages into the inflamed intestinal mucosa disrupting barrier function. In animal models of colitis, blocking CD40L prevents disease onset [114,115]. In human disease, CD40L and CD40 have been reported to be increased in both the circulation and the gut mucosa in IBD [116–119]. Furthermore, in the inflamed bowel, CD40 is strongly over expressed in both endothelial and mesenchymal cells within the mucosa and submucosa [120–122] and CD40 expression has been shown to associate with clinical activity of UC [119]. CD40L is over expressed by lamina propria T cells from CD and UC mucosa [118]. CD40L from activated T cells, platelets or soluble CD40L have all been reported to be biologically active and able to trigger inflammatory responses, such as upregulation of chemokines, and cytokines from intestinal microvascular endothelial cells of IBD patients, suggesting a direct link to the pathophysiology of disease [117,120,123].

4.2.5. Autoimmune skin diseases (Skin)

There are several autoimmune skin diseases, where infiltrating lymphocytes, especially activated T cells, are believed to play a key role in tissue pathogenesis. In the skin, CD40 is expressed in healthy keratinocytes [124], but can be greatly upregulated in autoimmune skin disease such as Systemic sclerosis (SSc) and SLE. In SLE, but not healthy donors, $CD40L^{+}$ infiltrating lymphocytes are present in skin lesional biopsies [125].

CD40 is greatly upregulated in skin fibroblasts from SSc, where ligation with CD40L was shown to induce the fibroblasts to produce proinflammatory cytokines and chemokines such as IL-6, IL-8, and MPC-1 as well as induce CD80 expression [126]. CD40L is also overexpressed by blood T cells and in lesional skin in SSc [127,128], and soluble CD40L has been found to associate with limited cutaneous SSc [129], and vascular complications [130]. Lastly, CD40L can also contribute to SSc disease through the differentiation of autoantibodies to DNA topoisomerase I [131].

4.2.6. Type 1 diabetes (Pancreas)

In humans, both pancreatic duct cells and islet beta cells and have been shown to express CD40. Upon ligation with CD40L, these cells produce pro-inflammatory cytokines, that may contribute to the pathogenesis of T1D and islet graft rejection upon encounter with activated T cells [132–134]. Patients with T1D have also been shown to have significantly higher levels of sCD40L as well as platelet surface CD40L expression [135,136], suggesting other sources of ligation for these CD40-expressing pancreatic cells.

CD40L is predominantly expressed on activated T cells, however, in both mice and humans, CD40 has also been described to be T cell expressed in T1D (TCD40) [137]. These cells appear to be less susceptible to Treg-mediated inhibition [138], and in mice, it has been suggested that these TCD40 effector cells are directly diabetogenic, as they have the ability to transfer disease in nonobese diabetic (NOD).SCID recipients [139]. In NOD mice, CD40 interactions have been shown to be required for the development of T1D. Anti-CD40L treatment starting at 3 weeks-until 12 weeks of age, completely inhibited the development of T1D, whereas starting treatment at 9–10 weeks of age showed no effect in NOD mice [140]. Moreover, we have recently shown that a single injection of anti-CD40L given to NOD mice early in life significantly reduces the development of insulitis and glucosuria out to 30 weeks of age [107], suggesting that transient blockade of this pathway is sufficient to inhibit the development of autoimmunity in this model.

4.2.7. Multiple sclerosis (CNS)

Multiple sclerosis (MS) is an autoimmune disorder in which the sheaths of nerve cells in the brain and the spinal cord are damaged by autoimmune mechanisms involving B and T cells [141]. One study demonstrated that there is a positive correlation between the albumin quotient (marker of blood-brain-barrier breakdown) and sCD40L levels in the serum of MS patients [142]. In relapsing-remitting MS patients, it was also demonstrated that B cells can be activated via CD40 in NFKB-dependent manner with hyperphoshorylation of p65 [143]. CD40-dependent activation of B cells has been shown to induce secretion of IL-15, which in turn results in increased granzyme B secretion by CD8 T cells [144]. In a contrary clinical study, researchers demonstrated that there was decreased amounts of sCD40L in the serum of MS patients and administration of Interferon beta and glatiramer acetate further reduced these levels [145]. Interestingly, an allele of CD40 and its reduced mRNA expression has been shown to be a risk factor for MS [146]. In conclusion, there is an indication that the CD40/CD40L pathway is altered in MS, and a better understanding of different mechanisms at play may result in effective treatment options for this disease.

