Rheumatoid arthritis (RA) is an autoimmune disease characterized by progressive disability caused by chronic inflammation (Cutolo et al., 2021). The immunopathology of RA is complex and involves different cell populations that are involved in immune/inflammatory responses and therefore, remains to be fully elucidated (Cutolo et al., 2015). Among the various inflammatory cells involved in RA, macrophages appear to play a central role in disease progression (Cutolo, 1999; Szekanecz and Koch, 2005; Mills, 2015). Macrophages invading synovial tissues release cytokines, mainly tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 (Mantovani et al., 2004; Udalova et al., 2016; Cutolo et al., 2021). The pro-inflammatory cytokines in the synovial tissues induce the activation of endothelial cells which in turn express adhesion molecules that promote the recruitment of inflammatory cells (Furuzawa-Carballeda et al., 2010; Cutolo and Nadler, 2013). The autoantigen in RA is unknown, but type II collagen (the main component of articular cartilage) is a candidate. In the disease process, the activation of T cells plays an important role, as activated T cells can activate monocytes, macrophages, synovial fibroblasts, and B cells (Goldring and Gravallese, 2000; Hoffman, 2001), which are involved in autoimmune diseases. Therefore, inhibiting T-cell activation is one of the current therapeutic strategies used in RA patients. Under physiological conditions, T-cell activation requires the binding of the T-cell receptor (TCR) to a specific major histocompatibility complex peptide on antigen-presenting cells (APCs). In addition, to produce an effective immune response, inhibiting T-cell activation plays an important role in RA. Therefore, inhibiting T-cell activation is one of the current therapeutic strategies. Cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4-Ig), also known as abatacept, reduces cytokine secretion by inhibiting T-cell activation. To achieve a homeostatic therapeutic effect, CTLA4-Ig has to be administered repeatedly over several weeks, which limits its applicability in RA treatment. To overcome this limitation, we increased the number of sialic acid-capped antennas by genetically engineering the CTLA4 region to increase the therapeutic effect of CTLA4-Ig. N-acetylgalcosaminyltransferase (GnT) and α2,6-sialyltransferase (α2,6-ST) were co-overexpressed in Chinese hamster ovary (CHO) cells to generate a highly sialylated CTLA4-Ig fusion protein, named ST6. The therapeutic and immunogenic effects of ST6 and CTLA4-Ig were compared. ST6 dose-dependently decreased paw edema in a mouse model of collagen-induced arthritis and reduced cytokine levels in a co-culture cell assay in a similar manner to CTLA4-Ig. ST6- and CTLA4-Ig-induced T cell-derived cytokines were examined in CD4 T cells isolated from peripheral blood mononuclear cells after cell killing through irradiation followed by flow- and magnetic-bead-assisted separation. Interestingly, compared to CTLA4-Ig, ST6 was substantially less immunogenic and more stable and durable. Our data suggest that ST6 can serve as a novel, less immunogenic therapeutic strategy for patients with RA.

Key Words: Rheumatoid arthritis, CTLA-4Ig (abatacept), Cytokine, Immunogenicity, ST6, Inflammation

A Novel Therapeutic Effect of a New Variant of CTLA4-Ig with Four Antennas That Are Terminally Capped with Sialic Acid in the CTLA4 Region

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the binding of different costimulatory molecules is required for the activation of T lymphocytes (Alegre et al., 2001). CD28 is the most characteristic and important costimulatory molecule expressed in naive and primed T cells (Sharpe, 2009).

