

FUSION PROTEINS FOR USE IN TREATMENT OF THYROID EYE DISEASE

FIELD OF THE INVENTION

The present disclosure relates to methods of treatment of thyroid eye disease (TED) by administering a fusion protein, such as a fusion protein as defined herein, to subjects in need thereof. The disclosure provides treatment of pathophysiological parameters of TED, including both systemic and local pathophysiological parameters, whereby the disease is efficiently treated.

BACKGROUND OF THE INVENTION

Autoimmune diseases include a wide range of clinical disorders, which are defined by a pathologic response to self- or autoantigens and results in an immune system-mediated injury. In general, autoimmune diseases are characterized by the presence of abnormal lymphocyte (T and B cells) activation, with non-lymphoid cells also playing an important role in disease pathogenesis, deriving by a break in immune tolerance and, at least in part, resulting in the dysregulated antibody production.

Autoimmune diseases can be either systemic or tissue-specific in nature: systemic autoimmune disorders include systemic rheumatic diseases (like systemic lupus erythematosus), while tissue/organ-specific diseases include endocrine and neurologic disorders (like autoimmune thyroiditis and multiple sclerosis).

Thyroid eye disease (TED) is a painful, disabling, and disfiguring autoimmune condition commonly associated with Graves' disease (a form of autoimmune thyroiditis resulting in hyperthyroidism) and can lead to visual impairment, including diplopia and loss of vision. (Joseph SS et. Al 2015; Bartalena L. et al. 2020). TED can also present in patients with other autoimmune disorders, including Hashimoto's thyroiditis (a form of autoimmune thyroiditis resulting in hypothyroidism). (Patel A. et al 2019) TED is characterized by the presence of proptosis (bulging out of the eye bulb), that represents the major debilitation for the patients. Moderate-to-severe disease may develop in up to one-third of patients and can be sight-threatening.

The pathophysiology of TED is complex and is comprised by several parameters including both a systemic pathophysiological component and local inflammatory orbitopathy, as here described. The active phase of TED is characterized by orbital inflammation and infiltration of

different immune cells, causing extensive orbital remodeling. The triggering cause of TED is likely due to the loss of self-tolerance to thyroid stimulating hormone receptor (TSHR) and the production of stimulating autoantibodies. The orbital fibroblasts present in the retro-orbital tissue express TSHR, and the binding of this receptor by anti-TSHR antibodies leads to cell
5 activation. Thereafter, orbital fibroblasts differentiate into mature adipocytes and myofibroblasts, which increases hyaluronic acid and proinflammatory cytokine production. (Weiler DL. 2017; Jain AP. et al 2021) Histopathological findings of the orbit include extensive deposition of hyaluronan between muscle fibers leading to enlargement of extraocular muscles and widespread inflammatory infiltrate with associated interstitial oedema. (Joseph SS et al.
10 2015; Patel A. et al. 2019; Hwang CJ. et al. 2009) Local activation of these cells and production of inflammatory molecules ultimately lead to the expansion and remodeling of periorbital and orbital tissues and to diplopia and proptosis.

Another cell population playing a pivotal role in TED are the circulating fibrocytes. (Douglas RS. Et al. 2014) Fibrocytes are mesenchymal cells that arise from monocyte precursors. They
15 can infiltrate injured organs and have both the inflammatory features of macrophages and the tissue remodeling properties of fibroblasts. Chronic inflammatory stimuli mediate the differentiation, trafficking, and accumulation of fibrocytes in conditions associated with autoimmunity. (Reilkoff RA. Et al. 2011) Fibrocyte precursors occur with increased frequency in the peripheral blood of patients with TED and express both TSHR and insulin-like growth
20 factor-1 receptor (IGF-1R) and can be activated directly by the stimulating autoantibodies. Activated fibrocytes express CD40.

Cytokines, including proinflammatory and anti-inflammatory cytokines, are also important players in the pathogenesis of TED and participate in different processes, for example by contributing to the initiation and propagation of local autoimmune inflammation, which plays
25 a pivotal role in sustaining autoimmune reaction within a specific tissue.

CD40/CD40L binding has been proposed to have a role on the pathophysiology of TED. On orbital fibroblasts and infiltrating fibrocytes, CD40L binding to CD40 causes upregulation and release of pro-inflammatory cytokines (e.g. IL-6 and IL-8), which induce genes coding for
30 prostaglandin H synthase-2, hyaluronan synthase, and uridine diphosphate glucose dehydrogenase, directly resulting in hyaluronan production, orbital inflammation, and eventually tissue remodeling. (Hwang CJ. et al. 2009; Smith TJ. et al. 2008)

IGF-1R has also been described in the pathophysiology of TED. IGF-1R and TSHR have been shown to form a physically and functionally interactive complex within orbital fibroblasts, and the stimulation of IGF-1R similarly leads to the activation of TSHR and contributes to the inflammatory reaction. (Jain AP. et al 2021)

- 5 IGF-1R inhibitors have been studied for the treatment of TED, however, despite a reported reduction in e.g., proptosis upon inhibition of IGF-1R, a relapse rate of around 30% has been reported for IGF-1R inhibitors. (Couch, SM. 2022)

Other treatments for TED include the use of i.v. glucocorticoids ($\pm$ statins), mycophenolate mofetil ($\pm$ steroids), CD20 inhibitors, IL-6 receptor inhibitors, and orbital radiation. These
10 treatments are aimed at reducing systemic and local inflammation or at decreasing the titer of autoantibodies. However, these treatments have been found to have only a minor impact on proptosis and diplopia. Therefore, despite several efforts for identifying treatment of TED, challenges still exist for effective treatment options. Thus, there exists an unmet need for new efficient treatment with prolonged effect and a low relapse rate.

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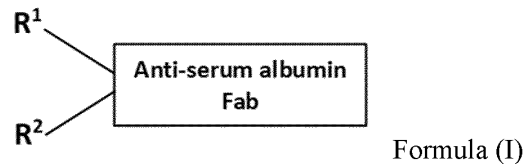
A number of biological agents targeting the CD40/CD40L axis have been developed and have shown efficacy in suppressing immune response in preclinical autoimmune disease models.

- The fusion proteins of the present disclosure have been previously described in WO2021/149015, wherein a blocking effect on the interactions between CD40L in the CD4+
20 T cell and CD40 in the B cell, resulting in selective inhibition of B cell maturation and immunoglobulin class switching was demonstrated. It was further described how the fusion proteins provided decrease in IgG antibody titer and decreased cellularity of the germinal center in the lymph nodes upon keyhole limpet hemocyanin (KLH) immune challenge in cynomolgus monkeys, proving an effect of the fusion protein on the T cell-dependent antibody response
25 (TDAR).

- Despite the proposed role of CD40/CD40L binding as part of the complex pathophysiology of TED, any potential therapeutic benefits resulting from blockade of the CD40/CD40L interaction, such as reduction of the fibrocyte induced systemic pathophysiology and/or
30 reduction in local inflammatory orbitopathy, has not been reported. Thus, it remains unknown if CD40L inhibition alone will have any effect on the various disease parameters, as described above.

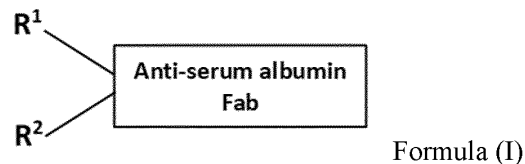
SUMMARY OF THE INVENTION

Disclosed herein are methods of treatment of thyroid eye disease (TED) comprising administering a therapeutically effective amount of a fusion protein comprising a structure according to formula (I):



to a subject in need thereof,
wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin antigen binding fragment (Fab), and
wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab.

Also disclosed herein are methods of treatment of Graves' disease comprising administering a therapeutically effective amount of a fusion protein comprising a structure according to formula (I):



to a subject in need thereof,
wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin antigen binding fragment (Fab), and
wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab.

In some embodiments, the R1 is linked to a heavy chain variable domain of the anti-serum albumin Fab, and R2 is linked to a light chain variable domain of the anti-serum albumin Fab.

In some embodiments, each of the R1 and R2 is an anti-CD40L hu5c8 scFv. In some embodiments, R1 and R2 each comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of

CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3);

5 CDR VL2: YASNLES (SEQ ID NO:4);

CDR VL3: QHSWEIPPT (SEQ ID NO:5);

CDR VH1: SYMY (SEQ ID NO:6);

CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and

CDR VH3: SDGRNDMDS (SEQ ID NO:8).

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In some embodiments, R1 and R2 each comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of QVQLVQSGAEVVKPGASVKLSCKASGYIFTSYYMYWVKQAPGQGLEWIGEINPSNG DTNFNEKFKSKATLTVDKSASTAYMELSSLRSEDVAVYYCTRS DGRNDMDSWGQG

15 TLVTVSS (SEQ ID NO:10).

In some embodiments, R1 and R2 each comprises a light chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHWYQQKPGQPPKLLIKYASNL
20 ESGVPARFSGSGSGTDFTLTISSEPEDFATYYCQHSWEIPPTFGGGTKLEIKR (SEQ ID NO:9).

In some embodiments, R1 and R2 each comprises a heavy chain variable domain comprising and amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID
25 NO:10 and a light chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:9.

In some embodiments, each of the R1 and R2 comprises an amino acid sequence having at least 80% identity to SEQ ID NO:11. In some embodiments, each of the R1 and R2 comprises
30 an amino acid sequence of SEQ ID NO:11. In some embodiments, each of the R1 and R2 comprises an amino acid sequence of SEQ ID NO:11 or SEQ ID NO:12.

In some embodiments, each of R1 and R2 is linked to the anti-serum albumin Fab by one or more linkers. In some embodiments, each linker comprises 1 to 20 amino acids. In some embodiments, each linker comprises an amino acid sequence having at least 90% identity to SEQ ID NO:23 or SEQ ID NO:24. In some embodiments, each linker comprises an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24. In some embodiments, the heavy chain variable domain and the light chain variable domain of R1 and R2 are linked by a (G4S)₃ linker comprising the amino acid sequence of SEQ ID NO:23.

In some embodiments, the anti-CD40L scFv is linked to the N-terminus of the heavy chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:23. In some embodiments, the anti-CD40L scFv is linked to the N-terminus of the light chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:24.

In some embodiments, a first anti-CD40L scFv is linked to the N-terminus of the heavy chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:23 and a second anti-CD40L scFv is linked to the N-terminus of the light chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:24.

In some embodiments, the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of
CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
CDR VL2: GASTGAT (SEQ ID NO:14);
CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3: ETVMAGKALDY (SEQ ID NO:18).

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of
QVQLVQSGGSPVQPGGSLRLSCAASGFMFRAYSMNWVRQAPGKGLEWVSSISSGR
YIHVADSVKGRFTISRDNKNSLYLQMNSLRRAEDTAVYYCARETVMAGKALDYWG
QGTLVTVSS (SEQ ID NO:19).

In some embodiments, the anti-serum albumin Fab comprises a light chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of DIVLTQSPGTLSPGETATLSCRASQSVGSNLAWYQQKPGQAPRLLIYGASTGATG VPARFSGSRSGTDFTLTITSLQPEDFATYYCQQYYSLAKTFGQGTQLEIKR (SEQ ID NO:20).

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:19 and a light chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:20.

In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:21 (VH-CH1 domain). In some embodiments, the anti-serum albumin Fab comprises a light chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:22 (VL-CL domain). In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:21 (VH-CH1 domain) and a light chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:22 (VL-CL domain).

In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:1. In some embodiments, the fusion protein comprises a light chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:2. In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:1 and a light chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:2.

In some embodiments, the fusion protein is administered in a pharmaceutical formulation. In some embodiments, the fusion protein is administered by transcutaneous, subcutaneous,

intravenous, or intramuscular administration. In some embodiments, the fusion protein is administered in an amount of about 0.01 mg/kg to about 100 mg/kg body weight of the subject.

In some embodiments, the treatment disclosed herein results in treatment of orbitopathy of TED. In some embodiments, the treatment of the orbitopathy of TED results in a reduction in inflammation in the periorbital space.

In some embodiments, the treatment disclosed herein results in treatment of systemic pathophysiology of TED. In some embodiments, the treatment of the systemic pathophysiology of TED results in deactivation of PBMCs and a decrease in cytokine expression. In some embodiments, the treatment of the systemic pathophysiology of TED results in a decrease in autologous immune cell infiltration in the periorbital space.

In some embodiments, the treatment disclosed herein results in treatment of the systemic pathophysiology of TED and in treatment of the orbitopathy of TED.

In some embodiments, the treatment results in a reduction of proptosis.

In some embodiments, the treatment results in a reduction of diplopia.

DESCRIPTION OF DRAWINGS

FIG. 1 shows the percentage change in the right ear thickness on day 8 and 9 relative to day 7 in KLH-immunized mice which were untreated (vehicle) or treated with m-Ab1 at the dose of 6.8 or 13.6 mg/kg. A decrease in ear thickness was observed over time as compared to untreated (vehicle) controls.

FIGS. 2A-2C show the levels of the pro-inflammatory cytokines TNF- α , IL-4 and IL-22 at the site of KLH-immunization in the right ear in mice which were untreated (Vehicle) or treated with m-Ab1 at the dose of 6.8 or 13.6 mg/kg. Treatment with m-Ab1 significantly reduced the levels of TNF- α compared to untreated (vehicle) controls (FIG. 2A). A reduced level was also observed for IL-4 (FIG. 2B) and IL-22 (FIG. 2C) following treatment with m-Ab1. Statistical significance: * $p < 0.05$, ** $p < 0.005$.

FIGS. 3A-3D show mRNA levels (relative expression $2^{-\Delta\Delta C_t}$) of CD40 (FIG. 3A), IL-6 (FIG. 3B), IL-8 (FIG. 3C), and RANTES (FIG. 3D) as monitored by real-time quantitative PCR (qPCR) in PBMCs which are untreated (Ctrl), treated with IFN- γ alone (IFN- γ), or treated with IFN- γ and 15 nM megaCD40L alone (15 nM CD40L), or in the presence of 3.75 or 124 nM h-Ab2 (3.75 nM h-Ab2), (124 nM h-Ab2) or in the presence of control antibody (3.75 nM Ctr-Ab), (124 nM Ctr-Ab). For statistical analysis, one-way ANOVA with Bonferroni correction was used, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

FIGS. 4A-4D show mRNA levels (relative expression $2^{-\Delta\Delta C_t}$) of CD40 (FIG. 4A), IL-6 (FIG. 4B), IL-8 (FIG. 4C), and RANTES (FIG. 4D) as monitored by real-time quantitative PCR (qPCR) in fibroblasts which are untreated (Ctrl), treated with IFN- γ alone (IFN- γ), or treated with IFN- γ and 32 nM human recombinant soluble CD40L alone (32 nM CD40L), or in the presence of 5, 135, or 1215 nM h-Ab1 (5 nM h-Ab1), (135 nM h-Ab1), (1215 nM h-Ab1) or in the presence of control antibody (5 nM Ctr-Ab), (135 nM Ctr-Ab), (1215 nM Ctr-Ab). For statistical analysis, one-way ANOVA with Bonferroni correction was used, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

FIGS. 5A-5C show mRNA levels (relative expression $2^{-\Delta\Delta C_t}$) of CD40 (FIG. 5A), IL-8 (FIG. 5B), and RANTES (FIG. 5C) as monitored by real-time quantitative PCR (qPCR) in myoblasts which are untreated (Ctrl), treated with IFN- γ alone (IFN- γ), or treated with IFN- γ and 32 nM human recombinant soluble CD40L alone (32 nM CD40L), or in the presence of 5, 135, or 1215 nM h-Ab1 (5 nM h-Ab1), (135 nM h-Ab1), (1215 nM h-Ab1) or in the presence of control antibody (5 nM Ctr-Ab), (135 nM Ctr-Ab), (1215 nM Ctr-Ab). For statistical analysis, one-way ANOVA with Bonferroni correction was used, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

FIGS. 6A-6C show mRNA levels (relative expression $2^{-\Delta\Delta C_t}$) of IL-6 (FIG. 6A), IL-8 (FIG. 6B), and RANTES (FIG. 6C) as monitored by real-time quantitative PCR (qPCR) in fibroblasts which were treated with IFN- γ and 32 nM human recombinant soluble CD40L in the presence of increasing concentrations of h-Ab1 or control antibody (0.56 nM to 3645 nM). Black closed circles: h-Ab1, grey triangles: Ctr-Ab. Data are expressed as mean $\pm$ standard error of the mean (SEM) of N=5 biological replicates, with n=2 technical replicates per experiment.

FIGS. 7A-7B shows relative protein levels of IL6 (FIG. 7A) and IL8 (FIG. 7B) as quantified by MSD assay in fibroblasts which were treated with IFN- γ and 32 nM human recombinant soluble CD40L in the presence of increasing concentrations of h-Ab1 or control antibody (0.56 nM to 3645nM). 100% is set as the protein levels found for fibroblasts stimulated with IFN- γ + CD40L alone. Black closed circles: h-Ab1, grey triangles: Ctr-Ab. Data are expressed as mean $\pm$ standard error of the mean (SEM) of N=5 biological replicates, with n=2 technical replicates per experiment.

FIGS. 8A-8D show mRNA levels (relative expression $2^{-\Delta\Delta Ct}$) of CD40 (FIG. 8A), IL-6 (FIG. 8B), IL-8 (FIG. 8C), and RANTES (FIG. 8D) as monitored by real-time quantitative PCR (qPCR) in myoblasts which were treated with IFN- γ and then 32 nM human recombinant soluble CD40L in the presence of increasing concentrations of h-Ab1 or control antibody (90 pM to 10 μ M). Black closed circles: h-Ab1, grey triangles: Ctr-Ab. Data are expressed as mean $\pm$ standard error of the mean (SEM) of N=4-5 biological replicates. For statistical analysis, one-way ANOVA with Dunnett's post-hoc test was used with 32 nM CD40L as reference ****p<0.0001.

FIGS. 9A-9B show relative protein levels of IL-6 (FIG. 9A) and IL-8 (FIG. 9B) as quantified by MSD assay in myoblasts which were treated with IFN- γ and then 32 nM human recombinant soluble CD40L in the presence of increasing concentrations of h-Ab1 or control antibody (90 pM to 10 μ M). 100% is defined as the protein levels released by myoblasts stimulated with IFN- γ + CD40L alone. Data are expressed as mean $\pm$ standard error of the mean (SEM) of N=4-5 biological replicates. For statistical analysis, one-way ANOVA with Dunnett's post-hoc test was used with 32 nM CD40L as reference ****p<0.0001.

FIGS. 10A-10B show (A) PCA plot illustrating the distinct transcriptomic profiles of PBMCs based on activation status and origin. PC1 effectively separates activated PBMCs from naive PBMCs, independent of their origin, while PC2 distinguishes between healthy individuals and those with TED, highlighting differences in their transcriptomic profiles. (B) Volcano plot displaying the DE genes between naïve TED PBMCs and healthy PBMCs. A total of 290 DE genes were identified, with 105 genes significantly downregulated and 185 genes significantly upregulated in the TED samples. Upregulated genes related to inflammatory cytokines and T-cell modulation are labelled.

