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ORIGINAL RESEARCH ARTICLE

Evaluation of Antibody Properties and Clinically Relevant Immunogenicity, Anaphylaxis, and Hypersensitivity Reactions in Two Phase III Trials of Tralokinumab in Severe, Uncontrolled Asthma

Mats Carlsson[1] · Martin Braddock[2] · Yuling Li[3] · Jihong Wang[3] · Weichen Xu[3] · Nicholas White[4] · Ayman Megally[5] · Gillian Hunter[6] · Gene Colice[5]

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Abstract

Introduction Tralokinumab is a monoclonal antibody (mAb) that neutralizes interleukin (IL)-13, a cytokine involved in the pathogenesis of asthma.

Objective The objectives of this study were to characterize the potential immunogenic properties of tralokinumab and report data for anti-drug antibodies (ADAs) and hypersensitivity reactions from two phase III clinical trials.

Methods The oligosaccharide structure of tralokinumab, Fab-arm exchange, and ADAs were characterized by standard techniques. Hypersensitivity adverse events (AEs) were evaluated in two pivotal clinical trials of tralokinumab in severe, uncontrolled asthma: STRATOS 1 and 2 (NCT02161757 and NCT02194699).

Results No galactose-α-1,3-galactose (α-Gal) epitopes were found in the Fab region of tralokinumab and only 4.5% of glycoforms contained α-Gal in the Fc region. Under non-reducing conditions, Fab-arm exchange did not take place with another immunoglobulin (Ig) G_4 mAb (mavrilimumab). However, following glutathione reduction, a hybrid antibody with monovalent bioactivity was detected. ADA incidences (titers) were as follows: STRATOS 1—every 2 weeks (Q2 W) 0.8% (26.0), every 4 weeks (Q4 W) 0.5% (26.0), placebo 0.8% (52.0); STRATOS 2—Q2 W 1.2% (39.0), placebo 0.8% (13.0). Participant-reported hypersensitivity AE rates were as follows: STRATOS 1—Q2 W 25.9%, Q4 W 25.0%, placebo 25.5%; STRATOS 2—Q2 W 13.2%, placebo 9.0%. External evaluation for anaphylaxis by Sampson criteria found no tralokinumab-related severe hypersensitivity or anaphylaxis reactions.

Conclusion Preclinical assessments suggested a low likelihood of immunogenicity for tralokinumab. In STRATOS 1 and 2, ADA incidence was low, no differences were found between tralokinumab-treated and placebo groups in reporting of hypersensitivity reactions, and there were no Sampson criteria-evaluated anaphylaxis events with tralokinumab treatment. Together, the results suggest that tralokinumab treatment would not increase the risk for severe hypersensitivity or anaphylactic reactions.

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✉ Mats Carlsson
Mats.Carlsson@astrazeneca.com

[1] Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal 431 83, Sweden

[2] Global Medicines Development, AstraZeneca, Granta Park, Great Abington, Cambridge CB21 6GH, UK

[3] Biopharmaceutical Development, MedImmune, One MedImmune Way, Gaithersburg, MD 20878, USA

[4] Clinical Pharmacology, Pharmacometrics and DMPK, MedImmune, Granta Park, Great Abington, Cambridge CB21 6GH, UK

[5] Global Medicines Development, AstraZeneca, One MedImmune Way, Gaithersburg, MD 20878, USA

[6] Biometrics and Information Sciences, AstraZeneca, Granta Park, Great Abington, Cambridge CB21 6GH, UK

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Key Points

In vitro characterization of the glycans of tralokinumab and its ability to undergo Fab-arm exchange did not suggest that there would be an increased risk for hypersensitivity and anaphylactic reactions during tralokinumab treatment.

The STRATOS 1 and 2 clinical findings were in line with these in vitro data. Low incidence of ADAs and neutralizing antibodies were reported, and no tralokinumab-related severe hypersensitivity or anaphylaxis reactions were observed according to Sampson criteria.