Human cytotoxic T-lymphocyte antigen 4-immunoglobulin (hCTLA4-Ig), a licensed drug prescribed for the treatment of RA known as abatacept, is a fusion protein that regulates T cell-costimulatory signaling via the CD28:CD80/86 pathway (Ruderman and Pope, 2005). Therefore, the CD28:CD80/86 costimulatory pathway, which appears to play a key role in T-cell activation following interaction with APCs, is a potential therapeutic target for the treatment of RA. However, CTLA4 is a protein receptor that functions as an immune checkpoint to downregulate the immune system. CTLA4 is expressed on the surface of T cells and acts as an “off” switch when bound to CD80 or CD86 on the surface of APCs (Cutolo et al., 2015). Interestingly, unexpected effects arising from the interaction between CTLA4-Ig and the CD80/86 complex have been observed (Cutolo and Nadler, 2013). Initially, it was suggested that CTLA4-Ig might exert its therapeutic effects by modulating effector cell-costimulatory signaling via the CD28:CD80 pathway. However, it was recently shown that CTLA4-Ig has effects on the surface of APCs (Cutolo et al., 2015).

The overexpression of antibodies containing CTLA4 carrying four antennas in a sialyltransferase (GnT) and α2,6-sialyltransferase (α2,6-ST) and named them CHO GnT-IV∙ST cells (GnT-IV refers to the number of antennas formed on GnT, which is four in this case). The cells were further engineered to overexpress a human form of CTLA4-Ig and cultured in ProCHO5 medium (Lonza, Verviers, Belgium) supplemented with 4 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA). We termed the recombinant human CTLA4-Ig protein, which has four antennas terminally capped with sialic acid, “ST6.” It should be noted that the N-glycan structure of the CTLA4 region of ST6 is different from that of abatacept, which mainly consists of bi-antennary (GnT-II) and tri-antennary (GnT-III) structures (Bora de Oliveira et al., 2017). The overexpression of α2,6-ST serves to enhance the sialylation of various glycoproteins, while the overexpression of α2,6-ST serves to mediate anti-inflammatory effects and facilitate human-like glycosylation (Chung et al., 2017). The sialic acid caps in the CTLA4 region improve the half-life of the protein (Ngantung et al., 2006). To enhance the therapeutic effect of CTLA4-Ig, CTLA4 has to be modified with an optimal number of antennas that are terminally capped with sialic acid (Lim et al., 2022).

In the present study, we investigated the anti-inflammatory effect of ST6 containing CTLA4 carrying four antennas in a co-culture assay of THP-1 macrophages and Jurkat T cells and compared the results with those of CTLA4-Ig (abatacept). In addition, we comparatively investigated the immunogenicity responses induced by CTLA4-Ig and ST6 in CD4 T cells isolated from human peripheral blood mononuclear cells (PBMCs) by measuring the levels of the T cell-derived cytokines IL-2, IFN-γ, and TNF-α. The purpose of our study was to investigate the direct effects of CTLA4-Ig and ST6 treatments on the T-cell phenotype and the therapeutic potential of ST6 for the treatment of patients with RA. Our data suggest that ST6 may exert a novel therapeutic effect in patients with RA.

MATERIALS AND METHODS

Reagents

CTLA4-Ig (abatacept) (2 mg/mL) and ST6 were kindly provided by Prof. D. I. Kim of Inha University (Incheon, Korea). For injection, all agents were diluted to the proper concentration in sterile phosphate-buffered saline (PBS) (Invitrogen/Gibco, Carlsbad, CA, USA). All chemicals used in the experiments were of analytical grade. CTLA4-Ig is a soluble fusion protein that consists of the extracellular domain of human CTLA4 linked to the modified Fc (hinge, CH2, and CH3 domains) portion of human immunoglobulin G1 (IgG1). It is produced using recombinant DNA technology with the CHO cell expression system. Its apparent molecular weight is 92 kDa. CTLA4-Ig is formulated as a sterile, white, preservative-free, lyophilized powder for intraperitoneal (i.p.) administration. Following reconstitution with 1 mL of sterile water, the solution is clear and colorless to pale yellow and has a pH of 7.4. Each single-use vial contains 20 mg CTLA4-Ig, 40 mg mannitol, 10 mg sucrose, and 1.2 mg Tris (hydroxymethyl) aminomethane.