FIGS. 11A-E show histograms with the percentage of different cell types following incubation of PBMCs with anti-CD3/CD28 beads for 24 hours. This incubation leads to efficient T-cell activation in both healthy and TED samples, evidenced by an increase in surface markers CD69 (C) and CD25 (A), and increased expression of CD40L (B and D), characteristic of T-effector cells, as demonstrated by FACS analysis. Additionally, a notable increase in B cells expressing CD40 is observed in TED samples, a change not seen in healthy PBMCs (E).

FIG 12 shows a heatmap illustrating transcriptional analysis of TED PBMCs following anti-CD3/CD28 stimulation. The analysis confirms broad cell activation, evidenced by increased expression of genes related to inflammation such as IFNG, TNF, IL2, IL4, IL6, and IL9, among others.

FIGS. 13A-C show (A) Violin plot showing that CD40 expression is downregulated in activated TED PBMCs following treatment with the CD40L inhibitor (h-Ab1), while unaltered following treatment with anti-TNP IgG (negative control). (B) From same samples as in A), data shown for dendritic cell released chemokines CXCL9, CXCL10, and CXCL11 post-treatment with CD40L inhibitor. (C) Volcano plot illustrating DE genes in response to CD40L inhibitor treatment. Key inflammation-associated genes, including CCL2, ACHE, and FCER2, are significantly downregulated.

DETAILED DESCRIPTION OF THE INVENTION

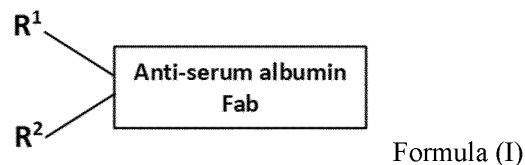
As disclosed herein, it has been determined that the fusion proteins as described herein provide effect on both the systemic autoimmune component and underlying cause of the disease as well as the local inflammatory orbitopathy component of thyroid eye disease (TED). Both of these are characterizing TED pathophysiology and treatment of both components can result in efficient treatment of TED, wherein both the symptoms of the local inflammatory orbitopathy of the disease as well as the underlying systemic cause and progression of the disease are treated.

As demonstrated by the data provided herein, the fusion proteins as described herein are able to reduce local and systemic inflammation. Specifically, a fusion protein disclosed herein was found to inhibit the production of pro-inflammatory cytokines produced by activated fibroblast and myoblasts, as well as by peripheral blood mononuclear cells (PBMC), cell populations

involved in the pathogenesis of TED. It was further found that the fusion proteins of the present disclosure enter the local inflammatory environment and demonstrate that administration of the fusion proteins result in reduction of the local inflammatory response associated with the infiltration of immune cells. Thus, administration of the fusion proteins disclosed herein can provide efficient treatment of TED by acting on both the underlying autoimmune cause of disease and the resulting local inflammatory condition resulting in symptoms of the disease. Accordingly, disclosed herein are methods of treatment of Graves' disease and/or thyroid eye disease (TED). In some embodiments, the present disclosure relates to methods of treatment of TED.

Fusion Proteins

The methods of treatment of the present disclosure relate to administration of a fusion protein comprising a structure according to formula (I):



Formula (I)

to a subject in need thereof,

wherein R^1 and R^2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and

wherein each R^1 and R^2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab.

In some embodiments, the fusion proteins of the present disclosure consist of a structure according to Formula (I), as herein defined.

The fusion proteins of the present disclosure are recombinant bispecific (scFv)₂-Fab fusion protein targeting the human CD40L and at the same time binding to human serum albumin (HSA). Binding to HSA is used to extend the half-life of the fusion proteins, as further described in previous disclosure of WO2021/149015. In some embodiments, the fusion protein is a CD40L inhibitor.

In some embodiments, R1 is linked to a heavy chain variable domain of the anti-serum albumin Fab, and R2 is linked to a light chain variable domain of the anti-serum albumin Fab.

In some embodiments, each of R1 and R2 is linked to the anti-serum albumin Fab by one or more linkers, such as wherein each linker comprises 1 to 20 amino acids.

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In some embodiments, each linker comprises an amino acid sequence having at least 90% identity to SEQ ID NO:23 or SEQ ID NO:24. In some embodiments, each linker comprises an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24. In some embodiments, each linker consists of an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24.

10 In some embodiments, the linker linking the anti-CD40L scFv to the N-terminus of the heavy chain of the anti-serum albumin Fab has an amino acid sequence of SEQ ID NO:23. In some embodiments, the linker linking the anti-CD40L scFv to the N-terminus of the light chain of the anti-serum albumin Fab has an amino acid sequence of SEQ ID NO:24.

In some embodiments, a first anti-CD40L scFv is linked to the N-terminus of the heavy chain
15 of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:23 and a second anti-CD40L scFv is linked to the N-terminus of the light chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:24.

Structure of anti-CD40L scFv

20 The anti-CD40L scFv of the fusion protein of the present disclosure provides binding of the fusion protein to CD40L and thereby inhibits binding of the CD40L target to CD40. The term single-chain variable fragment (scFv) as used herein refers to an antibody fragment composed of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a short linker peptide. The linker can either connect the N-terminus of the VH with the C-
25 terminus of the VL, or vice versa.

In some embodiments, each of the R1 and R2 is an anti-CD40L hu5c8 scFv, wherein the anti-CD40L scFv comprises the VH and VL fragments of hu5C8. The hu5C8 antibody is also known as ruplizumab (INN name) and has been assigned CAS No.:
30 220651-94-5. In some embodiments, R1 and R2 are different anti-CD40L scFvs. In some embodiments, R1 and R2 are same anti-CD40L scFvs.

In some embodiments, R1 and R2 each comprises complementarity determining domain (CDR) regions comprising the amino acid sequences having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to

- 5 CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3);
CDR VL2: YASNLES (SEQ ID NO:4);
CDR VL3: QHSWEIPPT (SEQ ID NO:5);
CDR VH1: SYMY (SEQ ID NO:6);
CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and
10 CDR VH3: SDGRNDMDS (SEQ ID NO:8).

In some embodiments, R1 and R2 each comprises heavy chain complementarity determining domain (CDR) regions comprising the amino acid sequences of

- CDR VH1: SYMY (SEQ ID NO:6);
15 CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and
CDR VH3: SDGRNDMDS (SEQ ID NO:8).

In some embodiments, R1 and R2 each comprises light chain complementarity determining domain (CDR) regions comprising the amino acid sequences of

- 20 CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3);
CDR VL2: YASNLES (SEQ ID NO:4); and
CDR VL3: QHSWEIPPT (SEQ ID NO:5).

In some embodiments, R1 and R2 each comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of

- 25 CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3);
CDR VL2: YASNLES (SEQ ID NO:4);
CDR VL3: QHSWEIPPT (SEQ ID NO:5);
CDR VH1: SYMY (SEQ ID NO:6);
30 CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and
CDR VH3: SDGRNDMDS (SEQ ID NO:8).

In some embodiments, R1 and R2 each comprises complementarity determining domain (CDR) regions consisting of the amino acid sequences of

CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3);

CDR VL2: YASNLES (SEQ ID NO:4);

5 CDR VL3: QHSWEIPPT (SEQ ID NO:5);

CDR VH1: SYMY (SEQ ID NO:6);

CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and

CDR VH3: SDGRNDMDS (SEQ ID NO:8).

In some embodiments, R1 and R2 each comprises a heavy chain variable domain comprising
10 an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to
QVQLVQSGAEVVKPGASVKLSCKASGYIFTSYYMYWVKQAPGQGLEWIGEINPSNG
DTNFNEKFKSKATLTVDKSASTAYMELSSLRSEDTAVYYCTRS DGRNDMDSWGQG
TLVTVSS (SEQ ID NO:10). In some embodiments, R1 and R2 each comprises a heavy chain
15 variable domain comprising the amino acid sequence of SEQ ID NO:10. In some embodiments, R1 and R2 each comprises a heavy chain variable domain consisting of the amino acid sequence of SEQ ID NO:10.

In some embodiments, R1 and R2 each comprises a light chain variable domain comprising an
20 amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to
DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHYQQKPGQPPKLLIKYASNL
ESGVPARFSGSGSGTDFTLTISSEPEDFATYYCQHSWEIPPTFGGGTKLEIKR (SEQ ID NO:9). In some embodiments, R1 and R2 each comprises a light chain variable domain
25 comprising the amino acid sequence of SEQ ID NO:9. In some embodiments, R1 and R2 each comprises a light chain variable domain consisting of the amino acid sequence of SEQ ID NO:9.

In some embodiments, R1 and R2 each comprises a heavy chain variable domain comprising
30 an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:10 and a light chain variable domain comprising an amino acid sequence having at least 80%, at

least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:9.

In some embodiments, R1 and R2 each comprises a heavy chain variable domain comprising an amino acid sequence of SEQ ID NO:10 and a light chain variable domain comprising an amino acid sequence of SEQ ID NO:9.

In some embodiments, R1 and R2 each comprises a heavy chain variable domain consisting of an amino acid sequence of SEQ ID NO:10 and a light chain variable domain consisting of an amino acid sequence of SEQ ID NO:9.

- 10 In some embodiments, the heavy chain variable domain and the light chain variable domain of R1 and R2 are linked by a linker, such as for example linked by a G4S linker, such as a (G4S)₃ linker comprising the amino acid sequence of SEQ ID NO:23. In some embodiments, the heavy chain variable domain and the light chain variable domain of R1 and R2 are linked by a (G4S)₃ linker consisting of the amino acid sequence of SEQ ID NO:23.
- 15 In some embodiments, the linker connects the C-terminus of the VL to the N-terminus of the VH.

- In some embodiments, each of the R1 and R2 comprises an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:11.

In some embodiments, each of the R1 and R2 comprises an amino acid sequence having at least 80% identity to SEQ ID NO:11.

- In some embodiments, each of the R1 and R2 comprises an amino acid sequence of SEQ ID NO:11 or SEQ ID NO:12. In some embodiments, each of the R1 and R2 comprises an amino acid sequence of SEQ ID NO:11. In some embodiments, each of the R1 and R2 consists of an amino acid sequence of SEQ ID NO:11.

Structure of anti-serum albumin Fab

- The anti-serum albumin Fab of the present disclosure is a Fab fragment which provides binding of the fusion protein to serum albumin. Binding to serum albumin provides extended half-life of the fusion protein.

As used herein, the term “Fab” refers to an antibody fragment that is the fragment antigen-binding region (Fab region) of an antibody, which is a region on an antibody that binds to

antigens. It is composed of one constant (C) and one variable (V) domains of each of the heavy and the light chains, such as composed of VH-CH1 from the antibody heavy chain and VL-CL from the antibody light chain.

- 5 In some embodiments, the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions comprising the amino acid sequences having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to
CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
- 10 CDR VL2: GASTGAT (SEQ ID NO:14);
CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3: ETVMAGKALDY (SEQ ID NO:18).
- 15 In some embodiments, the anti-serum albumin Fab comprises heavy chain complementarity determining domain (CDR) regions comprising the amino acid sequences of
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3 is ETVMAGKALDY (SEQ ID NO:18) or ETVAAGKALDY (SEQ ID NO:30).
- 20 In some embodiments, the anti-serum albumin Fab comprises heavy chain complementarity determining domain (CDR) regions comprising the amino acid sequences of
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3: ETVMAGKALDY (SEQ ID NO:18).
- 25 In some embodiments, the anti-serum albumin Fab comprises heavy chain complementarity determining domain (CDR) regions consisting of the amino acid sequences of
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3 is ETVMAGKALDY (SEQ ID NO:18) or ETVAAGKALDY (SEQ ID NO:30).
- 30 In some embodiments, the anti-serum albumin Fab comprises heavy chain complementarity determining domain (CDR) regions consisting of the amino acid sequences of
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and

CDR VH3: ETVMAGKALDY (SEQ ID NO:18).

In some embodiments, the anti-serum albumin Fab comprises light chain complementarity determining domain (CDR) regions comprising the amino acid sequences of

- 5 CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
CDR VL2: GASTGAT (SEQ ID NO:14); and
CDR VL3: QQYYSFLLAKT (SEQ ID NO:15).

In some embodiments, the anti-serum albumin Fab comprises light chain complementarity determining domain (CDR) regions consisting the amino acid sequences of

- 10 CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
CDR VL2: GASTGAT (SEQ ID NO:14); and
CDR VL3: QQYYSFLLAKT (SEQ ID NO:15).

In some embodiments, the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of

- 15 CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
CDR VL2: GASTGAT (SEQ ID NO:14);
CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);
CDR VH1: AYSMN (SEQ ID NO:16);
20 CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3 is ETVMAGKALDY (SEQ ID NO:18) or ETVAAGKALDY (SEQ ID NO:30).

In some embodiments, the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of

- 25 CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
CDR VL2: GASTGAT (SEQ ID NO:14);
CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3: ETVMAGKALDY (SEQ ID NO:18).

- 30 In some embodiments, the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions consisting of the amino acid sequences of

CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
CDR VL2: GASTGAT (SEQ ID NO:14);

CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3 is ETVMAGKALDY (SEQ ID NO:18) or ETVAAGKALDY (SEQ ID NO:30).

- 5 In some embodiments, the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions consisting of the amino acid sequences of

CDR VL1: RASQSVGSNLA (SEQ ID NO:13);
CDR VL2: GASTGAT (SEQ ID NO:14);
CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);

- 10 CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHVADSVKG (SEQ ID NO:17); and
CDR VH3: ETVMAGKALDY (SEQ ID NO:18).

- In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain
15 comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to
QVQLVQSGGPGVKKPGGSLRLSCAASGFMFRAYSMNWVRQAPGKGLEWVSSISSGR
YIHVADSVKGRFTISRDNKNSLYLQMNSLRAEDTAVYYCARETVMAGKALDYWG
QGTLVTVSS (SEQ ID NO:19).

- 20 In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:19 or SEQ ID NO:68.

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:19.

- In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain
25 comprising the amino acid sequence of SEQ ID NO:68.

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain consisting of the amino acid sequence of SEQ ID NO:19 or SEQ ID NO:68.

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain consisting of the amino acid sequence of SEQ ID NO:19.

- 30 In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain consisting of the amino acid sequence of SEQ ID NO:68.

- In some embodiments, the anti-serum albumin Fab comprises a light chain variable domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to DIVLTQSPGTLSPGETATLSCRASQSVGSNLA^WYQQKPGQAPRLLIYGASTGATG
- 5 VPARFSGSRSGTDFTLTITSLQPEDFATYYCQQYY^SFLAKTFGQGTQLEIKR (SEQ ID NO:20). In some embodiments, the anti-serum albumin Fab comprises a light chain variable domain comprising the amino acid sequence of SEQ ID NO:20. In some embodiments, the anti-serum albumin Fab comprises a light chain variable domain consisting of the amino acid sequence of SEQ ID NO:20.
- 10 In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:19 and a light chain variable domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%,
- 15 at least 98%, at least 99%, or 100% identity to SEQ ID NO:20. In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising the amino acid sequence of SEQ ID NO:19 and a light chain variable domain comprising the amino acid sequence of SEQ ID NO:20. In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain consisting of the amino acid sequence of SEQ ID NO:19 and a
- 20 light chain variable domain consisting of the amino acid sequence of SEQ ID NO:20.
- In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:21 (VH-CH1 domain). In some embodiments, the anti-serum albumin Fab
- 25 comprises a heavy chain domain comprising an amino acid sequence of SEQ ID NO:21 or SEQ ID NO: 69 (VH-CH1 domain). In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence of SEQ ID NO:21 (VH-CH1 domain). In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence of SEQ ID NO:69 (VH-CH1 domain). In some
- 30 embodiments, the anti-serum albumin Fab comprises a heavy chain domain consisting of an amino acid sequence of SEQ ID NO:21 or SEQ ID NO: 69 (VH-CH1 domain). In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain consisting of an amino acid sequence of SEQ ID NO:21 (VH-CH1 domain). In some embodiments, the anti-

serum albumin Fab comprises a heavy chain domain consisting of an amino acid sequence of SEQ ID NO:69 (VH-CH1 domain).

5 In some embodiments, the anti-serum albumin Fab comprises a light chain domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:22 (VL-CL domain). In some embodiments, the anti-serum albumin Fab comprises a light chain domain comprising an amino acid sequence of SEQ ID NO:22 (VL-CL domain). In some
10 embodiments, the anti-serum albumin Fab comprises a light chain domain consisting of an amino acid sequence of SEQ ID NO:22 (VL-CL domain).

In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to
15 SEQ ID NO:21 (VH-CH1 domain) and a light chain domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:22 (VL-CL domain). In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence of SEQ ID NO:21 or SEQ ID NO: 69 (VH-CH1 domain)
20 and a light chain domain comprising an amino acid sequence of SEQ ID NO:22 (VL-CL domain). In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence of SEQ ID NO:21 (VH-CH1 domain) and a light chain domain comprising an amino acid sequence of SEQ ID NO:22 (VL-CL domain). In some
25 embodiments, the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence of SEQ ID NO:69 (VH-CH1 domain) and a light chain domain comprising an amino acid sequence of SEQ ID NO:22 (VL-CL domain). In some
30 embodiments, the anti-serum albumin Fab comprises a heavy chain domain consisting of an amino acid sequence of SEQ ID NO:21 or SEQ ID NO: 69 (VH-CH1 domain) and a light chain domain consisting of an amino acid sequence of SEQ ID NO:22 (VL-CL domain). In some
embodiments, the anti-serum albumin Fab comprises a heavy chain domain consisting of an amino acid sequence of SEQ ID NO:21 (VH-CH1 domain) and a light chain domain consisting of an amino acid sequence of SEQ ID NO:22 (VL-CL domain). In some embodiments, the anti-serum albumin Fab comprises a heavy chain domain consisting of an amino acid sequence

of SEQ ID NO:69 (VH-CH1 domain) and a light chain domain consisting of an amino acid sequence of SEQ ID NO:22 (VL-CL domain).

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising

- 5 (a) a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of SYGIS (SEQ ID NO:47), a heavy chain CDR2 comprising the amino acid sequence of WINT YSGGTKYAQKF QG (SEQ ID NO:48), and a heavy chain CDR3 comprising the amino acid sequence of LGHCQRGICSDALDT (SEQ ID NO:49);
- 10 (b) a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of SYGIS (SEQ ID NO:47), a heavy chain CDR2 comprising the amino acid sequence of RINTYNGNTGYAQLQG (SEQ ID NO:50), and a heavy chain CDR3 comprising the amino acid sequence of LGHCQRGICSDALDT (SEQ ID NO:49);
- (c) a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid
15 sequence of NYGIH (SEQ ID NO:51), a heavy chain CDR2 comprising the amino acid sequence of SISYDGSNKYYADSVKG (SEQ ID NO:52), and a heavy chain CDR3 comprising the amino acid sequence of DVHYYGSGSYNAFDI (SEQ ID NO:53);
- (d) a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of SYAMS (SEQ ID NO:54), a heavy chain CDR2 comprising the amino acid
20 sequence of VISHDGGFQYYADSVKG (SEQ ID NO:55), and a heavy chain CDR3 comprising the amino acid sequence of AGWLRQYGMDV (SEQ ID NO:56);
- (e) a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of AYWIA (SEQ ID NO:57), a heavy chain CDR2 comprising the amino acid sequence of MIWPPDADARYSPSFQG (SEQ ID NO:58), and a heavy chain CDR3
25 comprising the amino acid sequence of LYSGSYSP (SEQ ID NO:59); or
- (f) a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of AYSMN (SEQ ID NO:16), a heavy chain CDR2 comprising the amino acid sequence of SISSSGRYIHYADSVKG (SEQ ID NO:17), and a heavy chain CDR3 comprising the amino acid sequence of ETVMAGKALDY (SEQ ID NO:18).