1 Introduction

Tralokinumab is an immunoglobulin (Ig) G_4 human monoclonal antibody (mAb) that potently and specifically neutralizes interleukin (IL)-13 [1] by preventing binding to IL-13 receptors (IL-13R) α1 and α2 [2]. IL-13 is a pleiotropic type-2 cytokine that has a central role in the pathogenesis of some types of asthma [3]. For example, in severe asthma, IL-13 promotes the production of IgE, leading to recruitment of eosinophils and airway remodeling via IL-4R α, IL-13Rα1, and/or IL-4α [4]. As part of the ATMOSPHERE late-stage clinical development program [5], tralokinumab was investigated in two pivotal phase III clinical trials, STRATOS 1 and 2 [6, 7], for the treatment of severe, uncontrolled asthma. There are safety challenges associated with developing a mAb for clinical use; for example, mAbs may present an immunogenic risk with the potential to induce severe hypersensitivity reactions such as anaphylaxis [8, 9]. Several factors have been identified that affect the immunogenicity of therapeutic mAbs and potentially increase the risk of hypersensitivity reactions; these include the nature of the mAb [10], the manufacturing process, stability of the mAb, drug excipients, mode of administration, and recipient predisposition [9]. Tralokinumab has two qualities that could hypothetically increase the risk of hypersensitivity reactions and of other adverse events (AEs): the presence of galactose-α-1,3-galactose (α-Gal) epitopes on the antibody, and the propensity of IgG_4 mAbs to undergo Fab-arm exchange and form bispecific antibodies.

The presence of α-Gal epitopes is one of the structural elements of a mAb that may be affected by the manufacturing process, and may influence the potential for developing anaphylactic or hypersensitivity reactions. During production in mammalian cells, mAbs typically undergo glycosylation, a post-transcriptional modification whereby enzymes attach glycans to proteins [10]. The type and the level of glycosylation are associated with the mAb characteristics, the production host cell line, and the cell culture conditions. A functional gene for α-1,3-galactosyltransferase, which is the enzyme required for α-Gal epitope synthesis, is absent in humans but present in mice [11]. As a result, mAbs that are manufactured using mouse cell lines may be glycosylated with glycans that contain the α-Gal epitope. These epitopes are seen as 'foreign' by the human immune system and may elicit a xenoreactive immune response. Such responses have previously been reported with cetuximab, a chimeric mouse–human IgG_1 mAb for the treatment of colorectal cancer, which is produced in the mouse cell line Sp2/0 and contains α-Gal epitopes [10]. Immune reactions including severe hypersensitivity and anaphylaxis reported in people taking cetuximab are thought to be connected to the α-Gal epitopes [10, 12]. Tralokinumab is produced using a mouse myeloma cell line (NS0) that has the ability to attach α-Gal to the mAb during glycosylation [13] and could, therefore, contain these epitopes. Consequently, the question arose whether tralokinumab, like cetuximab, might be potentially associated with an increased risk of hypersensitivity or anaphylaxis compared with other mAbs.

Another structural characteristic of a mAb that can affect its immunogenic potential is its IgG subclass. The IgG_4 mAb has been an isotype of choice for therapeutic mAbs due to limited effector functions [14, 15] and lack of allotypes [16], which both theoretically reduce the potential for hypersensitivity reactions. However, unlike IgG_1, IgG_2, and IgG_3 isotypes, the core-hinge region sequence of IgG_4 allows the inter-heavy chain disulfide bonds in the hinge region to form intra-heavy chain disulfide bonds, resulting in half-antibody molecules [14, 17]. This half-antibody formation can lead to Fab-arm exchange with other IgG_4 antibodies. The resulting IgG_4 antibodies are functionally monovalent (i.e., unable to cross-link identical antigens) but structurally bispecific (i.e., able to bind two different antigens) [18]. These functionally bispecific IgG_4 antibodies have been proposed to contribute to AEs due to potential undesired specificity [14, 19, 20]. As an example, it has been suggested that Fab-arm exchange of natalizumab (a humanized IgG4κ anti-α4 subunit of integrin mAb) may be implicated in the pathogenesis of progressive multifocal leukoencephalopathy (PML), an opportunistic, life-threatening viral disease [19]. Tralokinumab has the typical structure of an IgG_4 molecule, with two identical heavy chains and two identical light chains [21], and therefore has the potential to form half-antibodies with endogenous IgG_4 antibodies [17].