5. Clinical trials

Given the broad spectrum of impact of CD40 signaling on the immune response and its potential role in a variety of disease settings, several molecules targeting this pathway have been generated and evaluated in clinical settings (Table I). Early trials provided biologic validation for blocking this pathway but also revealed unexpected safety complications associated with targeting of CD40L with a monoclonal antibody (mAb).

5.1. Early trials with anti-CD40L mAbs

5.1.1. Ruplizumab (BG9588)

One of the first molecules generated to target the CD40 pathway was a CD40L-specific humanized IgG1 mAb, BG9588. An open label phase II trial with BG9588 in patients with proliferative lupus nephritis highlighted the potential benefit of this approach in autoimmunity [147]. Among patients evaluated for efficacy in this study ($n = 18$ receiving ≥3 infusions), treatment with BG9588 resulted in a significant decrease in anti-dsDNA antibody titers and a significant increase in complement C3 concentration [147]. Additionally, treatment with BG5988 completely reduced hematuria in all patients with significant hematuria at baseline.

Analysis of lymphocyte subsets revealed a slight increase in total $CD19^+$ B cells 28 days post final dose of BG5988 which returned to baseline 1 month later [147]. Additional analysis on a small subset of patients within this study revealed treatment related reductions in spontaneously proliferating B cells [148]. Treatment with BG5988 also resulted in profound reductions in the frequency of circulating $CD38^{hi}$ plasma cells by flow cytometry [148] and IgM and IgG anti-DNA secreting cells by ELISPOT [149]. In this small subset of patients evaluated, decreases in anti-dsDNA antibodies were also associated with improvement in SLE disease activity index (SLEDAI) [148].

While this was a small open-label study, it suggested that blockade of the CD40 pathway can modulate key clinical parameters of lupus and highlighted the immunomodulatory potential of CD40-CD40L blockade in autoimmunity. Unfortunately, clinical evaluation of BG9588 was halted due to safety concerns, with reports of thromboembolic events occurring in several independent studies involving this monoclonal antibody [150].

5.1.2. Toralizumab (IDEC-131)

A second anti-CD40L mAb, IDEC-131 was also evaluated in number of early clinical trials, including Phase II studies in SLE, multiple sclerosis and CD [151,152]. Efficacy of IDEC-131 was not demonstrated in a cohort of mild-moderate SLE patients [152]. However thromboembolic complications were also observed in three patients in two separate trials involving IDEC-131, resulting in the discontinuation of development of this molecule.

5.1.3. Thromboembolic complications with CD40L-specific mAbs

Following the safety issues in these early clinical trials, much effort was focused on understanding the mechanism driving the thromboembolic events in response to anti-CD40L mAb treatments. One potential explanation for the unanticipated safety issues in clinical trials is linked to the expression pattern of FcγRIIa (or CD32a), which is found on human, but not mouse, platelets [153]. CD40L is highly upregulated on activated platelets [154], and concurrent antibody-mediated binding to both CD40L and FcγRIIa on adjacent platelets is believed to lead to platelet aggregation resulting in thrombosis.

Importantly, the Fc portion of the anti-CD40L mAb has been definitively linked with its toxicity, where immune complexes (ICs) comprised of anti-CD40L antibody and recombinant CD40L activate platelets in vitro, in an FcγRIIa-dependent manner [155]. Administration of pre-formed anti-CD40L ICs leads to thrombosis in mice transgenic for human FcγRIIa, but not in mice that do not express human FcγRIIa [156]. Additionally, thrombosis was not observed in human FcγRIIa transgenic mice receiving aglycosylated anti-CD40L which does not engage FcγR. These observations support the possibility that thromboembolic complications in anti-CD40L mAb treated subjects are the result of platelet aggregation triggered by anti-CD40L ICs, in an Fc-dependent manner.

5.2. Ongoing trials targeting CD40-CD40L pathway

To avoid the safety complications associated with mAb based targeting of CD40L, a number of alternate approaches to block CD40-CD40L interactions are being evaluated (Table I).

5.2.1. Active CD40L-targeted therapies

5.2.1.1. Dapirolizumab pegol. Dapirolizumab pegol (CDP7657) is an anti-CD40L Fab' antibody fragment conjugated to polyethylene glycol (PEG)

Table I
CD40-CD40L antagonistic therapeutics evaluated in clinical trials for autoimmune indications.