30

In some embodiments, the anti-serum albumin Fab comprises a light chain variable domain comprising

- (a) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSISSRYLN (SEQ ID NO:33), a light chain CDR2 comprising the amino acid sequence of GASRLES (SEQ ID NO:34), and a light chain CDR3 comprising the amino acid sequence of QQSDSVPVT (SEQ ID NO:35);
- 5 (b) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSISSRYLN (SEQ ID NO:36), a light chain CDR2 comprising the amino acid sequence of AASSLQS (SEQ ID NO:37), and a light chain CDR3 comprising the amino acid sequence of QQSYSTPPYT (SEQ ID NO:38);
- (c) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid
10 sequence of RASQSIFNYVA (SEQ ID NO:39), a light chain CDR2 comprising the amino acid sequence of DASNRAT (SEQ ID NO:40), and a light chain CDR3 comprising the amino acid sequence of QQRSKWPPTWT (SEQ ID NO:41);
- (d) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASETVSSRQLA (SEQ ID NO:42), a light chain CDR2 comprising the amino
15 acid sequence of GASSRAT (SEQ ID NO:43), and a light chain CDR3 comprising the amino acid sequence of QQYGSSPRT (SEQ ID NO:44);
- (e) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSVSSSSLA (SEQ ID NO:45), a light chain CDR2 comprising the amino acid sequence of GASSRAT (SEQ ID NO:43), and a light chain CDR3 comprising the amino
20 acid sequence of QKYSSYPLT (SEQ ID NO:46); or
- (f) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSVGSNLA (SEQ ID NO:13), a light chain CDR2 comprising the amino acid sequence of GASTGAT (SEQ ID NO:14), and a light chain CDR3 comprising the amino acid sequence of QQYYSFLLAKT (SEQ ID NO:15).

25

In some embodiments, the anti-serum albumin Fab comprises

- (a) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSISSRYLN (SEQ ID NO:31), a light chain CDR2 comprising the amino acid sequence of GASRLES (SEQ ID NO:32), and a light chain CDR3 comprising the amino acid
30 sequence of QQSDSVPVT (SEQ ID NO:33) and
- a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of SYGIS (SEQ ID NO:45), a heavy chain CDR2 comprising the amino acid

- sequence of WINT YSGGTKYAQKF QG (SEQ ID NO:46), and a heavy chain CDR3 comprising the amino acid sequence of LGHCQRGICSDALDT (SEQ ID NO:47);
- (b) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSISSYLN (SEQ ID NO:34), a light chain CDR2 comprising the amino acid sequence of AASSLQS (SEQ ID NO:35), and a light chain CDR3 comprising the amino acid sequence of QQSYSTPPYT (SEQ ID NO:36) and
- a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of SYGIS (SEQ ID NO:45), a heavy chain CDR2 comprising the amino acid sequence of RINTYNGNTGYAQLQG (SEQ ID NO:48), and a heavy chain CDR3 comprising the amino acid sequence of LGHCQRGICSDALDT (SEQ ID NO:47);
- (c) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSIFNYVA (SEQ ID NO:37), a light chain CDR2 comprising the amino acid sequence of DASNRAT (SEQ ID NO:38), and a light chain CDR3 comprising the amino acid sequence of QQRSKWPPTWT (SEQ ID NO:39) and
- a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of NYGIH (SEQ ID NO:49), a heavy chain CDR2 comprising the amino acid sequence of SISYDGSNKYYADSVKG (SEQ ID NO:50), and a heavy chain CDR3 comprising the amino acid sequence of DVHYYGSGSYYNAFDI (SEQ ID NO:51);
- (d) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASETVSSRQLA (SEQ ID NO:40), a light chain CDR2 comprising the amino acid sequence of GASSRAT (SEQ ID NO:41), and a light chain CDR3 comprising the amino acid sequence of QQYGSSPRT (SEQ ID NO:42) and
- a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of SYAMS (SEQ ID NO:52), a heavy chain CDR2 comprising the amino acid sequence of VISHDGGFQYYADSVKG (SEQ ID NO:53), and a heavy chain CDR3 comprising the amino acid sequence of AGWLRQYGMDV (SEQ ID NO:54);
- (e) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSVSSSSLA (SEQ ID NO:43), a light chain CDR2 comprising the amino acid sequence of GASSRAT (SEQ ID NO:41), and a light chain CDR3 comprising the amino acid sequence of QKYSSYPLT (SEQ ID NO:44) and
- a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of AYWIA (SEQ ID NO:55), a heavy chain CDR2 comprising the amino acid

sequence of MIWPPDADARYSPSFQG (SEQ ID NO:56), and a heavy chain CDR3 comprising the amino acid sequence of LYSGSYSP (SEQ ID NO:57); or
(f) a light chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of RASQSVGSNLA (SEQ ID NO:13), a light chain CDR2 comprising the amino acid sequence of GASTGAT (SEQ ID NO:14), and a light chain CDR3 comprising the amino acid sequence of QQYYSFLAKT (SEQ ID NO:15) and
a heavy chain complementarity determining domain 1 (CDR1) comprising the amino acid sequence of AYSMN (SEQ ID NO:16), a heavy chain CDR2 comprising the amino acid sequence of SISSSGRYIHVADSVKG (SEQ ID NO:17), and a heavy chain CDR3 comprising
the amino acid sequence of ETVMAGKALDY (SEQ ID NO:18).

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to SEQ ID NO:63, 64, 65, 66, 67, 68, or 19. In some embodiments, the anti-serum albumin Fab comprises a light chain variable domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to SEQ ID NO:58, 59, 60, 61, 62, or 20.

In some embodiments, the anti-serum albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to SEQ ID NO:63, 64, 65, 66, 67, 68, or 19, and a light chain variable domain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% to SEQ ID NO:58, 59, 60, 61, 62, or 20, respectively.

Structure of Fusion protein

In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:1. In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence of SEQ ID NO:1 or SEQ ID NO: 29. In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence of SEQ ID NO:1. In some embodiments, the

fusion protein comprises a heavy chain comprising an amino acid sequence of SEQ ID NO:29. In some embodiments, the fusion protein comprises a heavy chain consisting of an amino acid sequence of SEQ ID NO:1 or SEQ ID NO: 29. In some embodiments, the fusion protein comprises a heavy chain consisting of an amino acid sequence of SEQ ID NO:1. In some
5 embodiments, the fusion protein comprises a heavy chain consisting of an amino acid sequence of SEQ ID NO:29.

In some embodiments, the fusion protein comprises a light chain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least
10 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:2. In some embodiments, the fusion protein comprises a light chain comprising an amino acid sequence of SEQ ID NO:2. In some embodiments, the fusion protein comprises a light chain consisting of an amino acid sequence of SEQ ID NO:2.

15 In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to SEQ ID NO:1 and a light chain comprising an amino acid sequence having at least 80%, at least 85%, at least 90%, at least 93%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity
20 to SEQ ID NO:2. In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence of SEQ ID NO:1 or SEQ ID NO: 29 and a light chain comprising an amino acid sequence of SEQ ID NO:2.

In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence of SEQ ID NO:1 and a light chain comprising an amino acid sequence of SEQ ID
25 NO:2. In some embodiments, the fusion protein comprises a heavy chain comprising an amino acid sequence of SEQ ID NO:29 and a light chain comprising an amino acid sequence of SEQ ID NO:2. In some embodiments, the fusion protein comprises a heavy chain consisting of an amino acid sequence of SEQ ID NO:1 or SEQ ID NO: 29 and a light chain consisting of an amino acid sequence of SEQ ID NO:2. In some embodiments, the fusion protein comprises a
30 heavy chain consisting of an amino acid sequence of SEQ ID NO:1 and a light chain consisting of an amino acid sequence of SEQ ID NO:2. In some embodiments, the fusion protein comprises a heavy chain consisting of an amino acid sequence of SEQ ID NO:29 and a light chain consisting of an amino acid sequence of SEQ ID NO:2.

As used herein, the terms “variable region” and “variable domain” are used interchangeably and are common in the art. The variable region typically refers to a portion of an antibody, generally, a portion of a light or heavy chain, typically about the amino-terminal 110 to 120 amino acids in the mature heavy chain and about 90 to 115 amino acids in the mature light chain, which differ extensively in sequence among antibodies and are used in the binding and specificity of a particular antibody for its particular antigen. The variability in sequence is concentrated in those regions called complementarity determining regions (CDRs) while the more highly conserved regions in the variable domain are called framework regions (FR). Without wishing to be bound by any particular mechanism or theory, it is believed that the CDRs of the light and heavy chains are primarily responsible for the interaction and specificity of the antibody with antigen.

The terms “VL” and “VL domain” are used interchangeably to refer to the light chain variable region of an antibody. The terms “VH” and “VH domain” are used interchangeably to refer to the heavy chain variable region of an antibody.

The term “Kabat numbering” and like terms are recognized in the art and refer to a system of numbering amino acid residues in the heavy and light chain variable regions of an antibody, or an antigen-binding portion thereof. In certain aspects, the CDRs of an antibody can be determined according to the Kabat numbering system (see, e.g., Kabat EA & Wu TT (1971) Ann NY Acad Sci 190: 382-391 and Kabat EA et al., (1991) Sequences of Proteins of Immunological Interest, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). In some embodiments, the CDRs of the antibodies described herein have been determined according to the Kabat numbering scheme.

In some embodiments, the position of one or more CDRs along the VH (e.g., CDR1, CDR2, or CDR3) and/or VL (e.g., CDR1, CDR2, or CDR3) region of an antibody described herein can vary by one, two, three, four, five, or six amino acid positions so long as immunospecific binding to an antigen is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). For example, the position defining a CDR of an antibody described herein can vary by shifting the N-terminal and/or C-terminal boundary of the CDR by one, two, three, four, five, or six amino acids, relative to the CDR position of a multispecific antibody described herein, so long as immunospecific binding to the antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at

least 60%, at least 70%, at least 80%, at least 90%, at least 95%). In other embodiments, the length of one or more CDRs along the VH (e.g., CDR1, CDR2, or CDR3) and/or VL (e.g., CDR1, CDR2, or CDR3) region of an antibody described herein can vary (e.g., be shorter or longer) by one, two, three, four, five, or more amino acids, so long as immunospecific binding to the antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). [0106] In some embodiments, a VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and/or VH CDR3 described herein can be one, two, three, four, five or more amino acids shorter than one or more of the CDRs described herein so long as immunospecific binding to the antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). In other embodiments, a VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and/or VH CDR3 described herein can be one, two, three, four, five or more amino acids longer than one or more of the CDRs described herein so long as immunospecific binding to the antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). In other embodiments, the amino terminus of a VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and/or VH CDR3 described herein can be extended by one, two, three, four, five or more amino acids compared to one or more of the CDRs described herein so long as immunospecific binding to the antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). In other embodiments, the carboxy terminus of a VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and/or VH CDR3 described herein can be extended by one, two, three, four, five or more amino acids compared to one or more of the CDRs described herein so long as immunospecific binding to the antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). In other embodiments, the amino terminus of a VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and/or VH CDR3 described herein can be shortened by one, two, three, four, five or more amino acids compared to one or more of the CDRs described herein so long as immunospecific binding to the antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). In some embodiments, the carboxy terminus of a VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and/or VH CDR3 described herein can be shortened by one, two, three, four, five or more amino acids compared to one or more of the CDRs described herein so long as immunospecific binding to the

antigen(s) is maintained (e.g., substantially maintained, for example, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%). Any method known in the art can be used to ascertain whether immunospecific binding to the antigen(s) is maintained.

- The determination of percent identity between two sequences (e.g., amino acid sequences or
5 nucleic acid sequences) can also be accomplished using a mathematical algorithm. A specific, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin S & Altschul SF (1990) PNAS 87: 2264-2268, modified as in Karlin S & Altschul SF (1993) PNAS 90: 5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul SF et al., (1990) J Mol Biol 215: 403.
- 10 BLAST nucleotide searches can be performed with the NBLAST nucleotide program parameters set, e.g., for score=100, wordlength=12 to obtain nucleotide sequences homologous to nucleic acid molecules described herein. BLAST protein searches can be performed with the XBLAST program parameters set, e.g., to score 50, wordlength=3 to obtain amino acid sequences homologous to a protein molecule described herein. To obtain gapped alignments
15 for comparison purposes, Gapped BLAST can be utilized as described in Altschul SF et al., (1997) Nuc Acids Res 25: 3389 3402. Alternatively, PSI BLAST can be used to perform an iterated search which detects distant relationships between molecules (Id). When utilizing BLAST, Gapped BLAST, and PSI Blast programs, the default parameters of the respective programs (e.g., of XBLAST and NBLAST) can be used (see, e.g., National Center for
20 Biotechnology Information (NCBI) on the worldwide web, ncbi.nlm.nih.gov). Another specific, nonlimiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4:11 17. Such an algorithm is incorporated in the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a
25 PAM 120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. The percent (%) identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.
- 30 As used herein, a “conservative amino acid substitution” is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid,

glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). In certain embodiments, one or more amino acid residues within a CDR(s) or within a framework region(s) of an antibody can be replaced with an amino acid residue with a similar side chain.

Fusion proteins disclosed herein can be produced by any method known in the art for the synthesis of antibodies, for example, by chemical synthesis or by recombinant expression techniques, such as by the methods disclosed in WO2021/149015, incorporated herein by reference in its entirety. Suitable methods include conventional techniques in molecular biology, microbiology, genetic analysis, recombinant DNA, organic chemistry, biochemistry, PCR, oligonucleotide synthesis and modification, nucleic acid hybridization, and related fields within the skill of the art.

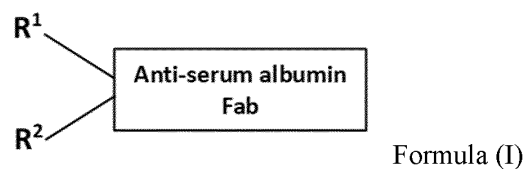
In some embodiments, the fusion proteins are administered in a pharmaceutical formulation, such as but not limited to, in a liquid pharmaceutical formulation. The pharmaceutical compositions can be formulated with pharmaceutically acceptable excipients in accordance with conventional techniques such as those disclosed in Remington, "The Science and Practice of Pharmacy", 22nd edition (2012), Edited by Allen, Loyd V., Jr. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed. The fusion proteins can be administered by administration routes known to the medical practitioner, such as be administered via transcutaneous, subcutaneous, intravenous, or intramuscular administration routes. In one embodiment, the fusion protein is administered via intravenous route.

In some embodiments, the fusion proteins are administered to a subject at a dose of about 0.01 to about 100 mg/kg of body weight of the recipient subject. The typical daily dosage might range from about 0.01 mg/kg to about 100 mg/kg or more, depending on several factors, e.g., the particular mammal being treated, the clinical condition of the individual subject, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

Methods of Treatment

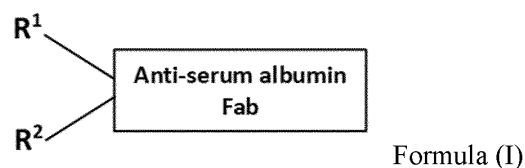
Disclosed herein are methods of treatment of Graves' disease and/or thyroid eye disease (TED) in subjects in need thereof. In some embodiments, the present disclosure relates to methods of treatment of TED in subjects in need thereof. TED may also be referred to as thyroid-associated ophthalmopathy (TAO) or Graves' ophthalmopathy (GO). In some embodiments, the present disclosure relates to methods of treatment of Graves' disease in subjects in need thereof.

In some embodiments, the methods of the present disclosure relate to administration of a fusion protein comprising a structure according to formula (I):



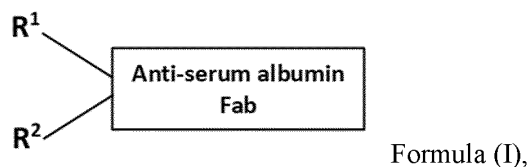
to a subject in need thereof,
wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and
wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab.

In some embodiments, the methods of the present disclosure relate to administration of a fusion protein of formula (I):



to a subject in need thereof,
wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and
wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab.

In some embodiments, the present disclosure relates to fusion proteins comprising a structure according to formula (I):



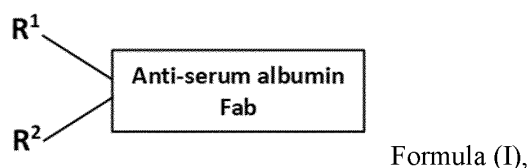
5

wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and

wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab

10 for use in the treatment of TED.

In some embodiments, the present disclosure relates to fusion proteins of formula (I):



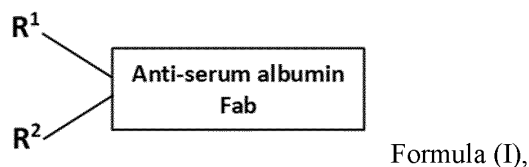
wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and

15

wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab

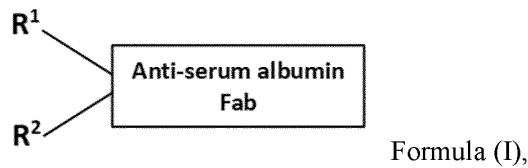
for use in the treatment of TED.

20 In some embodiments, the present disclosure relates to use of fusion proteins comprising a structure according to formula (I):



wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and
wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab
5 for the manufacture of a medicament for the treatment of TED.

In some embodiments, the disclosure relates to use of fusion proteins of formula (I):



wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an
10 N-terminus of the anti-serum albumin Fab, and
wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab
for the manufacture of a medicament for the treatment of TED.

15 In some embodiments, the subjects suffer from TED and Graves' disease. In some
embodiments, the subjects suffer from TED and Hashimoto's thyroiditis. In some
embodiments, the subject suffering from TED is diagnosed with Graves' disease. In some
embodiments, the subject suffering from TED is diagnosed with Hashimoto's thyroiditis. In
some embodiments, the method of the present disclosure relates to treatment of a TED patient
20 who has a co-morbid Graves' disease disorder. In some embodiments, the method of the
present disclosure relates to treatment of a TED patient who has a co-morbid Hashimoto's
thyroiditis disorder.

In some embodiments, the subjects suffer from acute or chronic TED. In some embodiments,
25 the subject is suffering from acute TED. A subject suffering from acute TED refers to a patient
wherein the disease progression is evolving with increasing symptoms and severity and has not
yet reached a plateau, according to the Rundle's curve of TED disease progression, such as a
patient with ophthalmologic symptom onset, e.g., less than 6 to less than 24 months ago or less
than 12 months ago. In some embodiments, acute TED refers to ophthalmologic symptom onset
30 from 0 to 18 months ago, from 6 to 18 months ago, from 6 to 15 months ago, from 6 to 12

months ago, or from 0 to 12 months ago. In some embodiments, the subject is suffering from chronic TED. Chronic TED refers to a state in the disease where disease progression has reached a plateau with no major changes in severity, according to the Rundle's curve of TED disease progression.