Therapeutic mAbs can elicit anti-drug antibodies (ADAs) when administered, which are another sign of immunogenicity. The ADAs bind to the mAb, forming immune complexes, which can lead to reduced pharmacological activity, reduced exposure to the mAb, or hypersensitivity reactions. The presence of large, cross-linked

ADA–mAb immune complexes in the circulation may lead to type III hypersensitivity reactions when these complexes are deposited in tissues such as the kidneys and synovial membranes [22].

The objectives of this analysis were four-fold: (1) to characterize the glycosylation of tralokinumab and the presence of α-Gal epitopes in the Fab and Fc domains in vitro; (2) to evaluate the development of tralokinumab Fab-arm exchange and half-antibody formation and their effects on safety and immunogenicity in vitro; (3) to report rates of ADAs in the clinical trials; and (4) to identify possible AEs of anaphylaxis, severe hypersensitivity reactions, and other AEs in the STRATOS 1 and 2 phase III pivotal clinical trials of tralokinumab in participants with severe, uncontrolled asthma.

2 Methods

2.1 Characterization of Glycosylation and the Galactose-α-1,3-Galactose (α-Gal) Epitope

Oligosaccharide profile analysis of tralokinumab was conducted to determine the type and abundance of N-linked oligosaccharide species [23]. Release of N-linked glycans from tralokinumab was conducted by incubation with PNGase F (Promega, Madison, WI, USA) at 37 °C for 16 h. The released N-glycans were then labeled with 2-aminobenzamide (2-AB). The labeled glycans were cleaned using GlykoClean SPE Cartridges (Prozyme, Hayward, CA, USA). The labeled glycans were separated by hydrophilic interaction liquid chromatography (HILIC) using a BEH 300 Å 1.7 µm glycoprotein column, 2.1 × 150 mm (Waters, Taunton, MA, USA) in an ultra-performance liquid chromatography (UPLC) system, eluted with a gradient of acetonitrile and 50 mM ammonium formate, pH 4.4, and detected using fluorescence absorbance. The identity of glycan peaks, present at 0.2% or greater of those detected, was confirmed by in-line electrospray ionization ion-trap mass spectrometry (ESI–MS; Orbitrap Fusion, Thermo Fisher Scientific, Waltham, MA, USA). Identity of 2-AB-labeled glycans was also verified by digestion with exo-glycosidases (α-mannosidase, α-galactosidase, β-galactosidase, and sialidase) at 37 °C overnight and analyzed by UPLC using the HILIC column already described. The linkages of the terminal monosaccharide units were identified by the changes between the 2-AB oligosaccharide profiles by HILIC before and after digestion with exo-glycosidase.

The methods for determination of the sialic acid content of tralokinumab, characterization of Fab and Fc glycosylation, the reporter gene bioactivity assay, and the neonatal Fc receptor (FcRn) binding assay can be found in Electronic Supplementary Material 1.

2.2 Half-Antibody Characterization

Methods for Lys-C peptide mapping to determine tralokinumab disulfide bonds and free thiols, characterization of different levels of reduction of tralokinumab, and Fab-arm exchange with another readily available IgG_4 therapeutic mAb (mavrilimumab) are described in Supplementary Electronic Material 1.

Biological activity of tralokinumab half-antibodies was measured using a human umbilical vein endothelial cells (HUVEC) assay [1], with tralokinumab preventing the IL-13-induced expression of vascular cell adhesion molecule-1 (VCAM-1) on the HUVECs. Full details of this methodology have been published previously [1]. HUVECs were obtained from Lonza (Basel, Switzerland) and IL-13 from Peprotech (Rocky Hill, NJ, USA).

Ion exchange chromatography (IEC) fractions enriched with half-antibody were prepared from tralokinumab incubated with PNGase F and carboxypetidase B, and immediately alkylated with N-ethylmaleimide (NEM). Bioactivity of the starting material and the IEC fractions was measured using the HUVEC ELISA (enzyme-linked immunosorbent assay) [1]. IC_{50} (half-maximal inhibitory concentration) values representing the concentration of tralokinumab at which half-maximal VCAM-1 expression was observed were generated using a four-parameter logistical curve fitted for the tralokinumab reference standard and test samples.