Target	Drug name	Format	Indications	Stage of development/clinical trial status	Citations
CD40L	Ruplizumab (BG9588)	Humanized IgG1 mAb	LN, transplantation	Discontinued after Phase 2 (safety issues): NCT00001789	Boumpas 2002 Grammer 2003
	Toralizumab (IDEC-131)	Humanized IgG1 mAb	CD, MS, SLE, ITP	Discontinued after Phase 2 (safety issues)	Davis 2000 Kalunian 2001 Kuwana 2004
	Dapirolizumab pegol	Fab' fragment	SLE	Phase 2b ongoing (SLE): NCT02804763	Chamerlain 2017
	Letolizumab (BMS-986004)	Fc-modified Human IgG1 fusion protein	ITP	Phase 1/2 completed (ITP): NCT02273960	Kim 2017 (NHP)
	VIB4920 (MEDI4920)	Tn3 Fusion protein	RA	Phase 1b ongoing (RA): NCT02780388	Oganesyan 2013
CD40	CFZ533	Fc-modified Human IgG1 mAb	pSS, RA, SLE, MG, transplantation	Phase 2 ongoing (pSS): NCT02291029	Cordoba 2015 Fisher 2017
	Bleselumab (ASKP1240)	Human IgG4 mAb	Psoriasis, transplantation, FSGS	Phase 2a completed (psoriasis): NCT01585233 Phase 2 ongoing (kidney transplant and FSGS): NCT02921789	Goldwater 2013 Okimura 2014
	BI-655064	Humanized IgG1 mAb	RA, ITP, LN	Phase 2 ongoing (LN): NCT03385564	Albach FN 2018
	ch5D12	Human IgG4 mAb	CD	Phase 1/2 completed (CD)	Kasran 2005
	FFP104	mAb	PBC, CD	Phase 1/2 ongoing (PBC): NCT02193360 Phase 2 ongoing (CD): NCT02465944	

Abbreviations - LN: lupus nephritis; CD: crohn's disease; MS: multiple sclerosis; SLE: systemic lupus erythematosus; ITP: immune thrombocytopenia; pSS: primary Sjogren's syndrome; RA: Rheumatoid arthritis; MG: myasthenia gravis; FSGS: focal segmental glomerulosclerosis; PBC: primary biliary cirrhosis.

[157]. Preclinical studies demonstrate that Dapirolizumab can inhibit humoral immune responses to the T-dependent antigen tetanus toxoid in cynomolgus monkeys with no evidence of thromboembolic complications in non-human primates with weekly dosing up to 200 mg/kg for three months [157]. In a phase I study, Dapirolizumab pegol was well tolerated in a cohort of 68 patients with SLE, with no serious treatment emergent adverse events (TEAE) reported [158]. Importantly, there was greater improvement in the clinical measures of disease activity in the dapirolizumab pegol group versus placebo, including improvements from baseline in various SLE activation indexes including SELENA, SLEDAI as well as in the proportion of BICLA and SRI4–5 responders. Consistent with proposed mechanism of action of CD40L blockade, improvements in disease activity scores in response to Dapirolizumab pegol coincided with mRNA expression changes in B cell- and PC-related genes [158]. Top-line results were recently announced from a randomized, double blind placebo-controlled Phase IIb study of Dapirolizumab pegol in patients with moderately to severely active SLE [159]. In this study, the primary endpoint to demonstrate a dose response on the British Isles Lupus Assessment Group (BILAG)-based Composite Lupus Assessment (BICLA) at 24-weeks was not met ($p = .06$). Additional data is still being evaluated including impact of treatment on various secondary clinical endpoints as well as biomarker data.

5.2.1.2. Letolizumab (BMS-986004). Letolizumab is dimeric antibody construct that targets CD40L and was generated to express a mutated IgG1 that lacks effector functions, including Fc binding and complement fixation [160]. Letolizumab does not induce platelet activation in vitro and did not affect coagulation factors or platelet counts/function following repeat dosing in non-human primates. Additionally, higher doses of letolizumab resulted in prolonged graft survival in Rhesus macaques following renal transplant [160]. The safety and efficacy of this molecule was evaluated in an open label Phase I/II trial in immune thrombocytopenia (ITP), with results from this trial currently pending.