Experimental animals and model establishment

Six-week-old female Dark Agouti (DA) rats (200-220 g weight) were purchased from Samtako (Osan, Korea). The animals were housed under specific pathogen-free conditions and a 12-hr light-dark cycle. All animal experiments were performed according to the National Research Council’s Guidelines for Animal Experiments of Chung-Ang University (Seoul, Korea) and were approved by the university committee for animal experiments. The animals were allowed to acclimate for more than one week before use in the experiments. For collagen-induced arthritis (CIA) induction, the mice were weighed (day –1) and the following day (day 0), they were immunized subcutaneously (s.c.) with 300 µL of an emulsion containing immunization-grade bovine type II collagen (Col II) and incomplete Freund’s adjuvant (IFA) (Sigma-Aldrich) at the base of the tail. The swelling of each hind paw was evaluated every three days. Drug administration was started on day 1, and 100 µL of CTLA4-Ig or ST6 was injected via the i.p. route every three days, for a total of 15 times. On the final day of the experiment, the animals were sacrificed and blood was sampled and the hind ankles of the rats were collected in 10% formalin.

Histological examination

The formalin-fixed samples were decalcified, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H&E) as described previously (Liu et al., 2019) for histological examination. Sample preparation was outsourced to the Korea Pathology Technical Center (Cheonji, Korea).

Enzyme-linked immunosorbent assay (ELISA)

IL-6 and TNF-α cytokine levels secreted into culture media and collagen-specific IgG and total IgG levels in blood were determined using the Quantikine® ELISA kit (R&D Systems, MN, USA) per the manufacturer’s instructions. THP-1 and Jurkat cells were seeded in triplicate into 6-well plates for 1:1 co-culture at 1×10⁶ cells of each line per well in 2 mL of medium. The cells were incubated at 37°C in a 5% CO₂ atmosphere for 24 h. After 1, 3, and 24 h of incubation, 1 µg/mL LPS and 10 µg/mL or 100 µg/mL of CTLA4-Ig or ST6 was added to each
well. After 24 h, the cells were centrifuged at 10,000×g at 4°C for 5 min. Secreted IL-6 and TNF-α cytokine levels in the supernatants were determined using the Quantikine® kit (R&D Systems). The absorbance at 450 nm was measured using a FlexStation® 3 Multi-Mode Microplate Reader (Molecular Devices, San Jose, CA, USA). To measure serum Ig levels, the blood samples were centrifuged at 10,000×g at 4°C for 5 min. Collagen-specific IgG and total IgG levels were assayed using the Quantikine® kit (R&D Systems).

**Cell culture**

To determine the most appropriate stimulus for inducing a pro-inflammatory condition, cultured THP-1 human monocytic leukemia cells were differentiated into macrophages by treating them with phorbol myristate acetate (PMA, 0.5 µg/mL, Sigma-Aldrich) for 48 h. Then, they were treated with 1 µg/mL lipopolysaccharide (LPS) (Sigma-Aldrich) for 1, 3, or 24 h. The THP-1 cells were cultured in Roswell Park Memorial Institute medium (RPMI) 1640 medium (Thermo Scientific, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Waltham, MA, USA) and supplemented with 0.055 mM 2-mercaptoethanol (Gibco) at 37°C in humidified air with 5% CO2. Jurkat T cells (clone E6-1) were purchased from the American Type Cell Culture (Manassas, VA, USA) and cultured in RPMI 1640 medium (Thermo Scientific) supplemented with 10% FBS, 1% penicillin/streptomycin (Gibco), and 10 mM HEPES buffer (Sigma-Aldrich) at 37°C in humidified air with 5% CO2. The cells were cultured as previously reported (Guse et al., 1999). Cultured Jurkat T cells were stimulated to differentiate into T cells by treating them with concanavalin A (5 µg/mL, Sigma-Aldrich) for 24 h. Then, the cells were treated with 1 µg/mL LPS (Sigma-Aldrich) for 1, 3, or 24 h. Cultured THP-1 and Jurkat cells were seeded in triplicate in 6-well plates for 1:1 co-culture. Co-culture supernatants, containing IL-1β, were added to HEK-Blue™ IL-1β cells (InvivoGen, San Diego, CA, USA), which are highly responsive to IL-1β, and then cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. In the presence of IL-1β, HEK-Blue™ IL-1β cells activate the NF-κB pathway and subsequently express secreted embryonic alkaline phosphatase (SEAP). The amount of SEAP in HEK-Blue™ IL-1β cells supernatants was measured using QUANTI-Blue™ solution.