5

In some embodiments, the subject is suffering from active TED. Activity of TED can be assessed by methods known to the skilled person, such as by assessing different inflammation parameters, e.g., spontaneous retrobulbar pain, gaze evoked orbital pain, redness of the eyelids, redness of the conjunctiva, swelling of the eyelids, inflammation of the caruncle and/or plica, and/or conjunctival oedema. Activity measures of TED can be presented in a clinical activity score (CAS) based on the listed inflammation parameters wherein 1 point is appointed for each parameter present. In some embodiments, active TED can be represented by a CAS score of equal to or higher than 3 on a 7-point CAS scale, such as a CAS score of 3 to 7. (Burch et al. 2022). In some embodiments, the subject is suffering from active TED and has a CAS score of 3 or higher, such as a CAS score of 4 or higher, for example a CAS score of 5 or higher, such as a CAS score of 6 or higher, for example a CAS score of 7. In some embodiments, the subject is suffering from inactive TED, such as has a CAS score of equal to or lower than 2 on a 7-point CAS scale.

20 In some embodiments, the subject is suffering from moderate-to-severe TED. Severity of TED can be assessed by methods known to the skilled person, such as by assessing different severity measures, e.g., lid aperture, swelling of the eyelids, redness of the eyelids, redness of the conjunctiva, conjunctiva oedema, inflammation of the caruncle or plica, proptosis, diplopia, eye muscle involvement, corneal involvement, and/or optic nerve involvement (Burch et al. 25 2022). In some embodiments, the subject is suffering from active, moderate to severe TED.

In some embodiments, the subject treated is euthyroid or has only mild hypo- or hyperthyroidism, such as having free triiodothyronine (FT3) and free thyroxine (FT4) levels not differing from the normal limits by more than +/-50%.

30

Disclosed herein are methods of treatment of TED comprising administering a therapeutically effective amount of a fusion protein as disclosed herein to a subject in need thereof.

In some embodiments, the treatment results in treatment of orbitopathy of TED. In some embodiments, the treatment of orbitopathy of TED results in a reduction in inflammation in the periorbital space, such as but not limited to, deactivation of orbital fibroblasts and/or myoblasts resulting in decreased cytokine expression.

- 5 In some embodiments, the treatment results in treatment of systemic pathophysiology of TED. In some embodiments, the treatment of the systemic pathophysiology of TED results in deactivation of PBMCs and decreased cytokine expression, resulting in a reduction in autologous immune cell infiltration in periorbital space.

- 10 In some embodiments, the treatment results in treatment of the systemic pathophysiology of TED and in treatment of the orbitopathy of TED.

- In some embodiments, the treatment results in a reduction of proptosis, such as but not limited to a reduction of proptosis of at least 2 mm. In some embodiments, the treatment results in a reduction of proptosis of at least 2 mm, at least 2.5 mm, at least 3 mm, at least 4 mm, at least 5 mm, or any ranges therein. In some embodiments, the treatment results in maximum proptosis reduction, wherein positioning of the eye-bulb returns to the pre-TED positioning of the eye-bulb, such as returns to the positioning corresponding to the standard positioning with respect to gender and ethnicity. In some embodiments, the treatment results in a reduction of proptosis in the range of 2 mm to maximum proptosis reduction, such as in the range of 3 mm to maximum proptosis reduction, for example in the range of 4 mm to maximum proptosis reduction, such as in the range of 5 mm to maximum proptosis reduction. In some embodiments, the reduction in proptosis is achieved following 6, 12, or 24 weeks of treatment. In some embodiments, the treatment results in a reduction of diplopia.

- 25 In some embodiments, the treatment results in a reduction in CAS score, such as a reduction in CAS score of 1, of 2, of 3, of 4, or of 5 on the 7-point CAS scale. In some embodiments, the treatment results in a reduction of at least 1 in CAS score, such as a reduction of at least 2, of at least 3, of at least 4, of at least 5 in CAS score on the 7-point CAS scale. In some embodiments, the treatment results in reduction in eyelid retraction. In some embodiments, the treatment results in a reduction of volume of the extra-orbital muscles and/or a reduction of volume of the extra-orbital fat tissue.

In some embodiments, the treatment results in a reduction in autoantibodies, such as a reduction in thyroid stimulating hormone-receptor auto antibodies (TSH-R-Ab), anti-thyroid peroxidase antibodies (TPO-Ab), and/or anti-thyroglobulin antibodies (Tg-Ab). In some embodiments, the treatment results in a reduction of thyroid dysfunction, such as a reduction in free triiodothyronine (FT3), free thyroxin (FT4), and/or thyroid stimulating hormone (TSH).

In some embodiments, the treatment results in an increase in Graves' Ophthalmopathy Quality of Life (GO-QOL) score, such as an increase in one or both of the sub-scores of GO-QOL of 1. measuring the consequences of double vision and decreased visual acuity on visual functioning, and 2. measuring the psychosocial consequences of a changed appearance (Terwee et al. 1998).

In some embodiments, the administration of the fusion protein results in treatment of the orbitopathy of TED. In some embodiments, the administration of the fusion protein results in reduction of orbital inflammation, such as reduction of the T cell-mediated local inflammation. In some embodiments, the administration of the fusion protein results in reduction of the inflammatory response of myoblasts and/or fibroblasts. In some embodiments, the administration of the fusion protein results in reduction of cytokine expression in the periorbital space, such as reduction of TNF- α , IL-4, IL-22, IL-6, IL-8, and/or RANTES expression in the periorbital space. In some embodiments, the administration of the fusion protein results in reduction of cytokine expression in the orbital fibroblasts and/or myoblasts, such as results in reduction of IL-6, IL-8, and/or RANTES expression in the orbital fibroblasts and/or myoblasts. In some embodiments, the treatment results in reduction in hyaluronic acid and/or GAG production in the periorbital space, ultimately resulting in a reduction or prevention of fibrosis. In some embodiments, the treatment results in a reduction of orbital fibroblasts differentiation into mature adipocytes and myofibroblasts, ultimately resulting in a reduction or prevention of hyaluronic acid and proinflammatory cytokine production.

In some embodiments, the administration of the fusion protein is resulting in treatment of the systemic pathophysiology. In some embodiments, the administration of the fusion protein results in a reduction in the activation of PBMCs, such as B cells, monocytes, dendritic cells and fibrocytes. In some embodiments, the treatment results in a reduction in the inflammatory response of PBMCs such as B cells, monocytes, dendritic cells and fibrocytes of the subject,

such as reduces inflammatory response in fibrocytes. In some embodiments, the treatment results in a reduction of activation and/or inflammatory response of PBMCs, such as reduction of IL-6, IL-8, and/or RANTES expression in the PBMCs. In some embodiments, the treatment result in a reduced CD40 expression in the PBMCs. In one embodiment, treatment results in
5 reduced CXCL9, CXCL10, and/or CXCL11 expression in dendritic cells. In one embodiment, treatment results in downregulation of inflammation-associated genes, such as downregulation of CCL2, ACHE, and/or FCER2 genes in PBMCs. Reduction of PBMC activation and inflammatory response can further result in reduction of infiltration of these activated PBMCs and/or cytokines therefrom in the orbital cavity, ultimately resulting in a reduced activation of
10 orbital fibroblasts and resulting local inflammation. In some embodiments, the administration of the fusion protein results in reduction and/or prevention of autologous immune cell infiltration in the periorbital space. In some embodiments, the administration of the fusion protein results in reduction of anti-TSHR autoantibodies and/or anti-IGF1R autoantibodies.

15 In some embodiments, the administration of the fusion protein is resulting in treatment of the systemic pathophysiology and of the orbitopathy of TED, such as resulting in treatment of the autoimmune component and in treatment of inflammation in the periorbital tissue of TED. In some embodiments, the administration of the fusion protein results in treatment of the underlying autoimmune condition to which the TED of the subject is associated, such as results
20 in treatment of the underlying Graves' disease or results in treatment of the underlying Hashimoto's thyroiditis to which the TED is associated.

Without being bound by theory, treatment of the systemic pathophysiology and of the orbitopathy of TED result in improvement of symptomatology and progression of TED,
25 thereby resulting in efficient treatment with low relapse rates. In some embodiments, the treatment results in no relapse of disease in the treated subject following the last dose, such as no relapse 28 weeks following the last dose.

In some embodiments, the treatment results in a reduction of proptosis, such as but not limited
30 to a reduction of proptosis of at least 2 mm as compared to baseline proptosis pre-treatment. The term proptosis (also known as exophthalmos) as used herein refers to the protrusion of the eye bulb. Proptosis can be evaluated using different methods known to the skilled person, such

as for example through measurement using a Hertel exophthalmometer or through measurements using magnetic resonance imaging (MRI).

In some embodiments, the treatment results in reduction of diplopia. In some embodiments, the treatment results in reduction of proptosis and diplopia. The term diplopia as used herein refers to double vision causing the subject to see two images potentially due to ocular misalignment. Diplopia can be evaluated by methods known to the skilled person, such as by the Bahn-Gorman scale (Grade 0-IV) or the Gorman score (Grade 0-III). Reduction of diplopia referred to herein can be a reduction of 1 or greater on the Bahn-Gorman scale or Gorman score, such as a reduction of 1 or 2 on the Bahn-Gorman scale or Gorman score. In one embodiment, reduction of diplopia result in Grade 0 (no diplopia) to II (inconstant diplopia) diplopia according to the Bahn-Gorman scale or Gorman score, such as a Grade II (inconstant diplopia) or lower according to the Bahn-Gorman scale or Gorman score, for example Grade I (intermittent diplopia) or lower according to the Bahn-Gorman scale or Gorman score, such as a Grade 0 (no diplopia) according to the Bahn-Gorman scale or Gorman score.

In some embodiments, the present disclosure relates to methods of treatment of Graves' disease comprising administering a therapeutically effective amount of a fusion protein as disclosed herein to a subject in need thereof. In some embodiments, the present disclosure relates to fusion proteins as disclosed herein for use in the treatment of Graves' disease. In some embodiments, the present disclosure relates to use of a fusion protein as disclosed herein for the manufacture of a medicament for the treatment of Graves' disease.

As used herein, the term "therapeutically effective amount" of a compound means an amount sufficient to alleviate, arrest, partly arrest, remove, ameliorate or delay the clinical manifestations of a given disease and its complications in a therapeutic intervention comprising the administration of said compound. An amount adequate to accomplish this is defined as a "therapeutically effective amount". Effective amounts for each purpose will depend on the severity of the disease or injury as well as the weight and general state of the subject.

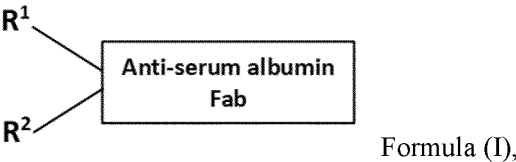
As used herein, the term "treatment" or "treating" is intended to indicate the management and care of a subject for the purpose of alleviating, arresting, partly arresting, removing, reducing, decreasing, ameliorating or delaying progress or prevention of the clinical manifestation of the

disease compared to a subject without the treatment disclosed herein. The subject to be treated can be a mammal, in particular a human or a nonhuman mammal.

As used herein, the terms “subject” and “patient” are used interchangeably. The subject can be an animal. In some embodiments, the subject is a mammal such as a non-primate (e.g., cow, pig, horse, cat, dog, rat, etc.) or a primate (e.g., monkey or human), or a human. In some embodiments, the subject is a cynomolgus monkey or a marmoset. In certain embodiments, such terms refer to a non-human animal (e.g., a non-human animal such as a pig, horse, cow, cat, or dog). In some embodiments, such terms refer to a pet or farm animal. In specific embodiments, such terms refer to a human.

Fusion Proteins

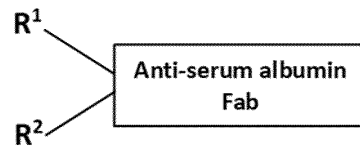
Disclosed herein are fusion proteins comprising a structure according to formula (I):



wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab;
wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab, and
wherein R1 and R2 each comprises complementarity determining domain (CDR) regions of CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3); CDR VL2: YASNLES (SEQ ID NO:4); CDR VL3: QHSWEIPPT (SEQ ID NO:5); CDR VH1: SYMY (SEQ ID NO:6); CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and CDR VH3: SDGRNDMDS (SEQ ID NO:8), and
wherein the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions of CDR VL1: RASQSVGSNLA (SEQ ID NO:13); CDR VL2: GASTGAT (SEQ ID NO:14); CDR VL3: QQYYSFLAKT (SEQ ID NO:15); CDR VH1: AYSMN (SEQ ID NO:16); CDR VH2: SISSSGRYIHYADSVK (SEQ ID NO:17); and CDR VH3 ETVAAGKALDY (SEQ ID NO:30).

Numbered embodiments

E1. A method of treatment of thyroid eye disease (TED) comprising administering a therapeutically effective amount of a fusion protein comprising a structure according to formula (I):



Formula (I)

to a subject in need thereof,

wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and

wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab.

E2. The method according to embodiment E1, wherein R1 is linked to a heavy chain variable domain of the anti-serum albumin Fab, and wherein R2 is linked to a light chain variable domain of the anti-serum albumin Fab.

E3. The method according to any one of the preceding embodiments, wherein each of the R1 and R2 is an anti-CD40L hu5c8 scFv.

E4. The method according to any one of the preceding embodiments, wherein R1 and R2 each comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of

CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3);

CDR VL2: YASNLES (SEQ ID NO:4);

CDR VL3: QHSWEIPPT (SEQ ID NO:5);

CDR VH1: SYMY (SEQ ID NO:6);

CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and

CDR VH3: SDGRNDMDS (SEQ ID NO:8).

E5. The method according to any one of the preceding embodiments, wherein R1 and R2 each comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of

QVQLVQSGAEVVKPGASVKLSCKASGYIFTSYYMYWVKQAPGQGLEWIGEINPSNG
5 DTNFNEKFKSKATLTVDKSASTAYMELSSLRSEDVAVYYCTRSDGRNDMDSWGQG
TLVTVSS (SEQ ID NO:10).

E6. The method according to any one of the preceding embodiments, wherein R1 and R2 each comprises a light chain variable domain comprising an amino acid sequence having at least
10 80% identity to the amino acid sequence of

DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHWYQQKPGQPPKLLIKYASNL
ESGVPARFSGSGSGTDFLTITSSVEPEDFATYYCQHSWEIPPTFGGGTKLEIKR (SEQ ID
NO:9).

15 E7. The method according to any one of the preceding embodiments, wherein R1 and R2 each comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:10 and a light chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of
20 SEQ ID NO:9.

E8. The method according to any one of the preceding embodiments, wherein the heavy chain variable domain and the light chain variable domain of R1 and R2 are linked by a (G4S)₃ linker comprising the amino acid sequence of SEQ ID NO:23.

25 E9. The method according to any one of the preceding embodiments, wherein each of the R1 and R2 comprises an amino acid sequence having at least 80% identity to SEQ ID NO:11.

E10. The method according to any one of the preceding embodiments, wherein each of the R1 and R2 comprises an amino acid sequence of SEQ ID NO:11 or SEQ ID NO:12.

30 E11. The method according to any one of the preceding embodiments, wherein each of the R1 and R2 comprises an amino acid sequence of SEQ ID NO:11.

E12. The method according to any one of the preceding embodiments, wherein each of R1 and R2 is linked to the anti-serum albumin Fab by one or more linkers.

5 E13. The method according to embodiment E12, wherein each linker comprises 1 to 20 amino acids.

E14. The method according to any one of embodiments E12 and E13, wherein each linker comprises an amino acid sequence having at least 90% identity to SEQ ID NO:23 or SEQ ID NO:24.
10

E15. The method according to any one of embodiments E12 to E14, wherein each linker comprises an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24.

15 E16. The method according to any one of the preceding embodiments, wherein the anti-CD40L scFv is linked to the N-terminus of the heavy chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:23.

E17. The method according to any one of the preceding embodiments, wherein the anti-CD40L scFv is linked to the N-terminus of the light chain of the anti-serum albumin Fab by a linker
20 having an amino acid sequence of SEQ ID NO:24.

E18. The method according to any one of the preceding embodiments, wherein a first anti-CD40L scFv is linked to the N-terminus of the heavy chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:23 and a second anti-CD40L scFv is
25 linked to the N-terminus of the light chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:24.

E19. The method according to any one of the preceding embodiments, wherein the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions comprising the
30 amino acid sequences of

CDR VL1: RASQSVGSNLA (SEQ ID NO:13);

CDR VL2: GASTGAT (SEQ ID NO:14);

CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);

CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHYADSVKG (SEQ ID NO:17); and
CDR VH3: ETVMAGKALDY (SEQ ID NO:18).

5 E20. The method according to any one of the preceding embodiments, wherein the anti-serum
albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence
having at least 80% identity to the amino acid sequence of
QVQLVQSGGPGVPKPGGSLRLSCAASGFMFRAYSMNWVRQAPGKGLEWVSSISSSGR
YIHYADSVKGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCARETVMAGKALDYWG
10 QGTLVTVSS (SEQ ID NO:19).

E21. The method according to any one of the preceding embodiments, wherein the anti-serum
albumin Fab comprises a light chain variable domain comprising an amino acid sequence
having at least 80% identity to the amino acid sequence of
15 DIVLTQSPGTLSPGETATLSCRASQSVGSNLAWYQQKPGQAPRLLIYGASTGATG
VPARFSGSRSGTDFTLTITSLQPEDFATYYCQQYYSFLAKTFGQGTQLEIKR (SEQ ID
NO:20).

E22. The method according to any one of the preceding embodiments, wherein the anti-serum
20 albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence
having at least 80% identity to the amino acid sequence of SEQ ID NO:19 and a light chain
variable domain comprising an amino acid sequence having at least 80% identity to the amino
acid sequence of SEQ ID NO:20.

25 E23. The method according to any one of the preceding embodiments, wherein the anti-serum
albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at
least 80% identity to the amino acid sequence of SEQ ID NO:21 (VH-CH1 domain).

E24. The method according to any one of the preceding embodiments, wherein the anti-serum
30 albumin Fab comprises a light chain domain comprising an amino acid sequence having at least
80% identity to the amino acid sequence of SEQ ID NO:22 (VL-CL domain).

E25. The method according to any one of the preceding embodiments, wherein the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:21 (VH-CH1 domain) and a light chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:22 (VL-CL domain).

E26. The method according to any one of the preceding embodiments, wherein the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:1.

E27. The method according to any one of the preceding embodiments, wherein the fusion protein comprises a light chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:2.

E28. The method according to any one of the preceding embodiments, wherein the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:1 and a light chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:2.

E29. The method according to any one of the preceding embodiments, wherein the fusion protein is administered in a pharmaceutical formulation.

E30. The method according to any one of the preceding embodiments, wherein the fusion protein is administered by transcutaneous, subcutaneous, intravenous, or intramuscular administration.

E31. The method according to any one of the preceding embodiments, wherein the fusion protein is administered in an amount of about 0.01 mg/kg to about 100 mg/kg body weight of the subject.