5.2.1.3. VIB4920. VIB4920 (formerly MEDI4920) is a novel CD40L binding protein comprised of two Tn3 proteins fused to human serum albumin [161]. Tn3, derived from the third fibronectin type III domain of human tenascin-C, is a small protein scaffold that possesses immunoglobulin-like folds, including loops structurally analogous to antibody complementarity-determining regions, which can be engineered to confer binding specificity. VIB4920 targets CD40L but does not possess an Fc domain and therefore is unlikely to induce adverse platelet responses. In a Phase 1a clinical trial in healthy subjects VIB4920 demonstrated an acceptable safety and tolerability profile while showing dose-dependent inhibition of T-dependent antibody responses [162,163]. VIB4920 was recently evaluated in a Phase 1b clinical trial in patients with adult-onset RA ([164] and manuscript in preparation). In this study, VIB4920 significantly reduced RA disease activity, as measured by DAS28-CRP, at day 85 as well additional clinical parameters including tender/swollen joint counts, CRP, and patient and physician global assessments [164]. Improvements in clinical activity in this trial were accompanied by reductions in Rheumatoid Factor autoantibodies of approximately 50% at the higher doses and significant reductions in Vectra DA, a composite measure of 12 circulating biomarkers associated with pathways that drive RA disease activity [164]. Phase II trials for VIB4920 are currently being planned.

5.2.2. Molecules targeting CD40

5.2.2.1. CFZ533. CFZ533, a fully human antagonist anti-CD40 monoclonal IgG1 antibody, contains a modified Fc domain to render it unable to mediate Fcγ-dependent effector functions [165,166]. This mutant anti-CD40 antibody demonstrated that blocking CD40 using a non-depleting antibody promotes allograft survival in non-human primates following kidney transplantation [165]. Splenic GCs and alloantibodies were also absent in CFZ533-treated transplanted animals, highlighting the impact of CD40 blockade on humoral responses in this setting. At a 6-month interim analysis in a Phase I/II study in de novo renal transplant, CFZ533 in combination with mycophenolate mofetil (MMF) and corticosteroids (CS) demonstrated comparable efficacy to tacrolimus, MMF and CS in the composite endpoint of biopsy proven graft rejection, graft loss or death. However, compared to tacrolimus, CFZ533 demonstrated improved renal function with fewer serious adverse events and infection complications, highlighting the potential for reduced nephrotoxicity with this approach [167]. CFZ533 also demonstrated efficacy in a Phase IIa randomized controlled trial in patients with primary Sjogren's syndrome (pSS) [168]. The authors reported a significant improvement in clinical disease activity as measured by improvement in ESSDAI in patients receiving CFZ533 (ΔESSDAI: 6.35 in 10 mg/kg CFZ533 group versus ΔESSDAI 1.27 in the placebo group). CFZ533 has also been evaluated in a number of other clinical trials including Graves' disease, RA, and myasthenia gravis.

5.2.2.2. Bleselumab (ASKP1240). Bleselumab (ASKP1240) is a fully human IgG4 monoclonal antibody that targets CD40. Numerous studies in non-human primates have demonstrated the efficacy of this molecule in promoting allograft survival. When administered as a maintenance monotherapy, ASKP1240 efficiently suppressed both cellular and humoral alloimmune responses and prevented rejection of hepatic allografts [169] and also prolongs renal allograft survival [170]. ASKP1240 promotes survival of pancreatic islet allograft in transplanted cynomolgus monkeys and this effect was associated with decreases in IFNγ secreting alloreactive peripheral T cells and suppression of anti-donor IgM and IgG antibodies [171]. ASKP1240 was safe and well tolerated in a Phase I study of healthy volunteers with no evidence of thromboembolic events [172]. Bleselumab recently reported results from a Phase IIa randomized, placebo-controlled multiple ascending dose study in patients with moderate-to-severe plaque psoriasis [173]. Bleselumab demonstrated a favorable safety profile in psoriasis patients, including no clinically significant infusion reactions, cytokine-release syndrome, or thromboembolic events reported. However, there was no evidence of clinical benefit, as measured by Psoriasis Area and Severity Index (PASI) score, Physician Static Global Assessment (PSGA) score or percent body surface area at the primary endpoint (week 8) with doses up to 3.0 mg/kg [173]. The study in plaque psoriasis was not powered for efficacy measurements, however, and evaluation of bleselumab is ongoin in a Phase II study in kidney transplantation and FSGS.