**Flow-cytometric analysis**

After culturing human PBMCs in a T75 flask, CD4 T cells were isolated using CD4 T cell beads via magnetic-activated cell sorting. The remaining cells were collected and exposed to 10-Gy γ-rays to inactivate the B cells. The isolated CD4 T cells were seeded in 6-well plates at 1×10⁵ cells per well and cultured for 24 h, and then incubated with 5 µg/mL CTLA4-Ig or 5, 10, or 20 µg/mL of ST6 for 24 h. The cells were harvested, washed with PBS, first stained with human BD Fc BlockTM (anti-CD16/32; BD Biosciences, Franklin Lakes, NJ), and then cultured for 24 h, and then incubated with 5 µg/mL LPS (Sigma-Aldrich) for 1, 3, or 24 h. Cultured THP-1 and Jurkat cells were seeded in triplicate in 6-well plates for 1:1 co-culture. Co-culture supernatants, containing IL-1β, were added to HEK-Blue™ IL-1β cells (InvivoGen, San Diego, CA, USA), which are highly responsive to IL-1β, and then cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% FBS and 1% penicillin/streptomycin. In the presence of IL-1β, HEK-Blue™ IL-1β cells activate the NF-κB pathway and subsequently express secreted embryonic alkaline phosphatase (SEAP). The amount of SEAP in HEK-Blue™ IL-1β cells supernatants was measured using QUANTI-Blue™ solution.

**Fig. 1.** CTLA4-Ig reduces collagen IgG in CIA mice and protects against CIA. (A) Timeline of the animal experiment. Female DA rats of 6 weeks of age were used in the experiments. CIA was induced as described in the Materials and Methods. (B) H&E staining of the hind paws. The joint structure was nearly normal (black arrows) in the normal control group. Synovial inflammation (yellow arrows) and narrow joint spaces (green arrows) were observed in the control group. No obvious cartilage erosion was observed in the dexamethasone (DEX) groups, but mild synovial inflammation (blue arrows) was observed in the CTLA4-Ig group. Representative images of histological sections of the paws acquired using an optical microscope (200×) are shown. (C) Effects of the treatments on body weight. (D) Effects of the treatments on edema scores. (E, F) Serum IgG1 levels (E) and total IgG (F) after the treatments as determined by ELISA. Data are the mean ± SD, n=six mice per group, ***p<0.0001, **p<0.001, *p<0.05. vs. the control group. The results show that CTLA4-Ig reduces RA swelling and suppresses serum IgG levels.
USA) to block the Fc receptor and then with 5 mM carboxyfluorescein succinimidyl ester (CFSE) using a CFSE cell proliferation kit (American Expression Vector, Carlsbad, CA, USA) to assess cell proliferation. The cells were washed three times with fluorescence-activated cell sorting (FACS) buffer (PBS) and resuspended in 500 µL of FACS buffer. The cells were analyzed by flow cytometry using a BD Flow Cytometer (BD Biosciences, Erembodegem, Belgium).

**Immunogenicity assay**

Human PBMCs were seeded in triplicate in 6-well plates at 1×10⁶ cells per well in 2 mL of medium. The cells were cultured in RPMI 1640 medium containing 10% human AB serum (Gibco) supplemented with 1% penicillin/streptomycin at 37°C in a 5% CO₂ atmosphere for 24 h. Then, T cells and B cells were separated using a magnetic cell separation column. The B cells were inactivated using 10-Gy γ-ray radiation at the Korea Institute of Radiological & Medical Sciences (KIRMS, Seoul, Korea) (Walcher et al. 2021). Inactivated B cells and isolated T cells were co-cultured for 24 h and then incubated with CTLA4-Ig (5 µg/mL) or ST6 (5, 10, 20 µg/mL) for 24 h. Then, the cells were centrifuged at 500×g for 5 min. α at 4°C for 5 min.