The potency of tralokinumab samples was determined by dividing the IC_{50} of each test sample by the IC_{50} of the tralokinumab reference standard and multiplying by 100.

2.3 The STRATOS 1 and 2 Phase III Trials

STRATOS 1 and 2 (ClinicalTrials.gov identifiers NCT02161757 and NCT02194699) were randomized, double-blind, placebo-controlled, parallel-group, phase III clinical trials, which evaluated tralokinumab in participants with severe asthma that was inadequately controlled despite treatment with inhaled corticosteroids plus a long-acting β_2 agonist. Participants aged 12–75 years received subcutaneous tralokinumab 300 mg every 2 weeks (Q2 W STRATOS 1 and 2), tralokinumab 300 mg every 4 weeks (Q4 W; STRATOS 1 only), or matching placebo. The primary endpoint for both trials was the annualized asthma exacerbation rate reduction at Week 52 [6, 7].

2.4 Safety Analyses in STRATOS 1 and 2

All safety summaries and ADA analyses in both trials were based on the safety analysis sets, defined as all participants

in each trial who received at least one dose of placebo or tralokinumab. In the safety analysis set, participants were assigned to treatment groups based on the actual treatment they received, regardless of which group they were randomized to.

The *Medical Dictionary for Regulatory Activities* (MedDRA®) version 20.0 was used to code AEs in both trials. AEs in STRATOS 1 were initially coded in MedDRA® version 19.1, but were then recoded in MedDRA® version 20.0 when follow-up data were included.

All AEs were reviewed by study and safety physicians on an ongoing basis. In addition, all safety information was reviewed by an independent Data Safety Monitoring Board.

2.4.1 Assessment of Anti-Drug Antibodies (ADAs) and Immunogenicity

Pre-dose serum samples for measuring the presence of ADAs were collected in the STRATOS 1 and 2 trials at baseline (before the first administration of tralokinumab) and at Weeks 26, 56 (6–8 weeks after the final tralokinumab dose), and 72 (22–24 weeks after the final tralokinumab dose). The ADA evaluable population included all participants in the safety population who had non–missing baseline ADA results and at least one non–missing post-baseline ADA result.

The presence and titer of ADAs in serum was detected using a Meso Scale Discovery bridging assay (Meso Scale Discovery, Gaithersburg, MD, USA). Briefly, serum samples were acid dissociated to release any ADAs complexed with free drug. Neutralized samples were mixed with biotinylated tralokinumab and Sulfo-tagged tralokinumab, to allow formation of an antibody–complex bridge. Following incubation, samples were added to a streptavidin-coated plate, and unbound material was washed away. Buffer containing tripolyamine was added, and an electric voltage applied to produce a chemiluminescent signal. Positive results were confirmed by competitive binding with excess tralokinumab to assess the specificity of binding.

For ADA titer determination, the assay was performed using serial dilutions of samples in human serum, with two replicates conducted. The titer was calculated as the reciprocal of the product of the assay's minimum required dilution (1 in 13) multiplied by the greatest serial dilution that yielded a response greater than the assay cut-point. In low concentration samples below the assay cut-point, titer will be reported as < 13. Consistent with statistical methods mandated by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA), the cut-point and normalization factor for the ADA screening assay was set to allow a 5% false-positive rate (determined by a 90% one-sided lower confidence interval [CI] for the 95th percentile of the negative control population, with a 90% confidence level) [24, 25]. Similarly, the ADA confirmatory assay cut-point was set to a 1% false-positive rate (determined using an 80% one-sided lower CI for the 99th percentile of the negative control population, with an 80% confidence level) [24, 25].

Confirmed ADA-positive samples were also tested for the presence of neutralizing antibodies (nAbs). Murine anti-human IL-13 mAb, clone 32116, (R&D Systems, Minneapolis, MN, USA) was immobilized to Meso Scale Discovery High-Bind plates. Samples and controls were incubated in the presence of acidic conditions, and neutralized and incubated in the presence of a known ruthenylated tralokinumab concentration overnight. Recombinant human IL-13 (R&D Systems, Minneapolis, MN, USA) was added to the blocked assay plates, incubated, and washed to remove unbound material. The sample–tralokinumab complex was then added to the assay plates and unbound material was washed away. Buffer containing tripolyamine was added, and an electric voltage applied to produce a chemiluminescent signal. An inhibition of signal, relative to a non-neutralizing control, was indicative of a neutralizing sample.