5.2.2.3. BI-655064. BI-655064 is a humanized antagonistic IgG1 mAb that binds CD40 and was designed with Fc mutations to prevent Fc-mediated effector functions including cytotoxicity and platelet activation [174]. In studies with cynomolgus monkeys, protracted dosing of BI-655064 resulted in decreased B cell levels and reduction in lymphoid GCs, both of which were reversed following cessation of treatment, without alterations in platelet aggregation or function [175]. In a Phase I study with 72 healthy male volunteers, a single dose of BI-665064 up to 120 mg (i.v. or s.c.) demonstrated a good safety profile, with the proportion of subjects having adverse events similar between active and placebo treatments. In a double-blind, randomized Phase I trial, patients with RA received either weekly BI-655064 (120 mg) or placebo as add-on therapy to methotrexate [176]. Patients receiving BI-655064 demonstrated higher ACR20/50 response rates (68.2%, 36.4%) compared to placebo (45.5%, 18.2%) at the primary endpoint (week 12). Treatment with BI-655064 also reduced the frequency of activated B cells in circulation as well as levels of total IL-6, IgM, IgG and Rheumatoid Factor autoantibodies [176]. A Phase II study was recently initiated to evaluate the efficacy of BI-665064 at three dose levels as an add-on therapy to standard of care in lupus nephritis.

5.3. Targeting CD40-CD40L: Molecules in pre-clinical development

In addition to the molecules highlighted above, there are a number of approaches to target the CD40-CD40L axis still being investigated pre-clinically. Blockade of the CD40L pathway delayed paralysis and extended survival in a mouse model of amyotrophic lateral sclerosis (ALS) [177] and an anti-CD40L monoclonal antibody (AT-1501, Anelixis Therapeutics) is currently in pre-clinical development for this indication. Several small molecule and peptide inhibitors of the CD40 pathway have also been generated [178–183]. A small molecule, BIO8898, has been described which inhibits the binding of sCD40L to CD40-Ig and blocks CD40L-mediated cellular effects in vitro [179]. A peptide inhibitor was shown to prevent hyperglycemia in NOD mice as well as reverse new-onset hyperglycemia in this preclinical setting [180]. Small interfering RNA (siRNA) approaches have also been employed pre-clinically to disrupt CD40 signaling [184,185]. Systemic delivery of siRNA to knockdown CD40 significantly reduced disease severity in a mouse model of collagen induced arthritis [185] and reduced graft inflammation in a rat model of kidney transplant [184].

6. Summary, future perspectives and outlook

Therapeutics that target the CD40-CD40L axis have the potential to broadly modulate multitude of responses influenced by this pathway, including both antibody-mediated and cellular immune processes. Much excitement around this pathway led to the development of two mAbs that were tested in the clinic and terminated due to unanticipated thrombotic complications. Novel engineering approaches have since been identified which aim to harness the centrality of this pathway for therapeutic purposes while minimizing the safety risks associated with platelet expressed CD40L. Currently, there are clinical trials ongoing in SLE, GVHD, transplant rejection, LN, FSGS and primary biliary cirrhosis that target this pathway. Future studies may bring trials in small orphan autoimmune and inflammatory conditions with great unmet need. Additionally, targeting this axis has great potential to expand beyond the autoimmune/inflammatory space, where having safe, efficacious drugs will be key.

While the CD40 pathway was initially described over 30 years ago, there are still novel aspects of its biology being uncovered. In recent years, new binding partners for CD40L, including several integrins, have been identified. While not reviewed extensively here due to limited insights into the contribution of these binding partners to the activity of CD40L, future work should focus on better defining the involvement of these interactions in the inflammatory potential of CD40L in health and disease. Identifying the contribution of alternate CD40L binding partners to disease pathogenesis could provide insight into differentiation between therapeutic targeting of CD40 versus CD40L. Additionally, more evidence continues to emerge to support a role for CD40L-mediated inflammation by non-hematopoietic cells. It will be important for future studies to explore the impact of CD40-CD40L blocking agents on both the humoral and non-humoral aspects of CD40 biology.

Conflicts of interest

JLK and RE were employees at MedImmune and owners of *AstraZeneca* stock. They are currently employees at and shareholders of Viela Bio. SR and RK are employees of MedImmune and owners of AstraZeneca stock.

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