**RESULTS**

**CTLA4-Ig suppresses the production of anti-collagen IgG1 and reduces total anti-collagen IgG in CIA model mice**

First, the effect of CTLA4-Ig (abatacept) on RA was investigated using a CIA mouse model. CIA was induced by immunization with Col II emulsified in IFA on day –1 (Brand et al., 2007). As of day 1, the CIA mice were injected i.p. with 2 mg/kg CTLA4-Ig or 3 mg/kg dexamethasone every two days. The thickness of the hind paws of the mice was first observed after two days and was subsequently measured once every two days. The timeline of the animal experiment is shown in Fig. 1A. Histological analysis of the ankle joints showed that IFA challenge caused typical inflammation characterized by cartilage erosion, synovial hyperplasia, and infiltration of multiple types of inflammatory cells, when compared with no treatment (Fig. 1B). Treatment with CTLA4-Ig significantly alleviated arthritis in mice. Dexamethasone was efficacious, but induced weight loss in mice as compared with no treatment. CTLA4-Ig was similarly effective, but did not affect body weight (Fig. 1C).

**Statistical analysis**

Data are presented as the mean ± SD of at least three independent experiments performed in triplicate. Means were compared using one-way analysis of variance. p-values<0.05 were considered statistically significant.

![Image](https://doi.org/10.4062/biomolther.2022.071)
Table 1. List of edema scores data

|       | Normal               | Control               | Dexamethasone          | CTLA4-Ig (0.5 mg/kg) | CTLA4-Ig (2 mg/kg) | ST6 (0.5 mg/kg)  |
|-------|----------------------|-----------------------|-----------------------|----------------------|-------------------|-----------------|
| Day   | Mice 1 | Mice 2 | Mice 3 | Mice 4 | Mice 5 | Mice 6 | Mice 1 | Mice 2 | Mice 3 | Mice 4 | Mice 5 | Mice 6 | Mice 1 | Mice 2 | Mice 3 | Mice 4 | Mice 5 | Mice 6 | Mice 1 | Mice 2 | Mice 3 | Mice 4 | Mice 5 | Mice 6 |
| 11    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 14    | 0      | 0      | 0      | 0      | 0      | 0      | 2      | 1      | 2      | 3      | 3      | 2      | 3      | 3      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      |
| 17    | 0      | 0      | 0      | 0      | 0      | 0      | 2      | 1      | 1      | 2      | 1      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      |
| 20    | 0      | 0      | 0      | 0      | 0      | 0      | 2      | 1      | 1      | 2      | 1      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      |
| 23    | 0      | 0      | 0      | 0      | 0      | 0      | 2      | 1      | 1      | 2      | 1      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      |
| 26    | 0      | 0      | 0      | 0      | 0      | 0      | 2      | 1      | 1      | 2      | 1      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      | 2      |
| 11    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 14    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 17    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 20    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 23    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 26    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 11    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 14    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 17    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 20    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 23    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 26    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 11    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 14    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 17    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 20    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 23    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
| 26    | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      | 0      |
In the control group, swelling of the hind paw peaked on day 25, but both CTLA4-Ig and dexamethasone suppressed the swelling (Fig. 1D). This finding was consistent with the lower levels of inflammation in the treated mice as revealed by histological analysis (Fig. 1B). As the CIA model is characterized by a potent antigen-specific humoral immune response (Song et al., 2015), we next measured serum levels of collagen-specific antibodies. The results showed that treatment with CTLA4-Ig and dexamethasone at a 100-fold dilution significantly reduced the serum levels of collagen-specific IgG and total IgG ($p<0.05$) (Fig. 1E, 1F). These results indicated that treatment with CTLA4-Ig and dexamethasone significantly inhibits the humoral immune response in CIA model mice.