E32. The method according to any one of the preceding embodiments, wherein the treatment results in treatment of orbitopathy of TED.

- E33. The method according to embodiment E32, wherein the treatment of the orbitopathy of TED results in a reduction in inflammation in the periorbital space.
- E34. The method according to any one of the preceding embodiments, wherein the treatment
5 results in treatment of systemic pathophysiology of TED.
- E35. The method according to embodiment E34, wherein the treatment of the systemic pathophysiology of TED results in deactivation of PBMCs and a decrease in cytokine expression.
- 10 E36. The method according to embodiment E34, wherein the treatment of the systemic pathophysiology of TED results in a decrease in autologous immune cell infiltration in the periorbital space.
- 15 E37. The method according to any one of the preceding embodiments, wherein the treatment results in treatment of the systemic pathophysiology of TED and in treatment of the orbitopathy of TED.
- E38. The method according to any one of the preceding embodiments, wherein the treatment
20 results in a reduction of proptosis.
- E39. The method according to any one of the preceding embodiments, wherein the treatment results in a reduction of diplopia.

25

Table 1. Sequences

SEQ ID NO:	Sequence	Description
1	DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHW YQQKPGQPPKLLIKYASNLESGVPARFSGSGSGTDFTLT ISSVEPEDFATYYCQHSWEIPPTFGGGTKLEIKRGGGGS GGGSGGGGSQVQLVQSGAEVVKPGASVKLSCKASG YIFTSYYMYWVKQAPGQGLEWIGEINPSNGDTNFK	CD40L inhibitor (h-Ab1) heavy chain

	KSKATLTVDKSASTAYMELSSLRSEDNAVYYCTRSDGR NDMDSWGQGTLLTVSSGGGSGGGGSGGGGSQVQLV QSGGGPVKPGGSLRLSCAASGFMFRAYSMNWVRQAP GKGLEWVSSISSSGRYIHVADSVKGRFTISRDNKNSLY LQMNSLRAEDNAVYYCARETMAGKALDYWGQGTLL TVSSASTKGPSVFPLAPSSKSTSEGTAAALGCLVKDYFP EPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTP SSSLGTQTYICNVNHKPSNTKVDKKVEPKSS	
2	DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMH YQQKPGQPPKLLIKYASNLESGVPARFSGSGSGTDFTLT ISSVEPEDFATYYCQHSWEIPPTFGGGTKLEIKRGGGGS GGGGSGGGGSQVQLVQSGAEVVKPGASVKLSCKASG YIFTSYMYWVKQAPGQGLEWIGEINPSNGDTNFNEKF KSKATLTVDKSASTAYMELSSLRSEDNAVYYCTRSDGR NDMDSWGQGTLLTVSSGSTSGSGKPGSGEGSTKGDIV LTQSPGTLSLSPGETATLSCRASQSVGSNLA WYQQKPGQAPRLLIYGASTGATGVPARFSGSRSGTDFTLTITSLQPE DFATYYCQQYYSLAKTFTGQGTQLEIKRTVAAPSVFIFP PSDEQLKSGTASVCLNNFYFPREAKVQWKVDNALQS GNSQESVTEQDSKSTYSLSNLTLSKADYEKHKVYAC EVTHQGLSSPVTKSFNRGES	CD40L inhibitor (h-Ab1 and h-Ab2) light chain
3	RASQRVSSSTYSYMH	Anti-CD40L scFv (CDR VL1)
4	YASNLES	Anti-CD40L scFv (CDR VL2)
5	QHSWEIPPT	Anti-CD40L scFv (CDR VL3)
6	SYMY	Anti-CD40L scFv (CDR VH1)
7	EINPSNGDTNFNEKFKS	Anti-CD40L scFv (CDR VH2)
8	SDGRNDMDS	Anti-CD40L scFv (CDR VH3)

9	DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHW YQQKPGQPPKLLIKYASNLESGVPARFSGSGSGTDFTLT ISSVEPEDFATYYCQHSWEIPPTFGGGTKLEIKR	Anti-CD40L scFv light chain variable domain
10	QVQLVQSGAEVVKPGASVKLSCKASGYIFTSYYMYWV KQAPGQGLEWIGEINPSNGDTNFNEKFKSKATLTVDKS ASTAYMELSSLRSEDNAVYYCTRSDGRNDMDSWGQG TLVTVSS	Anti-CD40L scFv heavy chain variable domain
11	DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHW YQQKPGQPPKLLIKYASNLESGVPARFSGSGSGTDFTLT ISSVEPEDFATYYCQHSWEIPPTFGGGTKLEIKRGGGGS GGGSGGGGSQVQLVQSGAEVVKPGASVKLSCKASG YIFTSYYMYWVKQAPGQGLEWIGEINPSNGDTNFNEKF KSKATLTVDKSASTAYMELSSLRSEDNAVYYCTRSDGR NDMDSWGQGTTLVTVSS	Anti-CD40L scFv
12	DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHW YQQKPGQPPKLLIKYASNLESGVPARFSGSGSGTDFTLT ISSVEPEDFATYYCQHSWEIPPTFGQGTKLEIKRGGGGS GGGSGGGGSQVQLVQSGAEVVKPGASVKLSCKASG YIFTSYYMYWVKQAPGQGLEWIGEINPSNGDTNFNEKF KSKATLTVDKSASTAYMELSSLRSEDNAVYYCTRSDGR NDMDSWGQGTTLVTVSS	Anti-CD40L scFv
13	RASQSVGSNLA	anti-serum albumin Fab (CDR VL1)
14	GASTGAT	anti-serum albumin Fab (CDR VL2)
15	QQYYSFLAKT	anti-serum albumin Fab (CDR VL3)
16	AYSMN	anti-serum albumin Fab (CDR VH1)
17	SISSSGRYIHVADSVKG	anti-serum albumin Fab (CDR VH2)
18	ETVMAGKALDY	anti-serum albumin Fab (CDR VH3)

19	QVQLVQSGGGPVKPGGSLRLSCAASGFMFRAYSMNW VRQAPGKGLEWVSSISSSGRYIHYSVKGRTISRDN AKNSLYLQMNSLRAEDTAVYYCARETMAGKALDY WGQGTLVTVSS	anti-serum albumin Fab heavy chain variable domain (VH)
20	DIVLTQSPGTLSPGETATLSCRASQSVGSNLAWYQQ KPGQAPRLLIYGASTGATGVPARFSGSRSGTDFLTITS LQPEDFATYYCQYYSFLLAKTFGQGTQLEIKR	anti-serum albumin Fab light chain variable domain (VL)
21	QVQLVQSGGGPVKPGGSLRLSCAASGFMFRAYSMNW VRQAPGKGLEWVSSISSSGRYIHYSVKGRTISRDN AKNSLYLQMNSLRAEDTAVYYCARETMAGKALDY WGQGTLVTVSSASTKGPSVFPLAPSSKSTSEGTAALGC LVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSL SSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSS	anti-serum albumin Fab heavy chain domain (VH-CH1 domain)
22	DIVLTQSPGTLSPGETATLSCRASQSVGSNLAWYQQ KPGQAPRLLIYGASTGATGVPARFSGSRSGTDFLTITS LQPEDFATYYCQYYSFLLAKTFGQGTQLEIKRTVAAPS VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDN ALQSGNSQESVTEQDSKSTYSLSNTLTLSKADYEKHK VYACEVTHQGLSSPVTKSFNRGES	anti-serum albumin Fab light chain domain (VL-CL domain)
23	GGGGSGGGSGGGGS	(G4S) ₃ Linker
24	GSTSGSGKPGSGEGSTKG	Linker
25	DIVLTQSPSSLAVSAGDKVTINCKSSQSLSGGYNYLA WYQQKTGQSPKLLIYFTSTRHTGVPDRFIGSGSGTDFLT TINSFQTEDLDYYCQHHYGTPLTFGDGKLEIKRGGG GSGGGSGGGGSQVQLKQSGAEFVKPGASVKISCKTS GYTFTDGYMNWVEQKPGQGLEWIGRIDPDSGDTRYNQ KFQGKATLTRDKSSSTVYMDLRSLTSEDSAVYYCARA PYIADIGEAFDYWGQGMVTVSSGSTSGSGKPGSGEGS TKGQVQLVQSGGGPVKPGGSLRLSCAASGFMFRAYSM NWVRQAPGKGLEWVSSISSSGRYIHYSVKGRTISR DNAKNSLYLQMNSLRAEDTAVYYCARETMAGKALD YWGQGTLVTVSSASTKGPSVFPLAPSSKSTSEGTAALG CLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSS	Mouse CD40L inhibitor (m-Ab1) Heavy chain

26	DIVLTQSPSSLAVSAGDKVTINCKSSQSLLSGGYNYLA WYQQKTGQSPKLLIYFTSTRHTGVPDRFIGSGSGTDFTL TINSFQTEDLGDIYYCQHHYGTPLTFGDGTKLEIKRGGG GSGGGGSGGGGSQVQLKQSGAEFVKPGASVKISCKTS GYTFTDGYMNVWEQKPGQGLEWIGRIDPDSGDTRYNQ KFQGKATLTRDKSSSTVYMDLRSLTSEDSAVYYCARA PYIADIGEAFDYWGQGTMTVTVSSGSTSGSGKPGSGEGS TKGDIVLTQSPGTLSLSPGETATLSCRASQSVGSNLAW YQQKPGQAPRLLIYGASTGATGVPARFSGSRSGTDFTL TITSLQPEDFATYYCQYYSFLLAKTFGQGTQLEIKRTVA APSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWK VDNALQSGNSQESVTEQDSKDYSLNTLTLSKADYE KHKVYACEVTHQGLSSPVTKSFNRGES	Mouse CD40L inhibitor (m-Ab1) Light chain
27	DIVMTQTPLSLSVTPGQPASISCRSSQSLLHSNGNTYLH WYLQKPGQSPQLLIYKVSNRFSGVPDRFSGSGSGTDFT LKISRVEAEDVGVYYCSQSTHVPWTFGQGTKVEIKRGG GGSGGGGSGGGGSQVQLQESGPGLVKPSETLSLTCTVS GGSISSGYWNWIRQPPGKGLEWIGTISYSGDTYYNPSL KSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARYGSY VFDYWGQGTITVTVSSGGGGSGGGGSGGGGSQVQLVQ SGGGPVKPGGSLRLSCAASGFMFRAYSMNWVRQAPG KGLEWVSSISSGRYIHYADSVKGRFTISRDNANKSLYL QMNSLRAEDTAVYYCARETMAGKALDYWGQGTLV TVSSASTKGPSVFPLAPSSKSTSEGTAAALGCLVKDYFPE PVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SSLGTQTYICNVNHKPSNTKVDKKVEPKSS	Control Ab (Ctr-Ab) Heavy chain
28	DIVMTQTPLSLSVTPGQPASISCRSSQSLLHSNGNTYLH WYLQKPGQSPQLLIYKVSNRFSGVPDRFSGSGSGTDFT LKISRVEAEDVGVYYCSQSTHVPWTFGQGTKVEIKRGG GGSGGGGSGGGGSQVQLQESGPGLVKPSETLSLTCTVS GGSISSGYWNWIRQPPGKGLEWIGTISYSGDTYYNPSL KSRVTISVDTSKNQFSLKLSSVTAADTAVYYCARYGSY VFDYWGQGTITVTVSSGSTSGSGKPGSGEGSTKGDIVLT QSPGTLSLSPGETATLSCRASQSVGSNLAWYQQKPGQA PRLLIYGASTGATGVPARFSGSRSGTDFTLTITSLQPEDF	Control Ab (Ctr-Ab) Light chain

	ATYYCQQYYSFLLAKTFGQGTQLEIKRTVAAPSVFIFPPS DEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSG NSQESVTEQDSKDSTYLSNTLTLSKADYEKHKVYACE VTHQGLSSPVTKSFNRGES	
29	DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHW YQQKPGQPPKLLIKYASNLESGVPARFSGSGSGTDFTLT ISSVEPEDFATYYCQHSWEIPPTFGGGKLEIKRGGGGS GGGSGGGGSQVQLVQSGAEVVKPGASVKLSCKASG YIFTSYMYWVKQAPGQGLEWIGEINPSNGDTNFNEKF KSKATLTVDKSASTAYMELSSLRSEDATVYYCTRSDGR NDMDSWGQGTLLTVSSGGGSGGGGSGGGGSQVQLV QSGGGPVKPGGSLRLSCAASGFMFRAYSMNWVRQAP GKGLEWVSSISSGRYIHYADSVKGRFTISRDNKNSLY LQMNSLRAEDTAVYYCARETVAAGKALDYWGQGTLLV TVSSASTKGPSVFPLAPSSKSTSEGTAALGCLVKDYFPE PVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPS SSLGTQTYICNVNHKPSNTKVDKKVEPKSS	CD40L inhibitor (h-Ab2) Heavy chain
30	ETVAAGKALDY	anti-serum albumin Fab (CDR VH3) (h-Ab2)
31	RASQISRYLN	anti-serum albumin Fab (CDR VL1)
32	GASRLES	anti-serum albumin Fab (CDR VL2)
33	QQSDSVPVT	anti-serum albumin Fab (CDR VL3)
34	RASQISSYLN	anti-serum albumin Fab (CDR VL1)
35	AASSLQS	anti-serum albumin Fab (CDR VL2)
36	QQSYSTPPYT	anti-serum albumin Fab (CDR VL3)
37	RASQSIFNYVA	anti-serum albumin Fab (CDR VL1)

38	DASNRAT	anti-serum albumin Fab (CDR VL2)
39	QQRSKWPPTWT	anti-serum albumin Fab (CDR VL3)
40	RASETVSSRQLA	anti-serum albumin Fab (CDR VL1)
41	GASSRAT	anti-serum albumin Fab (CDR VL2)
42	QQYGSSPRT	anti-serum albumin Fab (CDR VL3)
43	RASQSVSSSLA	anti-serum albumin Fab (CDR VL1)
44	QKYSSYPLT	anti-serum albumin Fab (CDR VL3)
45	SYGIS	anti-serum albumin Fab (CDR VH1)
46	WINTYSGGTKYAQKFQG	anti-serum albumin Fab (CDR VH2)
47	LGHCQRGICSDALDT	anti-serum albumin Fab (CDR VH3)
48	RINTYNGNTGYAQLQG	anti-serum albumin Fab (CDR VH2)
49	NYGIH	anti-serum albumin Fab (CDR VH1)
50	SISYDGSNKYYADSVKG	anti-serum albumin Fab (CDR VH2)
51	DVHYYGSGSYNAFDI	anti-serum albumin Fab (CDR VH3)
52	SYAMS	anti-serum albumin Fab (CDR VH1)
53	VISHDGGFQYYADSVKG	anti-serum albumin Fab (CDR VH2)
54	AGWLRQYGMDV	anti-serum albumin Fab (CDR VH3)

55	AYWIA	anti-serum albumin Fab (CDR VH1)
56	MIWPPDADARYSPSFQG	anti-serum albumin Fab (CDR VH2)
57	LYSGSYSP	anti-serum albumin Fab (CDR VH3)
58	ELVLTQSPSS LSASVGDRVIT ITCRASQSI YLNWYQQKPGKAPKLLIYG ASRLESGVPS RFSGSGSGTDFTLTISLQPEDFATYYCQQ SDSVPVTFGQGTRLEIKR	anti-serum albumin Fab light chain variable domain (VL)
59	DIVLTQSPSS LSASVGDRVIT ITCRASQSI SYLNWYQQKPGKAPKLLIYA ASSLQSGVPS RFSGSGSGTDFTLTISLQPEDFATYYCQQ SYSTPPYTFGQGTKLEIKR	anti-serum albumin Fab light chain variable domain (VL)
60	ELVLTQSPGTLSLSPGERATLSCRASQSI NYVAWYQQKPGQAPRLIYDASNRATGIPA RFSGSGSGTDFTLTISLQPEDFAVYYCQQ RSKWPPTWTFGQGTRVDIKR	anti-serum albumin Fab light chain variable domain (VL)
61	ELVLTQSPGTLSLSPGERATLSCRASETVS SRQLAWYQQKPGQAPRLIYGASSRATGIP DRFSGSGSGTDFTLTISRLEPEDSAVFYCC QYGSSPRTFGGGTKLEIKR	anti-serum albumin Fab light chain variable domain (VL)
62	ELVLTQSPGTLSLSPGERATLSCRASQSVS SSSLAWYQQKPGQAPRLIYGASSRATGIP DRFSGSGSGTDFTLTISLQPEDAATYYCQ KYSSYPLTFGQGTKLEIKR	anti-serum albumin Fab light chain variable domain (VL)
63	QVQLLQSGAEVKKPGASVKVSCKASGYTFT SYGISWVRQA PGQGLEWVGRINTYSGGTKY AQKFQGRVTMTDTSISTVYMELSGLKSD TAVYYCARLGHCQRGICSDALDTWGQGT LVTVSS	anti-serum albumin Fab heavy chain variable domain (VH)
64	EVQLLQSGAEVKEPGASVKVSCKASGYTFS SYGISWVRQA PGQGLEWVGRINTYNGNTGY AQLRQGRVTMTDTSISTIAYMEVRSLSRSD TAVYYCARLGHCQRGICSDALDTWGQGT TMV	anti-serum albumin Fab heavy chain variable domain (VH)

	TVSS	
65	QVQLVQSGGG VVQTGGSLRL SCAASGFTFR NYGIHWVRQA PGKGLEWVAS ISYDGSNKYY ADSVKGRFTI SRDNSRNTVH VQMDSLRGGD TAVYYCARDV HYYGSGSYYN AFDIWQGCTL VTVSS	anti-serum albumin Fab heavy chain variable domain (VH)
66	QVQLVQSGGG LVQPGGSLRL SCAASGFTFS SYAMSWVRQA PGKGLEWLSV ISHDGGFQYY ADSVKGRFTV SRDNSKNTLY LQMNSLRAED TAVYYCARAG WLRQYGMDVW GQGTTLTVSS	anti-serum albumin Fab heavy chain variable domain (VH)
67	EVQLVQSGTE VKKPGESLKI SCKISGYSFT AYWIAWVRQM PGKGLEWMGM IWPPDADARY SPSFQGGVTF SVDKSISTAY LQWHSKLTSD TAVYYCARLY SGSYSPWGQG TLTVSS	anti-serum albumin Fab heavy chain variable domain (VH)
68	QVQLVQSGGGPVKPGGSLRLSCAASGFMFRAYSMNW VRQAPGKGLEWVSSISSSGRYIHYADSVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCARETVAAGKALDYW GQGTTLTVSS	anti-serum albumin Fab heavy chain variable domain (VH) (h-Ab2)
69	QVQLVQSGGGPVKPGGSLRLSCAASGFMFRAYSMNW VRQAPGKGLEWVSSISSSGRYIHYADSVKGRFTISRDN AKNSLYLQMNSLRAEDTAVYYCARETVAAGKALDYW GQGTTLTVSSASTKGPSVFPLAPSSKSTSEGTAALGCLV KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS VVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSS	anti-serum albumin Fab heavy chain domain (VH-CH1 domain) (h-Ab2)
70	QVQLQESGPGLVKPSSETLSLTCTVSGGSISSGYWNWIR QPPGKGLEWIGTISYSGDTYYNPSLKSRTISVDTSKNQ FSLKLSSVTAADTAVYYCARYGSYVFDYWQGTTVT SSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPV TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSL GTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPA PEAAGGSPVFLFPPKPKDTLMISRTPEVTCVVVDVSHED PEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT VLHQDWLNGKEYKCAVSNKALPAPIEKTISKAKGQPR EPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWE	anti-TNP IgG heavy chain (negative control)

	SNGQPENNYKTTTPVLDSGSSFFLYSKLTVDKSRWQQ GNVFSCSVMHEALHNHYTQKSLSLSPGK	
71	DIVMTQTPLSLSVTPGQPASISCRSSQSLLHSNGNTYLH WYLQKPGQSPQLLIYKVSNRFSQVPDRFSGSGSGTDFT LKISRVEAEDVGVYYCSQSTHVPWTFGGGTKVEIKRTV AAPS VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQW KVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADY EKHKVYACEVTHQGLSSPVTKSFNRGEC	anti-TNP IgG light chain (negative control)

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EXAMPLES

Example 1 – effect of CD40L inhibition on local inflammation in the mouse model of dermal delayed-type hypersensitivity

To understand the effect of CD40L inhibition on reducing T cell-mediated local inflammation, we tested the pharmacodynamic response of m-Ab1 on the dermal delayed-type hypersensitivity (DTH) model in mouse. Molecular and cellular assessment of DTH responses is a useful approach for evaluating the mechanism of action (MOA) of immunomodulatory agents. As h-Ab1 does not cross react with rodent CD40L, the mouse specific m-Ab1 (SEQ ID NOs: 25 and 26) was used in this study. M-Ab1 differs from h-Ab1 in that the anti-CD40L scFvs are derived from the MR1 antibody and thus capable of binding to mouse CD40L. Beside this modification, m-Ab1 shares the same structure as h-Ab1 and shares similar affinities and potency.