**Table 1.** Continued

| Day | Mice 1 | Mice 2 | Mice 3 | Mice 4 | Mice 5 | Mice 6 |
|-----|--------|--------|--------|--------|--------|--------|
| 11  | 0      | 0      | 0      | 0      | 0      | 0      |
| 14  | 0      | 0      | 0      | 0      | 0      | 0      |
| 17  | 0      | 0      | 0      | 0      | 0      | 0      |
| 20  | 0      | 0      | 0      | 0      | 0      | 0      |
| 23  | 0      | 0      | 0      | 0      | 0      | 0      |
| 26  | 0      | 0      | 0      | 0      | 0      | 0      |

**Fig. 3.** Construction of an anti-inflammatory efficacy evaluation method using THP-1, Jurkat, and HEK-Blue™ IL-1β cells. THP-1 cells were seeded in a 6-well plate at 1×10^5 cells/well in RPMI 1640 medium and incubated with PMA (0.5 µg/mL) at 37°C in the presence of 5% CO2 for 48 h to induce differentiation into macrophages. Jurkat cells, activated with concanavalin-A (5 µg/mL, 24 h), were added to the differentiated THP-1 cells at 1×10^5 cells/well. The cells were incubated with CTLA4-Ig or ST6 at the indicated concentrations for 24 h and then treated with 1 µg/mL LPS for 1, 3, or 24 h. Then, 500 µL of the medium was collected and transferred into a 6-well plate containing HEK-Blue™ IL-1β cells (1×10^5 per well). The cells were incubated at 37°C in the presence of 5% CO2 for 24 h. Then, the SEAP assay was performed to evaluate IL-1β production. IL-6 and TNF-α levels in the THP-1/Jurkat medium were measured by ELISA to ensure the release of inflammatory cytokines during the activation of the APCs.

**ST6 inhibits paw edema as effectively as CTLA4-Ig in CIA model mice**

To investigate whether ST6 has therapeutic potential, we examined its effects on RA in CIA model mice and compared the results with those of CTLA4-Ig and dexamethasone. First, the effects of the treatments on body weight were determined. Dexamethasone was used as a positive control as it was demonstrated to reduce mouse body weight. While dexamethasone reduced body weight in the CIA mice, mice treated with CTLA4-Ig or ST6 did not show any significant change in body weight as compared with control mice (Fig. 2A). Histological analysis of the ankle joints of the hind paws showed that CIA induction resulted in typical inflammation characterized by cartilage erosion, synovial hyperplasia, and infiltration of multiple types of inflammatory cells as compared with no treatment (Fig. 2B). CIA was scored according to Mouse CIA Scoring...
ST6 suppresses LPS-induced production of TNF-α and IL-6 in a co-culture system of THP-1 and Jurkat cells

Central to the pathogenesis of RA are several pro-inflammatory cytokines, such as TNF-α and IL-6, which drive the inflammatory and destructive processes that are characteristic of the disease (Smolen and Maini, 2006; Page et al., 2010). To examine whether ST6 affects the production of pro-inflammatory cytokines, we measured the levels of TNF-α and IL-6 produced in an in vitro co-culture system of THP-1 and Jurkat cells as previously reported (Anderson et al., 1996; Page et al., 2010) and we compared the results with those of CTLA4-Ig and dexamethasone. A schematic representation of the THP-1, Jurkat, and HEK-Blue™ IL-1β cell co-culture system used to evaluate anti-inflammatory cytokine production is shown in Fig. 3. Treatment with ST6 significantly suppressed the LPS-induced increases in the levels of TNF-α and IL-6 over time, as measured by ELISA (Fig. 4). ST6 was similarly effective as CTLA4-Ig in inhibiting the production of IL-6 and TNF-α. Both ST6 and CTLA4-Ig showed a dose-dependent effect, and the effects were similarly sustained for the indicated periods, irrespective of the duration of the treatment (Fig. 4).