In the present study, we characterized the effect of CD40L inhibition on DTH response induced by keyhole limpet hemocyanin (KLH). Efficacy was determined by ear thickness measurements, serum antigen-specific antibody concentrations and measurement of cytokine levels in the local area of immunization.

Materials and methods:

Model induction: Female C57BL6 mice (Charles River Laboratories) weighing 18-20 g were used in the study. Animals were given ad libitum access to Teklad 22/5 Rodent Diet (Envigo, Cat #8640) and water, housed under standard conditions, and allowed to acclimate for at least 1 week prior to use in the study.

KLH (MilliporeSigma, Cat #H7017, Lot #0000188788) was formulated fresh, immediately prior to dosing in Freund's complete adjuvant (FCA; MilliporeSigma, Cat #F5881, Lot #1003484857) added with M tuberculosis H37RA. KLH was administered subcutaneously (s.c.) on day 0 (sensitization) and day 7 (challenge) at a dose of 2 or 10 mg/mL respectively, and in volumes equal to 0.1 mL/mouse.

On study day 0, animals had both ears calipered for ear thickness and injected subcutaneously with the KLH emulsion. On day 7, animals had both ears calipered for ear thickness. After caliper measurements, animals were injected with 10µl of a 10mg/ml KLH emulsion into the pinna of the right ear. Caliper measures of ear thickness were performed using a spring-loaded

micrometer caliper (Mitutoyo America Corporation, Model #700-118). Baseline measurements of both ears were recorded pre-challenge on day 0 and day 7. Both ears were measured on day 8 and day 9 and the difference from the baseline was calculated.

- 5 On day 9, (48 hours post-challenge) mice were sacrificed with isoflurane followed by exsanguination and cervical dislocation. The right ear was collected, and the weight of the whole right ear was recorded. An 8-mm biopsy of the right ear was collected, weighed, placed in 10% neutral-buffered formalin (NBF), and submitted for histopathological processing. The remaining right ear tissue not included in the 8-mm biopsy was weighed, flash-frozen in liquid
- 10 N₂, and submitted for ex vivo analyses. The left ear was collected, flash-frozen in liquid N₂, and submitted for ex vivo analyses.

Whole blood (500 µL yielding 3 x 20 µL and 2 x 45 µL serum) was collected via retro-orbital puncture at the time of sacrifice and processed to produce serum. Serum samples were immediately flash-frozen in liquid N₂.

15

Test article and dosing: m-Ab1 was formulated once in vehicle (20mM L-Histidine-HCL with 50 mg/mL sucrose) and maintained at 4 °C. m-Ab1 was administered intraperitoneally (i.p.) once on day 7 at a dose of 3.4, 6.8, or 13.6 mg/kg and in volumes equal to 5 mL/kg. Vehicle control group animals were dosed with the vehicle starting from day 0.

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Ex Vivo Analyses: Anti-KLH antibody Enzyme-Linked Immunosorbent Assay | Serum samples were analyzed using an anti-KLH antibody enzyme-linked immunosorbent assay kit (ELISA; Life Diagnostics, Cat #KLHG-1, Lot #KLHG1132223) based on the manufacturer's specifications.

- 25 **Luminex Assay** | In order to measure the levels of pro-inflammatory cytokines at the site of immunization, supernatant from homogenized ear tissue samples were analyzed using a Luminex multiplex assay (Mouse TH17 Magnetic Bead Panel (MilliporeSigma, Cat #MTH17MAG-47K, Lot #3977881) based on the manufacturer's specifications. Supernatant from homogenized ear tissue samples were analyzed using a Pierce bicinchoninic acid (BCA)
- 30 protein assay (Thermo Fisher Scientific Inc., Cat #23225, Lot #XI351701) based on the manufacturer's specifications.

Statistical Analyses: Data were expressed as mean $\pm$ standard error of the mean (SEM). Statistical analyses were performed on the data generated from this study using GraphPad Prism 10 for Windows (GraphPad Software, Inc.). Statistical significance was set at $p < 0.05$. Only statistically significant observations are included in the Results.

5

Results:

m-Ab1 (CD40L inhibitor) dose/response on right ear thickness (FIG. 1):

m-Ab1 administered at 3.4 mg/kg show no differences in right ear thickness compared to vehicle-treated controls.

- 10 m-Ab1 administered at 6.8 mg/kg decreased right ear thickness over time (m-Ab1 day 8: 0.319 ± 0.007 mm; m-Ab1 day 9: 0.329 ± 0.007 mm; vs. Vehicle day 8: 0.383 ± 0.012 mm; Vehicle day 9: 0.393 ± 0.017 mm), the percentage change in right ear thickness from day 8 (m-Ab1: $39.41 \pm 3.42\%$ vs. Vehicle: $72.73 \pm 5.57\%$) to day 9 (m-Ab1: $43.84 \pm 3.83\%$ vs. Vehicle: $77.15 \pm 7.56\%$), as well as the associated area under the curve (AUC) from day 7 through day 9 (m-Ab1: 0.599 ± 0.001 vs. Vehicle: 0.691 ± 0.021), relative to vehicle-treated controls.

- 15 m-Ab1 administered at 13.6 mg/kg decreased right ear thickness over time (m-Ab1 day 8: 0.293 ± 0.005 mm; m-Ab1 day 9: 0.305 ± 0.004 mm; vs. Vehicle day 8: 0.383 ± 0.012 mm; Vehicle day 9: 0.393 ± 0.017 mm), the percentage change in right ear thickness from day 8 (m-Ab1: $27.12 \pm 1.57\%$ vs. Vehicle: $72.73 \pm 5.57\%$) to day 9 (m-Ab1: $32.64 \pm 1.62\%$ vs. Vehicle: $77.15 \pm 7.56\%$), as well as the associated area under the curve (AUC) from day 7 through day 9 (m-Ab1: 0.560 ± 0.006 vs. Vehicle: 0.691 ± 0.021), relative to vehicle-treated controls.

Local pro-inflammatory cytokine expression is reduced by m-Ab1 (CD40L inhibitor) treatment:

- 25 KLH mice treated with 3.4, 6.8 or 13.6 mg/kg m-Ab1 had significant decreased tumor necrosis factor alpha (TNF- α) levels compared to vehicle-treated controls (m-Ab1 3.4 mg/kg: 8.22 ± 1.56 pg/mL, $p=0.028$; 6.8 mg/kg: 6.43 ± 1.09 pg/mL, $p=0.005$; 13.6 mg/kg: 5.99 ± 1.19 pg/mL, $p=0.003$; vs. Vehicle: 16.10 ± 2.59 pg/mL) (FIG. 2A).

- Levels of Interleukin 4 (IL-4) were also drastically reduced by the treatment with 3.4, 6.8 or 13.6 mg/kg m-Ab1 (m-Ab1 3.4 mg/kg: 1.72 ± 0.44 pg/mL, $p=0.088$; 6.8 mg/kg: 1.95 ± 0.46 pg/mL, $p=0.112$; 13.6 mg/kg: 1.70 ± 0.19 pg/mL, $p=0.086$; vs. Vehicle: 6.15 ± 2.04 pg/mL), but the decrease was not statistically significant (FIG. 2B).

30

Similarly, levels of IL-22 showed not-statistically significant reduction by the treatment (m-Ab1 3.4 mg/kg: 25.78 ± 5.42 pg/mL, $p=0.140$; 6.8 mg/kg: 24.01 ± 6.21 pg/mL, $p=0.078$; 13.6 mg/kg: 27.21 ± 5.57 pg/mL, $p=0.116$; vs. Vehicle: 63.81 ± 20.80 pg/mL) (FIG. 2C).

5 Conclusion:

Intradermal KLH challenge of the ear pinna following subcutaneous antigen sensitization resulted in a pronounced skin inflammation that peaked at 48h. Administration of the mouse CD40L inhibitor m-Ab1 reduced the inflammatory profile. m-Ab1 significantly decreased the levels of TNF- α . A reduction in the level of the cytokines IL-4 and IL-22 was also observed.

- 10 The reduction in the local inflammation resulted in a reduction of the challenged ear thickness (i.e., swelling). These data support that the fusion protein of the present disclosure enters the local inflammatory environment and demonstrate that inhibition of CD40L result in reduction of the local inflammatory response associated with the infiltration of immune cells. As infiltration of T cells and macrophages, as well as local peri-orbital inflammation, are
15 characteristics of TED, the results of this study support that administration of the fusion protein of the present disclosure and thereby inhibiting CD40L provides treatment of the orbitopathy in TED.

Example 2 – effect of CD40L inhibitor on inflammatory response in PBMCs

- 20 In TED patients, PBMCs have been described to be activated and play a systemic role in TED pathogenesis. Activated T-cells express CD40L, while other cell types, part of the PBMC pool, express CD40 (i.e., B cells, monocytes, dendritic cells and fibrocytes). When activated, CD40+ PBMC cells produce pro-inflammatory cytokines. Both activated cells (e.g., fibrocytes) and cytokines can infiltrate the orbital cavity, activating resident fibroblasts and starting the local
25 inflammation involved in TED orbitopathy.

The aim of this example was to demonstrate the efficacy of CD40L inhibitor in inhibiting inflammatory cytokines production by PMBC through blockage of the CD40/CD40L interaction.

30 Materials and methods:

PBMC isolation and culture: PBMCs were isolated from whole blood obtained from healthy donors. 18 mL of blood was dispensed to 50 mL Leucosep tubes (Greiner) containing 15mL Lymphoprep™ Density gradient medium (StemCell Technologies) and centrifuged at 1900

rpm for 15 minutes at room temperature with deceleration speed set to 1. Then, the plasma layer was discarded and PBMC fraction collected into 50 mL falcon tubes (ThermoFisher Scientific). PBMCs were washed with 40 mL of DMEM (Gibco) and centrifuged at 1900 rpm for 10 mins at 4°C. Supernatant was discarded and 2 more washes were performed. PBMCs were then counted using Vi-Cell BLU cell counter (Beckman Coulter) and resuspended at 5×10^6 cells/mL. PBMCs were plated at 2.5×10^6 cells/cm² in DMEM with 10% FBS in Nunc™ Cell-Culture Treated 24wellplates (ThermoFisher).

PBMC stimulation and treatment: After 10 days of culture, medium was changed to DMEM with 1% FBS and PBMCs were stimulated with 200 ng/mL of IFN- γ (R&D systems) for 72 hours. Then, PBMCs were treated with 15 nM megaCD40L (Enzo Lifesciences) alone or in combination with 5 or 135 nM of h-Ab2 (CD40L inhibitor with sequence of anti-CD40L scFvs identical to h-Ab1 while having a single point mutation in the anti-serum albumin Fab as compared to h-Ab1. The identical anti-CD40L scFv and the lack of involvement of serum albumin in the present assay result in CD40L binding affinities and potency similar to h-Ab1) and control antibody Ctr-Ab (differs from h-Ab1 by having scFv specific for TNP with no binding to CD40L, while anti-serum albumin Fab being identical to h-Ab1) for 24 hours. megaCD40L was premixed with indicated concentrations of respective antibodies for 5 min at RT in culture media before added to the cells. As controls, cells were left untreated or treated with IFN- γ only.

mRNA quantification by RT-PCR: *CD40*, *IL-6* (Interleukin 6), *IL-8* (Interleukin 8) and *CCL5* (C-C Motif Chemokine Ligand 5, known as *RANTES*) mRNA levels were monitored by real-time quantitative PCR (qPCR). After treatment, cells were lysed with 150 μ L of Aurum Total RNA Lysis Solution and RNA was purified following manufacturer instructions (Bio-Rad Laboratories). cDNA was synthesized using TaqMan™ Reverse Transcription Kit (ThermoFisher Scientific). Quantitative PCR was performed on a CFX96 thermocycler (Bio-Rad Laboratories) using TaqMan™ Universal PCR Master Mix (ThermoFisher Scientific) and predesigned qPCR probes (Integrated DNA technologies). *GPI* (Glucose-6-Phosphate Isomerase) was used as the housekeeping gene and relative expression was calculated using the 15 nM megaCD40L condition as reference using the $\Delta\Delta C_t$ method (Livak et al. 2001).

Statistical Analyses: Data were expressed as mean $\pm$ standard error (SD) of n=2-4 replicates from one experiment. For statistical analysis, One-way ANOVA with Bonferroni correction was performed using GraphPad Prism 10 for Windows (GraphPad Software, Inc.) *p<0,05, **p<0,01, ****p<0,0001.

5

Results:

PBMCs expressed CD40 upon IFN- γ stimulation. While *CD40* was barely expressed in unstimulated PBMCs, it was upregulated by 2.5-fold upon 72h of IFN- γ stimulation and further doubled when PBMCs were treated with additionally 15 nM megaCD40L for 24 hours after
10 IFN- γ stimulation. There was no significant impact of any antibody treatment on the *CD40* mRNA level (FIG. 3A).

PBMCs produced inflammatory cytokines as a response to megaCD40L. *IL-6*, *IL-8* and *RANTES* expression was almost absent in unstimulated PMBCs and barely increased upon IFN- γ , however, after following stimulation with 15 nM megaCD40L there was a clear increase
15 in cytokine expression of *IL-6*, *IL-8* and *RANTES* (FIGS. 3B-3D).

Inflammatory cytokine expression can be blocked by treatment with CD40-L inhibitor. Pre-treatment of megaCD40L with h-Ab2 decreased *IL-6*, *IL-8* and *RANTES* expression a dose-dependent manner (FIGS. 3B-3D). *IL-6* relative expression was reduced by 60% or 95% of megaCD40L stimulation levels when treated with 5 nM or 124 nM of h-Ab2 respectively. *IL-8* relative expression was reduced by 72% and 83% of megaCD40L stimulation levels when
20 treated with 5 nM or 124 nM of h-Ab2 respectively. *RANTES* relative expression was reduced by 47% and 71% of megaCD40L stimulation levels when treated with 5 nM or 124nM of h-Ab2 respectively. This reduction in cytokine expression was absent when control antibody was used, demonstrating that the dose-dependent effect is mediated by direct blocking of CD40L
25 with h-Ab2.

Conclusion:

This example demonstrates the efficacy of h-Ab2 in reducing the production of inflammatory cytokines by PBMCs via blocking the CD40:CD40L interaction. Activated PBMC cells (e.g.,
30 fibrocytes) and cytokines therefrom are involved in TED pathogenesis by activating orbital fibroblasts or myoblasts in the orbital cavity and contribute to the local inflammation wherefore a positive impact on their inflammatory status is expected to have a therapeutic impact for TED patients.

Example 3 – effect of CD40L inhibitor on inflammatory response in fibroblasts

The activation of orbital fibroblasts is a key characteristic of TED pathogenesis. It occurs when CD40L, which is expressed on activated T cells that infiltrate the retro-orbital tissue in TED, binds to CD40, a receptor localized on fibroblasts. This interaction triggers a signaling cascade that leads to the expression and secretion of various cytokines, such as IL-6, IL-8 and RANTES, that have pro-inflammatory and chemoattractant effects and, thus, contributing to the local orbit inflammation observed in TED patients.

The aim of this example was to demonstrate the efficacy of h-Ab1 in inhibiting inflammatory cytokines production by fibroblasts in the orbit through blockage of the CD40:CD40L interaction.

Materials and methods:

Fibroblast culture and treatment: Human primary fibroblasts (Lonza) were seeded at 40.000 cells/cm² in FGM™-2 Fibroblast Growth Medium-2 commercial culture system (Lonza). After 2 days of culture the culture medium was changed to DMEM with 1% FBS and fibroblasts were stimulated with 200 ng/ml IFN- γ (R&D systems) for 72 hours. Then, fibroblasts were treated with 32 nM human recombinant soluble CD40L (Enzo Lifesciences) alone or in combination with 5 or 135 nM of h-Ab1 or Ctr-Ab for 24 hours. Human recombinant soluble CD40L was premixed with indicated concentrations of respective antibodies for 5 min at RT in culture media before added to the cells. As controls, cells were left untreated or treated with IFN- γ only.

mRNA quantification by RT-PCR: *CD40*, *IL-6*, *IL-8* and *RANTES* mRNA levels were monitored by real-time PCR. After treatment, cells were lysed with 100 μ L of homogenization buffer from Maxwell® RSC simplyRNA Cells Kit and RNA was purified following manufacturer instructions (Promega) using the Maxwell® RSC Instrument (Promega). cDNA was synthesized using TaqMan™ Reverse Transcription Kit (ThermoFisher Scientific). Quantitative PCR was performed on a CFX96 thermocycler (Bio-Rad Laboratories) using TaqMan™ Universal PCR Master Mix ThermoFisher Scientific) and predesigned qPCR probes (Integrated DNA technologies). *GPI* was used as the housekeeping gene and relative expression was calculated using the 32 nM human recombinant soluble CD40L condition as reference using the $\Delta\Delta$ Ct method (Livak et al. 2001).

Statistical Analyses: Data were expressed as mean $\pm$ standard error (SD) of n=2-4 replicates from one experiment. For statistical analysis, One-way ANOVA with Bonferroni correction was performed using GraphPad Prism 10 for Windows (GraphPad Software, Inc.) *p<0,05, **p<0,01, ****p<0,0001.