DISCUSSION

RA is a chronic disease that affects 0.5% to 1.0% of the global population. It is associated with pain, fatigue, disability, and functional loss, which lead to a substantial decrease in the patient’s health-related quality of life and to considerable economic and societal burdens (Blumenauer et al., 2003; Davidson et al., 2014; Davignon et al., 2018). Therefore, we evaluated the antigenicity of ST6 as a protein drug. Using ELISA, we measured the amounts of IL-2, IFN-γ, and TNF-α secreted into the culture medium by CD4 T cells treated with 20 µg/mL CTLA4-Ig or 20 µg/mL ST6. The levels of all three major cytokines were lower for cells treated with ST6 than for cells treated with CTLA4-Ig (Fig. 5C-5E), demonstrating that ST6 is superior to CTLA4-Ig in terms of immunogenicity, particularly in the short term (within three days). Together, these data imply that ST6 may be a suitable replacement drug for CTLA4-Ig that promises a safer treatment for RA patients.

ST6 induces lower immunogenicity than CTLA4-Ig in isolated CD4 T cells

Cell proliferation CD4 T cells isolated from human PBMCs was analyzed by flow cytometry following staining with CFSE. The gating strategy used is shown in Fig. 5A. The results showed that treatment with 5 µg/mL CTLA4-Ig or 5, 10, or 20 µg/mL ST6 did not affect the proliferation of the CD4 T cells (Fig. 5B). IL-2 is known to promote T-cell expansion (Smith, 1988; Whitfield et al., 2017). In addition, T cells are targeted by TNF-α, either directly, as all cells that express TNF-α receptors are, or indirectly, as a result of antigen presentation or co-stimulation. The immunomodulatory role of TNF-α receptor 2 on T-cell activity in the CIA model of arthritis has been described (McCann et al., 2014; Davignon et al., 2018). Therefore, we examined the antigenicity of ST6 as a protein drug. Using ELISA, we measured the amounts of IL-2, IFN-γ, and TNF-α secreted into the culture medium by CD4 T cells treated with 20 µg/mL CTLA4-Ig or 20 µg/mL ST6. The levels of all three major cytokines were lower for cells treated with ST6 than for cells treated with CTLA4-Ig (Fig. 5C-5E), demonstrating that ST6 is superior to CTLA4-Ig in terms of immunogenicity, particularly in the short term (within three days). Together, these data imply that ST6 may be a suitable replacement drug for CTLA4-Ig that promises a safer treatment for RA patients.
Lagana et al., 2009; Huang et al., 2021). Owing to the continuous development of disease-modifying antirheumatic drugs (DMARDs), significant success has been achieved in preventing and relieving disease activity in RA patients. Unfortunately, a proportion of patients show a limited response to DMARDs (Huang et al., 2021).

In this study, we compared the therapeutic potentials of CTLA4-Ig, known as abatacept, and ST6, a variant of the CTLA4-Ig fusion protein having more sialic acid caps, for RA treatment. ST6 is a novel fusion protein generated via overexpression of GnT, α2,6-ST, and CTLA4-Ig in CHO cells (Lim et al., 2022). CTLA4-Ig is widely regarded as the first in a new class of drugs for the treatment of RA and as a co-stimulation blocker. It was approved in the USA for use in RA treatment.
in 2005 (Ozen et al., 2019). The binding of CTLA4-Ig to CD80 and CD86 induces the production of indoleamine-2,3-dioxygenase by APCs, a process that is associated with the down-regulation of the inflammatory responses of T cells, dendritic cells, and macrophages (Mellor and Munn, 1999; Tian et al., 2002; Kremer et al., 2003).

TNF-α is considered the major inflammatory cytokine involved in the pathogenesis of RA as it is found in large quantities in patients with the disease. Inflammation is associated with the accumulation of inflammatory cells, predominantly type 1 helper T cells and macrophages (Choy and Panayi, 2001; Jang et al., 2021). IL-6 promotes T-cell differentiation and collaborates with TNF-α and IL-1 to induce a systemic inflammatory response (Kitaori et al., 2009; Mustafa et al., 2021). The role of IL-6 in conjunction with TNF-α has been studied in humans as well as in various animal models to elucidate the mechanism of autoimmunity (Li et al., 2017). For better treatment of autoimmune disorders, there is a need for new drugs that regulate cytokine signaling and thus reduce inflammatory responses (Mustafa et al., 2021). In a clinical trial of CTLA4-Ig administered s.c., treatment reintroduction after three-month discontinuation, immunogenicity was observed in approximately 10% of patients (Kaine et al., 2012). Thus, there is a clear need for drugs that are more stable and less immunogenic.

The immune checkpoint CTLA4 is induced upon T-cell activation, but degrades quickly. This degradation process has been targeted in the clinical therapy of advanced cancers and autoimmune diseases (Cui et al., 2020). However, it is unknown whether inhibiting CTLA4 degradation ameliorates RA. Recent research revealed that the T cells of lipopolysaccharide-responsive beige-like anchor protein (LRBA)-deficient patients undergo accelerated CTLA4 degradation, which leads to life-threatening infiltration and autoimmune diseases (Lo et al., 2015). CTLA4 expressed on T cells is highly endocytic. CTLA4 is expressed constitutively in regulatory T cells or induced via CD28 and TCR signaling following T-cell activation. LRBA and activator protein 1 (AP1) have been found to bind to CTLA4 motifs, which appear to impose different fates on CTLA4. LRBA may mediate the recycling of CTLA4 to the plasma membrane, whereas AP1 may mediate CTLA4 trafficking to lysosomal compartments for degradation (Rowshanravan et al., 2018). Several pathways exist for the degradation of CTLA4. The formation of the CTLA4-Ig antigen-antibody complex leads to the degradation of CTLA4 and the CTLA4-Ig protein itself induces antigenic action, leading to a reduction in or degradation of autologous CTLA4 activity.

To prevent the rapid degradation of CTLA4-Ig, we generated a genetically engineered ST6 protein by overexpressing GnT, α2,6-ST, and CTLA4-Ig in CHO cells. Sialylation generally determines a glycoprotein’s circulatory lifetime as sialic acid can prevent the recognition of the glycoprotein by asialoglycoprotein receptors (Weiss and Ashwell, 1989). Thus, the number of antennas and number of sialic acid residues in a glycoprotein play an important role in the clearance of CTLA4-Ig fusion protein (Jing et al., 2010). CTLA4-Ig primarily has two- to three-antennary structures in the CTLA4 region (Bora de Oliveira et al., 2017). Therefore, a recent study investigated the co-overexpression of GnT and α2,6-ST as a means to produce a highly sialylated CTLA4-Ig fusion protein (Lim et al., 2017; Lim et al., 2022). The authors successfully created a four-antenna-structured fusion protein with an increased number of sialic acids in the CTLA4 region by covering the antenna tips with sialic acids (Lim et al., 2017, 2022). We expected that ST6 would have a better therapeutic effect than CTLA4-Ig in RA treatment; however, the results showed that ST6 has similar effects as CTLA4-Ig when used at the same concentration. Interestingly, not only did ST6 not affect the proliferation of isolated CD4 T cells, it also showed less immunogenicity than CTLA4-Ig as indicated by the lower levels major inflammatory cytokine secretion in vitro. In addition, ST6 showed a similar therapeutic effect as CTLA4-Ig in animal experiments. However, at present, it is not known whether the observed beneficial effect is rendered by the sialic acids. ST6, a novel highly sialylated form of CTLA4-Ig, is a potentially better therapeutic option than CTLA4-Ig (abatacept) for the treatment of RA.

CONFLICT OF INTEREST

None of the authors have conflicts of interest to declare.

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