5

Results:

Fibroblasts expressed CD40 upon IFN- γ stimulation. While CD40 expression was absent in unstimulated human primary fibroblasts, it was upregulated by more than 15-fold upon 72h of IFN- γ stimulation. The increase in CD40 expression was further doubled when fibroblasts were
10 treated with 32 nM human recombinant soluble CD40L for an additional 24 hours after IFN- γ stimulation. There was no significant impact of any antibody treatment on the *CD40* mRNA level, except when fibroblasts were treated with 1215nM of h-Ab1 wherein *CD40* mRNA levels decreased (FIG. 4A).

Fibroblasts produced inflammatory cytokines as response to soluble CD40L. Treatment
15 with 32 nM human recombinant soluble CD40L increased expression of *IL-6*, *IL-8* and *RANTES* in CD40-expressing fibroblasts, which was absent in control and IFN- γ only treated fibroblasts, indicating a direct effect of CD40L:CD40 interaction (FIGS. 4B-4D). The human recombinant soluble CD40L was in monomeric form, yet an inflammatory response was induced in the fibroblasts following treatment, potentially due to spontaneous formation of
20 trimeric complexes.

Inflammatory cytokine expression can be blocked with h-Ab1 treatment. Cytokine expression was reduced in a dose-dependent manner when fibroblasts were treated with CD40L combined with h-Ab1 (FIGS. 4B-4D). *IL-6* relative expression was reduced by 36% and 73
25 when fibroblasts were treated with 135 nM and 1215 nM of h-Ab1 respectively. *IL-8* relative expression decreased 46% and 93% when fibroblasts were treated with 135 nM or 1215 nM of h-Ab1 respectively. This effect in *IL-6* and *IL-8* was absent when 5nM of h-Ab1 or Ctr-Ab at 5 or 135 nM were used. *RANTES* mRNA relative expression was reduced by 30%, 72% and 95% when fibroblasts were treated with 5 nM, 135 nM and 1215 nM of h-Ab1 respectively and no reduction was observed when Ctr-Ab were used. Taken together, these results demonstrate
30 that the dose-dependent effect is mediated by direct blocking of CD40L with h-Ab1.

Conclusion:

This example demonstrates the efficacy of h-Ab1 in reducing the production of inflammatory cytokines by activated fibroblasts via blocking the CD40:CD40L interaction. Activated fibroblasts are key drivers of TED pathogenesis in the orbital cavity and to a large extent responsible for the local inflammation wherefore a positive impact on their inflammatory status is expected to have a large therapeutic impact for TED patients.

Example 4 – effect of h-Ab1 on inflammatory response in myoblasts

Resident myoblasts in the orbital cavity express CD40 on their surface and can respond to CD40L stimulation with production of inflammatory cytokines. The production of cytokines by myoblasts upon CD40:CD40L interaction is implicated in TED where muscle cells contribute to the local inflammation in the orbital cavity.

The aim of this example was to demonstrate the efficacy of h-Ab1 in inhibiting inflammatory cytokines production by myoblasts in the orbit through blockage of the CD40:CD40L interaction.

Materials and methods:

Myoblasts culture and treatment: Human primary myoblasts (ThermoFisher Scientific) were seeded at a density of 50,000 cells/cm² in DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1 ng/ml TGF-beta1 (R&D systems). After 2 days of culture, the culture medium was changed to DMEM with 1% FBS and myoblasts were stimulated with 200 ng/ml IFN- γ (R&D systems) for 72 hours. Then, myoblasts were treated with 32nM human recombinant soluble CD40L (Enzo Lifescience) alone or in combination with 5nM or 135nM of h-Ab1 (CD40L inhibitor) and control antibody Ctr-Ab for 24 hours. Human recombinant soluble CD40L and respective antibody were premixed and incubated for 5 minutes before added to the cells. As controls, there was a group of myoblasts that did not receive any stimulation or treatment at all and another group that received only IFN- γ .

mRNA quantification by RT-PCR: *CD40*, *IL-6*, *IL-8* and *RANTES* mRNA levels were monitored by real-time PCR. After treatment, cells were lysed with 100 μ L of homogenization buffer from Maxwell® RSC simply RNA Cells Kit and RNA was purified following manufacturer instructions (Promega) using the Maxwell® RSC Instrument (Promega). cDNA was synthesized using TaqMan™ Reverse Transcription Kit (ThermoFisher Scientific). Quantitative PCR was performed on a CFX96 thermocycler (Bio-Rad Laboratories) using

TaqMan™ Universal PCR Master Mix ThermoFisher Scientific) and predesigned qPCR probes (Integrated DNA technologies). *GPI* was used as the housekeeping gene and relative expression was calculated using the 32 nM human recombinant soluble CD40L condition as reference using the $\Delta\Delta C_t$ method (Livak et al. 2001).

- 5 **Statistical Analyses:** Data were expressed as mean $\pm$ standard error (SD) of n=2-4 replicates from one experiment. For statistical analysis, One-way ANOVA with Bonferroni correction was performed using GraphPad Prism 10 for Windows (GraphPad Software, Inc.) *p<0,05, **p<0,01, ***p<0,001 and ****p<0,0001.

10 Results:

Myoblasts started expressing CD40 upon IFN- γ stimulation. While *CD40* was barely expressed in unstimulated myoblasts, it was upregulated by 13-fold upon 72h of IFN- γ stimulation and further doubled when myoblasts were treated with additionally 32 nM human recombinant soluble CD40L for 24 hours after IFN- γ stimulation. There was no significant impact of any antibody treatment on the *CD40* mRNA level (FIG. 5A).

- 15 **Myoblasts produced inflammatory cytokines as response to soluble CD40L.** Treatment with 32nM human recombinant soluble CD40L increased expression of *IL-8* and *RANTES* in CD40-expressing myoblasts, which was absent in control and IFN- γ only treated myoblasts, indicating is a direct effect of CD40L:CD40 interaction (FIGS. 5B-5C). However, *IL-6* transcripts were not detected in either stimulated or unstimulated myoblasts, indicating that human primary myoblasts were unable to produce this cytokine in *in-vitro* conditions. The human recombinant soluble CD40L was in monomeric form, yet an inflammatory response was induced in the myoblasts following treatment, potentially due to spontaneous formation of trimeric complexes.

- 25 **Inflammatory cytokine expression can be blocked with h-Ab1 treatment.** Cytokine expression was reduced in a dose-dependent manner when myoblasts were treated with CD40L combined with h-Ab1 (FIGS. 5B-5C). *IL-8* relative expression decreased 37%, 85% and 100% when myoblasts were treated with 5nM, 135nM or 1215nM of h-Ab1 respectively. *RANTES* mRNA relative expression decrease 50% when myoblasts were treated with 5nM or 135nM of h-Ab1 and 80% when myoblasts were treated 1215nM of h-Ab1. No reduction was observed when Ctr-Ab was used. These results demonstrate that the dose-dependent effect is mediated by direct blocking of CD40L with h-Ab1.
- 30

Conclusion:

This example demonstrates the efficacy of h-Ab1 in reducing the production of inflammatory cytokines by activated myoblasts via blocking the CD40:CD40L interaction. Activated myoblasts are involved in TED pathogenesis in the orbital cavity and contribute to the local inflammation wherefore a positive impact on their inflammatory status is expected to have a therapeutic impact for TED patients.

Example 5 – Dose-dependent effect of CD40L inhibitor on inflammatory response in fibroblasts

The aim of this example was to demonstrate the potency of h-Ab1 in inhibiting inflammatory cytokines production by fibroblasts through blockage of the CD40:CD40L interaction.

Materials and methods:

Fibroblast culture and treatment: Human primary fibroblasts (Lonza) were seeded at 40,000 cells/cm² in FGM™-2 Fibroblast Growth Medium-2 commercial culture system (Lonza). After 2 days of culture the culture medium was changed to DMEM with 1% FBS and fibroblasts were stimulated with 200 ng/ml IFN- γ (R&D systems) for 72 hours. Then, fibroblasts were treated with 32 nM human recombinant soluble CD40L (Enzo Lifesciences) alone or in combination with increasing 3-fold concentrations from 0.56 nM to 3645nM of h-Ab1 or Ctr-Ab for 24 hours. Human recombinant soluble CD40L was premixed with indicated concentrations of respective antibodies for 5 min at RT in culture media before added to the cells. As controls, cells were left untreated or treated with IFN- γ only.

mRNA quantification by RT-PCR: *CD40*, *IL-6*, *IL-8* and *RANTES* mRNA levels were monitored by real-time PCR. After treatment, cells were lysed with 100 μ L of homogenization buffer from Maxwell® RSC simplyRNA Cells Kit and RNA was purified following manufacturer instructions (Promega) using the Maxwell® RSC Instrument (Promega). cDNA was synthesized using TaqMan™ Reverse Transcription Kit (ThermoFisher Scientific). Quantitative PCR was performed on a CFX96 thermocycler (Bio-Rad Laboratories) using TaqMan™ Universal PCR Master Mix ThermoFisher Scientific) and predesigned qPCR probes (Integrated DNA technologies). *GPI* was used as the housekeeping gene and relative expression was calculated using the 32 nM human recombinant soluble CD40L condition as reference using the $\Delta\Delta$ Ct method (Livak et al. 2001). Based on a concentration range of h-Ab1 or Ctr-Ab, versus a fixed human recombinant soluble CD40L concentration, a Four Parameter

Logistic (4PL) regression curve can be fitted and an IC₅₀ (half-maximal inhibitory concentration) value of antibody inhibition of cytokine related gene expression can be determined.

Protein quantification by MSD assay: Supernatant was collected prior and after treatment with CD40L and antibodies and IL6 and IL8 protein production was quantified using a custom-made U-PLEX Custom Biomarker (hu) Assays, following manufacturer instructions (MSD). Relative cytokine production was calculated using the 32 nM human recombinant soluble CD40L condition as reference (100%). Based on a concentration range of h-Ab1 or Ctr-Ab, versus a fixed human recombinant soluble CD40L concentration, a Four Parameter Logistic (4PL) regression curve was fitted and the IC₅₀ (half-maximal inhibitory concentration) value of antibody inhibition of cytokine protein production was determined.

Statistical Analyses: Data were expressed as mean $\pm$ standard error of the mean (SEM) of N=5 biological replicates, with n=2 technical replicates per experiment. IC₅₀ values were represented as Geometrical mean and 95% confidence interval of IC₅₀s calculated on each experiment.

Results:

Inflammatory related gene expression can be blocked with h-Ab1 treatment in a dose-dependent manner. When fibroblasts were treated with CD40L combined with h-Ab1, there was a clear block of the relative expression of inflammation related genes (*IL6*, *IL8* and *RANTES*), showing maximum efficacy of 100% (Figures 6A-C). Cytokine expression was reduced in a dose-dependent manner with an IC₅₀ of 157.1 nM (56.1-439.8 nM), 94.2 nM (30.1-295.1 nM) and 29.6 nM (3.7-238.5 nM) for *IL6*, *IL8* and *RANTES*, respectively. No reduction was observed when Ctr-Ab were used. Taken together, these results demonstrate that the dose-dependent effect is mediated by direct blocking of CD40L with h-Ab1.

Inflammatory cytokine protein production can be blocked with h-Ab1 treatment in a dose-dependent manner. When fibroblasts were treated with CD40L combined with h-Ab1, there was a clear block of the cytokine protein production (*IL6* and *IL8*), showing maximum efficacy of 100% (Figures 7A-B). Cytokine expression was reduced in a dose-dependent manner, with an IC₅₀ value of 139.9 nM (50.9-383.9 nM) for *IL6* and 106.6 nM (34.1-333.4 nM) for *IL8*, respectively. No reduction was observed when Ctr-Ab were used.

Conclusion:

This example demonstrates the efficacy of h-Ab1 in reducing the production of inflammatory cytokines by activated fibroblasts via binding to human recombinant soluble CD40L and thus blocking the CD40:CD40L interaction. The gene expression and protein production was blocked in a dose dependent manner with maximum efficacy of 100%. Activated fibroblasts are key drivers of TED pathogenesis in the orbital cavity and to a large extend responsible for the local inflammation wherefore a positive impact on their inflammatory status is expected to have a large therapeutic impact for TED patients.

10 **Example 6 – Concentration-dependent effect of CD40L inhibitor on inflammatory response in myoblasts**

The aim of this example was to determine the potency of h-Ab1 inhibiting gene expression and secretion of pro-inflammatory cytokines in primary myoblast cells through the inhibition of CD40L interaction with its receptors.

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Materials and methods:

Myoblasts culture and treatment: Human primary myoblasts (Lonza) were plated at a density of 50,000 cells/cm² in complete SkGM-2 media (Lonza). After 3 days in culture, the cell media was changed to DMEM (Gibco) supplemented with 1% FBS (Gibco) and 1 ng/mL TGF-beta1 (R&D Systems) to prevent differentiation of the cells to myotubes. Myoblasts were stimulated with 200 ng/ml IFN-γ (R&D Systems) for 72 hours. Cells not treated with IFN-γ served as controls.

The myoblasts were then treated with 32 nM human recombinant soluble CD40L (Enzo Lifescience) either alone or in the presence of a concentration range of the CD40L inhibitor h-Ab1 (90 pM – 10 μM), or control molecule Ctr-Ab (90 pM – 10 μM) for 4 hours. The human recombinant soluble CD40L was briefly (~5 minutes) pre-mixed with the test compounds before addition to the cells. Cells that had been pre-treated with IFN-γ but did not receive CD40L served as a further control.

30

mRNA quantification by RT-PCR: *CD40*, *IL-6*, *IL-8* and *RANTES* mRNA levels were determined by real-time PCR. After treatment, cells were lysed with 100 μL Homogenization Solution (Promega) and RNA was purified using a MaxWell HT SimplyRNA kit following

manufacturer instructions (Promega). Quantitative PCR was performed on a CFX96 thermocycler (Bio-Rad Laboratories) using PrimeTime One-Step Broad Range Master Mix and pre-designed qPCR probes (Integrated DNA technologies). *GPI* was used as the housekeeping gene and relative expression was calculated using the IFN- γ only treated condition as reference using the $\Delta\Delta C_t$ method (Livak et al. 2001).

Protein quantification by MSD assay: Cell supernatant was collected before and after treatment with soluble CD40L. IL-6 and IL-8 protein present in the supernatant was quantified using a custom-made U-Plex Biomarker (hu) Assay following manufacturer instructions (Meso Scale Discovery).

Data processing and statistical analyses: All data are expressed as mean $\pm$ standard error of mean (SEM) of N=5 (IL-6, IL-8) or N=4 (RANTES) independent experiments, each performed with triplicate wells of cells for each condition. For normalisation of cytokine mRNA expression data, expression relative to the 32 nM CD40L treated condition was set as 100%, while cytokine expression in the IFN- γ only treated control was set as 0%. Similarly, for normalisation of cytokine protein data, cytokine present in the supernatant of cells treated with 32 nM CD40L was set at 100%, while cytokine present in the supernatant of cells treated with IFN- γ only was set at 0%. For comparison of CD40 mRNA expression, data was normalised to the expression in cells treated for 72 hr with IFN- γ and 4 hours with 32 nM CD40L.

Data was fitted to a four parameter logistic (4PL) equation to generate a regression curve in GraphPad Prism version 10 (GraphPad Software, Inc.). The regression curves were used to estimate the IC_{50} (half-maximal inhibitory concentration) value of h-Ab1. Statistical differences between relative expression levels of CD40 were determined by a one-way ANOVA with Dunnett's post-hoc test using GraphPad Prism version 10 (GraphPad Software).

Results:

Myoblasts up-regulate CD40 expression upon IFN- γ stimulation. Expression of CD40 mRNA was significantly ($P < 0.0001$) up-regulated by an average of 10-fold in myoblasts treated for 72 hr by IFN- γ compared to un-treated control levels (FIG 8A). CD40 expression did not significantly change following treatment with 32 nM CD40L either alone or in the

presence of any concentration of either h-Ab1 or Ctr-Ab tested (FIG 8A) (only data for the highest and lowest concentrations of h-Ab1 and Ctr-Ab tested are shown on the graph).

Effect of h-Ab1 treatment on gene expression of pro-inflammatory cytokines. Treatment of myoblasts that had been pre-treated for 72 hr with IFN- γ followed by 32 nM soluble CD40L resulted in the up-regulation of mRNA expression of the pro-inflammatory cytokines IL-6 and IL-8 and the chemokine RANTES (Fig 8B-D). The mRNA up-regulation of IL-6 (Fig 8B), IL-8 (Fig 8C) and RANTES (Fig 8D) was inhibited by h-Ab1 in a concentration-dependent manner. The pIC₅₀ values for inhibition by of mRNA expression h-Ab1 were estimated to be 6.55 $\pm$ 0.11 for IL-6 (mean $\pm$ s.e.m. N=5), 6.45 $\pm$ 0.37 for IL-8 (N=5) and 9.14 $\pm$ 0.21 for RANTES (N=4). This corresponds to IC₅₀ values for h-Ab1 of 280.3 nM (142.0 – 553.5 nM) for IL-6 inhibition, 353.5 nM (32.4 – 3855 nM) for IL-8 inhibition and 0.72 nM (0.15 – 3.49 nM) for RANTES inhibition. (geomean (95% confidence intervals)). No reduction in IL-6, IL-8 or RANTES mRNA expression was observed for the control Ctr-Ab (Fig 8B-D).

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Effect of h-Ab1 treatment on the release of pro-inflammatory cytokines. Treatment of myoblasts that had been pre-treated for 72 hr with IFN- γ followed by 32 nM soluble CD40L resulted in the release of the pro-inflammatory cytokines IL-6 and IL-8 (Fig 9A-B). The release of both IL-6 and IL-8 could be significantly inhibited by h-Ab1 in a concentration-dependent manner with a maximum efficacy of 100% inhibition. In the presence of 90 pM, 90 nM or 10 μ M h-Ab1, respectively, release of IL-6 was 88.2 $\pm$ 8.3% (mean $\pm$ s.e.m, N=4), 50.5 $\pm$ 6.3% (N=5, p<0.001) and -3.8 $\pm$ 2.3% (N=4, p<0.001) compared to release in response to 32 nM soluble CD40L alone (Fig 9A). Similarly, in the presence of 90 pM, 90 nM or 10 μ M h-Ab1, respectively, release of IL-8 was 106.1 $\pm$ 9.9% (mean $\pm$ s.e.m, N=4), 46.9 $\pm$ 5.2% (N=5, p<0.001) and -8.1 $\pm$ 1.1% (N=4, p<0.001) compared to release in response to 32 nM soluble CD40L alone (Fig 9B). No reduction in the release of either IL-6 or IL-8 was observed in the presence of the control, Ctr-Ab (Fig 9A-B).

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Conclusion:

This example demonstrates the efficacy of h-Ab1 in reducing the increased expression and secretion of inflammatory cytokines IL-6 and IL-8 and chemokine RANTES induced by soluble CD40L in myoblasts. By binding to soluble CD40L, h-Ab1 prevents the interaction of CD40L with its receptors. Activated myoblasts are involved in TED pathogenesis in the orbital

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cavity and contribute to the local inflammation. Therefore, a reduction of the inflammatory potential of the myoblasts is expected to have a positive therapeutic impact for TED patients.

Example 7 – CD40L inhibitor blocks innate immune responses in activated PBMCs from TED patients.

In patients with TED, PBMCs have been identified as active contributors to the systemic pathogenesis of the condition. Specifically, activated T-cells expressing CD40L play a crucial role in immune signalling. Other cell types within the PBMC pool, such as B cells, monocytes, and dendritic cells, express CD40 and can get activated by CD40L, to release pro-inflammatory cytokines that significantly impact surrounding tissues. The activated cells and their cytokine products can infiltrate the orbital cavity, where they interact with resident fibroblasts to trigger a cascade of local inflammatory responses, exacerbating the symptoms associated with TED orbitopathy. The aim of this example is to demonstrate the efficacy of a CD40L inhibitor in mitigating the production of inflammatory cytokines by PBMCs.

Materials and methods:

PBMC culture: Cryopreserved PBMCs from 3 TED patients and 3 Healthy volunteers were obtained from Cureline. PBMCs were thawed placing the cryovial in a 37°C water bath, gently agitating it until the contents were mostly thawed. Cells were then transferred to a tube containing 10mL of prewarmed DMEM with 10% FBS and centrifuged for 5 mins at 300 x G. Supernatant was removed and PBMCs were resuspended at 1×10^6 cells/ml. PBMCs were plated at 250.000 cells/cm² in DMEM with 10% FBS in Nunc™ Cell-Culture Treated 24-well plates.

PBMC stimulation and treatment: After 6 hours, PBMCs were stimulated with aCD3/CD28-coated beads (ThermoFisher Scientific) in a 1:1 cell to bead ratio to introduce polyclonal T-cell activation. Cells were stimulated with aCD3/CD28 beads alone or in the presence of 100 nM of either h-Ab1 (CD40L inhibitor) or anti-TNP IgG (negative control) for 24 hours. As controls, cells were left untreated (Naïve) or treated with beads only (Activated).

Flow cytometry analysis: PBMCs were harvested, beads were removed by magnetic separation, and cells were washed with PBS. After centrifugation for 2 minutes at 2600 RPM, 1 µL per million cells of LIVE/DEAD™ Fixable Near-IR Stain (except for FMO LD, where PBS was added) was added and incubated for 10 minutes at room temperature (protected from

light). Next, BD Fc Block™ was added to the cells and incubated for 10 minutes. Then, samples were washed with PBS, centrifuged for 2 mins at 2600 RPM, and BD OptiBuild™ Mouse Anti-Human antibodies (Table 2) were added and incubated for 20 minutes at 4°C and protected from light. Then, samples were washed with PBS twice and 100 µL BD Cytfix™ Fixation Buffer100 were added to the wells, mixed by pipetting, and incubated for 20 minutes at room temperature. Samples were then washed twice with PBS and analyzed in a NovoCyte Advanteon flow cytometer to assess their immunophenotype.

Table 2. Antibodies used for PBMC immunophenotyping by Flow cytometry.

Antigen	Fluorochrome	Dilution
CD40	BV786	1:200
CD40L (CD154)	APC	1:100
CD3	Pe-Cy7	1:300
CD25	BV421	1:200
CD69	PE-CD594	1:200
CD4	BV711	1:150
CD19	AF700	1:200

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RNA Extraction: After treatment, PBMCs were harvested, beads were removed by magnetic separation and cells were lysed with 100 µL of homogenization buffer from Maxwell® RSC simplyRNA Cells Kit. RNA was purified following the manufacturer instructions (Promega) using the Maxwell® RSC Instrument (Promega). RNA quality was assessed using The Agilent 2100 Bioanalyzer system (Agilent). All samples had a RIN value over 9. RNA sequencing was performed by Eurofins to evaluate transcriptional changes.

RNA sequencing analysis: Eurofins Genomics provided the files from RNA sequencing as FASTQ files. The FastQC software was used for assessing the quality of the data, all samples passed QC and were therefore included for downstream analysis. The STAR aligner was used for performing every step from indexing of the genome, mapping and read counting. The reads were mapped to the Homo sapiens reference genome (Ensembl release-101). The differential gene expression analysis was computed using DESeq2 with default parameters, filtering for Log2 fold-change > ±1 and p. adjust <= 0.05. The Variance Stabilizing Transformation (VST)

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was applied to the read counts for some of the downstream analyses and visualizations, using the DESeq2 package. Gene set enrichment analysis was performed using the R package gProfiler2 using as background the expressed genes in the dataset. All plots and downstream analyses were performed in R (v4.0.2).

Results:

Gene Expression Analysis Reveals Immune Activation in Naïve TED PBMCs compared to healthy donors:

RNA-seq analysis revealed distinct transcriptomic profiles among PBMCs, demonstrating significant differences in gene expression based on PBMC origin and activation status. The PCA plot (Figure10A) illustrates these findings, with the second principal component (PC2) distinguishing between healthy individuals and those with TED. Additionally, the first principal component (PC1) effectively separates activated PBMCs from naïve PBMCs, independently of their origin. This separation highlights the unique transcriptomic signatures associated with cellular activation. Examining the specific transcriptional signatures, naïve TED PBMCs exhibit 290 differentially expressed (DE) genes when compared to healthy PBMCs, including several upregulated inflammatory cytokine genes such as IL6, IL1A, IL1B, CXCR3, and CCL20, as well as T-cell modulation-related genes like CD274, PRF1, and ADGRG1 (Figure10 B). In accordance, Gene Set Enrichment Analysis (GSEA) revealed that naïve TED PBMCs demonstrate heightened immune system activation and an upregulation of various inflammation-related pathways, including the Gene Ontology pathway named as “Overview of proinflammatory and profibrotic mediators” which is relevant in TED pathophysiology (Table 3). Table 3 depicts significant pathways identified through GSEA associated with the DE genes in TED PBMCs. The analysis reveals increased immune system activation and upregulation of various inflammation-related pathways, particularly the Gene Ontology pathway "Overview of proinflammatory and profibrotic mediators," which is relevant to the pathophysiology of TED.

Table 3. Top enriched pathways for upregulated Genes in TED Naïve vs Healthy Naïve PBMCs

Pathway	Adjusted P-Value
Overview of proinflammatory and profibrotic mediators	0,001071087

T-cell modulation	0,003119653
GPCRs other	0,004800239
Cytokine and inflammation response	0,00648799
Signal transduction through IL1R	0,015064138
Prostaglandin signalling	0,015064138
Photodynamic therapy induced NFκB survival signalling	0,017041382
Cells and molecules involved in local acute inflammatory response	0,02280362

Incubation of PBMCs with anti-CD3/CD28 beads result in efficient T-cell activation leading to cytokine expression.

Incubation of PBMCs with anti-CD3/CD28 beads for 24h leads to efficient T-cell activation in both naïve and TED samples. This activation is evidenced by an increase in surface markers such as CD69 and CD25 and increased expression of CD40L, which are characteristic of T-effector cells, as demonstrated by FACS analysis. Additionally, activation led to a notable increase in B cells expressing CD40 in TED samples, a change not observed in healthy PBMCs (Figure 11A-E). Transcriptional analysis confirmed broad cell activation following antiCD3/CD28 stimulation (increase in expression level observed for IFNG, TNF, IL2, IL4, IL6, IL9, among others) (Figure 12)

Treatment with CD40L inhibitor blocks innate immune responses in activated PBMCs from TED patients.

While, as anticipated, transcriptional upregulation of CD40L following aCD3/CD28 activation was not blocked upon treatment with CD40L inhibitor (h-Ab1) (data not shown), CD40 expression was significantly downregulated (Figure 13A), potentially altering the interaction between T-cells and antigen-presenting cells. In accordance, treatment with CD40L inhibitor led to a significant reduction in cytokines released by dendritic cells. Specifically, the levels of chemokines CXCL9, CXCL10, and CXCL11 were markedly diminished (Figure 13B). This reduction suggests a potential therapeutic effect in preventing the migration of activated T-cells into the orbital cavity, which is particularly relevant for the pathophysiology of TED, as the infiltrating activated T-cells are associated with the characteristic symptoms such as swelling, proptosis, and ocular muscle involvement. Notably, the expression of T-cell released cytokines, such as IL9 and IL2, were unaffected by CD40L inhibitor treatment (data not shown),

indicating that while CD40L inhibitor effectively modulates dendritic cell activity and cytokine release, it does not impact the intrinsic cytokine production abilities of activated T-cells. Treatment with anti-TNP IgG (negative control) did not change any gene expression patterns.

5 The DE gene analysis showed that other relevant inflammation-associated genes were also significantly downregulated by CD40L inhibitor treatment, including CCL2 (a cytokine involved in the trafficking of activated T-cells to inflammatory sites), ACHE (gene associated with B-cell growth, differentiation, and IgE regulation) and FCER2 (which regulates IgE production, B-cell differentiation, and antigen uptake) (Figure 13C). To explore the pathways
10 related to these DE genes, GSEA showed that treatment of activated TED PBMCs with CD40L inhibitor lead to downregulation of immune system activation and inflammatory pathways (Table 4). GSEA revealed significant downregulation of pathways that play a crucial role in immune response and inflammation such as T cell modulation and complement system pathways.

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Table 4. Top enriched pathways for downregulated genes in activated TED PBMCs after CD40L inhibitor treatment.

Pathway	Adjusted P-Value
T-cell modulation	0,003916698
Complement system	0,009124913
Acetylcholine synthesis	0,083220852
Nod like receptor NLR signalling pathway	0,14972263

Conclusion:

20 These findings collectively highlight an aberrant immune phenotype of TED PBMC and reveals the potential of CD40L inhibition to modulate immune responses by selectively downregulating CD40 expression and reducing pro-inflammatory cytokine release from dendritic cells, thereby potentially limiting the pathological trafficking of T-cells to orbital cavity.

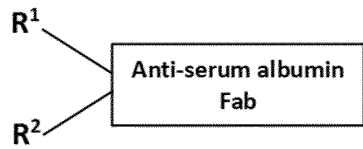
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It should be understood the that embodiments described herein should be considered in a descriptive sense only and not for purposes of limitation. Descriptions of features or aspects within each embodiment should typically be considered as available for other similar features or aspects in other embodiments. While one or more embodiments have been described with reference to the figures, it will be understood by those of ordinary skill in the art that various changes in form and details can be made therein without departing from the spirit and scope of the disclosure as defined by the following claims. All of the various aspects, embodiments, and options described herein can be combined in any and all variations.

All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be herein incorporated by reference.

What is Claimed is:

1. A fusion protein comprising a structure according to formula (I):



Formula (I),

- 5 wherein R1 and R2 are each an anti-CD40L single-chain variable fragment (scFv) linked to an N-terminus of the anti-serum albumin Fab, and wherein each R1 and R2 are linked to a heavy chain variable domain or a light chain variable domain of the anti-serum albumin Fab for use in treatment of thyroid eye disease (TED).

- 10 2. The fusion protein for use according to claim 1, wherein the fusion protein consists of a structure according to formula (I).

3. The fusion protein for use according to any one of the preceding claims, wherein R1 is linked to a heavy chain variable domain of the anti-serum albumin Fab, and wherein R2
15 is linked to a light chain variable domain of the anti-serum albumin Fab.

4. The fusion protein for use according to any one of the preceding claims, wherein each of the R1 and R2 is an anti-CD40L hu5c8 scFv.

- 20 5. The fusion protein for use according to any one of the preceding claims, wherein R1 and R2 each comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of

CDR VL1: RASQRVSSSTYSYMH (SEQ ID NO:3);

CDR VL2: YASNLES (SEQ ID NO:4);

- 25 CDR VL3: QHSWEIPPT (SEQ ID NO:5);

CDR VH1: SYMY (SEQ ID NO:6);

CDR VH2: EINPSNGDTNFNEKFKS (SEQ ID NO:7); and

CDR VH3: SDGRNDMDS (SEQ ID NO:8).

6. The fusion protein for use according to any one of the preceding claims, wherein R1 and R2 each comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of
QVQLVQSGAEVVKPGASVKLSCKASGYIFTSYYMYWVKQAPGQGLEWIGEINPSNG
5 DTNFNEKFKSKATLTVDKSASTAYMELSSLRSEDTA VYYCTRSDGRNDMDSWGQG
TLVTVSS (SEQ ID NO:10).

7. The fusion protein for use according to any one of the preceding claims, wherein R1 and R2 each comprises a light chain variable domain comprising an amino acid sequence
10 having at least 80% identity to the amino acid sequence of
DIVLTQSPATLSVSPGERATISCRASQRVSSSTYSYMHWYQQKPGQPPKLLIKYASNL
ESGVPARFSGSGSGTDFTLTISSVEPEDFATYYCQHSWEIPPTFGGGTKLEIKR (SEQ ID
NO:9).

15 8. The fusion protein for use according to any one of the preceding claims, wherein R1 and R2 each comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:10 and a light chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:9.

20 9. The fusion protein for use according to any one of the preceding claims, wherein the heavy chain variable domain and the light chain variable domain of R1 and R2 are linked by a (G4S)₃ linker comprising the amino acid sequence of SEQ ID NO:23.

25 10. The fusion protein for use according to any one of the preceding claims, wherein each of the R1 and R2 comprises an amino acid sequence having at least 80% identity to SEQ ID NO:11.

30 11. The fusion protein for use according to any one of the preceding claims, wherein each of the R1 and R2 comprises an amino acid sequence of SEQ ID NO:11 or SEQ ID NO:12.

12. The fusion protein for use according to any one of the preceding claims, wherein each of the R1 and R2 comprises an amino acid sequence of SEQ ID NO:11.

13. The fusion protein for use according to any one of the preceding claims, wherein each of R1 and R2 is linked to the anti-serum albumin Fab by one or more linkers.

5 14. The fusion protein for use according to claim 13, wherein each linker comprises 1 to 20 amino acids.

15 15. The fusion protein for use according to any one of claims 13 and 14, wherein each linker comprises an amino acid sequence having at least 90% identity to SEQ ID NO:23
10 or SEQ ID NO:24.

16. The fusion protein for use according to any one of claims 13 to 15, wherein each linker comprises an amino acid sequence of SEQ ID NO:23 or SEQ ID NO:24.

15 17. The fusion protein for use according to any one of the preceding claims, wherein the anti-CD40L scFv is linked to the N-terminus of the heavy chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:23.

20 18. The fusion protein for use according to any one of the preceding claims, wherein the anti-CD40L scFv is linked to the N-terminus of the light chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:24.

25 19. The fusion protein for use according to any one of the preceding claims, wherein a first anti-CD40L scFv is linked to the N-terminus of the heavy chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:23 and a second anti-CD40L scFv is linked to the N-terminus of the light chain of the anti-serum albumin Fab by a linker having an amino acid sequence of SEQ ID NO:24.

30 20. The fusion protein for use according to any one of the preceding claims, wherein the anti-serum albumin Fab comprises complementarity determining domain (CDR) regions comprising the amino acid sequences of

CDR VL1: RASQSVGSNLA (SEQ ID NO:13);

CDR VL2: GASTGAT (SEQ ID NO:14);

CDR VL3: QQYYSFLLAKT (SEQ ID NO:15);
CDR VH1: AYSMN (SEQ ID NO:16);
CDR VH2: SISSSGRYIHYADSVKG (SEQ ID NO:17); and
CDR VH3: ETVMAGKALDY (SEQ ID NO:18).

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21. The fusion protein for use according to any one of the preceding claims, wherein the anti-serum albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of QVQLVQSGGPGVKPGGSLRLSCAASGFMFRAYSMNWVRQAPGKGLEWVSSISSGR
10 YIHYADSVKGRFTISRDNALNSLYLQMNSLRAEDTAVYYCARETVMAGKALDYWG QGTLVTVSS (SEQ ID NO:19).

22. The fusion protein for use according to any one of the preceding claims, wherein the anti-serum albumin Fab comprises a light chain variable domain comprising an amino acid
15 sequence having at least 80% identity to the amino acid sequence of DIVLTQSPGTLSPGETATLSCRASQSVGSNLAWYQQKPGQAPRLLIYGASTGATG VPARFSGSRSGTDFTLTITSLQPEDFATYYCQQYYSFLLAKTFGQGTQLEIKR (SEQ ID NO:20).

20 23. The fusion protein for use according to any one of the preceding claims, wherein the anti-serum albumin Fab comprises a heavy chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:19 and a light chain variable domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:20.

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24. The fusion protein for use according to any one of the preceding claims, wherein the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:21 (VH-CH1 domain).

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25. The fusion protein for use according to any one of the preceding claims, wherein the anti-serum albumin Fab comprises a light chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:22 (VL-CL domain).

26. The fusion protein for use according to any one of the preceding claims, wherein the anti-serum albumin Fab comprises a heavy chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:21 (VH-CH1 domain) and a light chain domain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:22 (VL-CL domain).

27. The fusion protein for use according to any one of the preceding claims, wherein the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:1.

28. The fusion protein for use according to any one of the preceding claims, wherein the fusion protein comprises a light chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:2.

29. The fusion protein for use according to any one of the preceding claims, wherein the fusion protein comprises a heavy chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:1 and a light chain comprising an amino acid sequence having at least 80% identity to the amino acid sequence of SEQ ID NO:2.

30. The fusion protein for use according to any one of the preceding claims, wherein the fusion protein comprises a heavy chain comprising an amino acid sequence of SEQ ID NO:1 and a light chain comprising an amino acid sequence of SEQ ID NO:2.

31. The fusion protein for use according to any one of the preceding claims, wherein the fusion protein is administered in a pharmaceutical formulation.

32. The fusion protein for use according to any one of the preceding claims, wherein the fusion protein is administered by transcutaneous, subcutaneous, intravenous, or intramuscular administration.

33. The fusion protein for use according to any one of the preceding claims, wherein the fusion protein is administered in an amount of about 0.01 mg/kg to about 100 mg/kg body weight of the subject.

5 34. The fusion protein for use according to any one of the preceding claims, wherein the treatment results in treatment of orbitopathy of TED.

35. The fusion protein for use according to claim 34, wherein the treatment of the orbitopathy of TED results in a reduction in inflammation in the periorbital space.

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36. The fusion protein for use according to any one of the preceding claims, wherein the treatment results in treatment of systemic pathophysiology of TED.

15 37. The fusion protein for use according to claim 36, wherein the treatment of the systemic pathophysiology of TED results in deactivation of PBMCs and a decrease in cytokine expression.

38. The fusion protein for use according to any one of the preceding claims, wherein the treatment results in blocking of the innate immune response in PBMCs.

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39. The fusion protein for use according to claim 38, wherein the treatment results in a reduced CD40 expression in the PBMCs.

25 40. The fusion protein for use according to claim 38, wherein the treatment results in a reduced CXCL9, CXCL10, and/or CXCL11 expression in dendritic cells.

41. The fusion protein for use according to claim 38, wherein the treatment results in a downregulation of inflammation-associated genes in PBMCs, such as downregulation of CCL2, ACHE, and/or FCER2 genes.

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42. The fusion protein for use according to any one of claims 36 to 41, wherein the treatment of the systemic pathophysiology of TED results in a decrease in autologous immune cell infiltration in the periorbital space.

43. The fusion protein for use according to any one of the preceding claims, wherein the treatment results in treatment of the systemic pathophysiology of TED and in treatment of the orbitopathy of TED.

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44. The fusion protein for use according to any one of the preceding claims, wherein the treatment results in a reduction of proptosis.

45. The fusion protein for use according to any one of the preceding claims, wherein
10 the treatment results in a reduction of diplopia.