Case records of participants with a positive ADA response were reviewed for patterns of AEs.

2.4.2 Identification of Hypersensitivity Reactions and Possible Anaphylaxis Events

All AEs of hypersensitivity were identified using the MedDRA® Standardized MedDRA® Query (SMQ) for hypersensitivity, anaphylactic reaction, and anaphylactoid shock conditions. Within this group, a two-step process was used to identify and evaluate a subset comprising possible anaphylactic reactions or any AE for anaphylactoid shock or hypersensitivity. Using a programmatic approach, AEs were identified that occurred either the day of, or within 72 h of the day after, trial drug administration by a modified algorithm of the SMQ for anaphylactic reaction. The preferred terms (PTs) representing gastrointestinal events were also added to the algorithm. The PTs comprising the algorithm of this SMQ are provided in Table 1. If an AE was captured under the anaphylaxis SMQ, it was automatically considered as one possible anaphylaxis event. If two or more AEs were identified as occurring in two or more of the other categories (for respiratory, dermatological, cardiovascular, and gastrointestinal PTs), these AEs were also considered as one possible anaphylaxis event (Table 1). In addition to this programmatic approach, the blinded study physicians reviewed all AEs that occurred in STRATOS 1 and 2 individually and identified those that could possibly be anaphylaxis events.

2.4.3 Evaluation of Possible Anaphylaxis Events

The clinical evaluation of hypersensitivity reactions for possible anaphylaxis events followed the Sampson criteria [26]. Briefly, the diagnosis of anaphylaxis is highly likely when one of these three clinical scenarios is observed:

1. The acute onset of a reaction (minutes to hours) with involvement of the skin, mucosal tissue, or both, and at least one of the following:
   (a) respiratory compromise; or
   (b) reduced blood pressure or symptoms of end-organ dysfunction.
2. Two or more of the following occurring rapidly after exposure: involvement of the skin/mucosal tissue, respiratory compromise, reduced blood pressure, or associated symptoms and/or persistent gastrointestinal symptoms.
3. Reduced blood pressure after exposure to an allergen for that participant.

The list of possible anaphylaxis AEs was reviewed by an internal study physician and then referred to an independent, external, blinded evaluator (an allergist who was otherwise not involved in the study) for a participant-level review. The external evaluator was provided with the full profile, a narrative of the event, concomitant medications, medical history, respiratory history, and the dosing data for the participant. The evaluator was asked to determine whether the AE met the Sampson criteria for anaphylaxis. If these criteria were not met, the evaluator was then asked to determine whether the AE represented a non-anaphylactic hypersensitivity event. The evaluator could answer yes, no, or undetermined.

3 Results

3.1 Tralokinumab Glycosylation

The oligosaccharide profile of tralokinumab can be seen in Fig. 1. The most common oligosaccharides were biantennary glycans with a fucose residue at the core, without a terminal galactose G0f (43.4%), or with a single terminal galactose G1f(α1,6) (17.1%) and G1f(α1,3) (14.8%) (Table 2). Oligosaccharides containing the α-Gal epitope were present at 4.5% in total. Oligosaccharides containing terminal sialic acid residue (NGNA) were present at 10.9%. A high mannose-type oligosaccharide, M5, was present at a very low percentage (0.6%).

Fab glycosylation was found to be very low (< 1.0%) across 21 different manufactured lots of tralokinumab. Separation by size exclusion chromatography (SEC) allowed fractions enriched in glycosylated Fab to be prepared, and its presence was confirmed by intact mass and peptide mapping analysis. Peptide mapping identified two N-glycosylation sites in the heavy chain complementarity-determining region of the Fab, at the asparagine residues 31 and 59. The oligosaccharide profile of the Fab indicated that the major glycans found were sialylated glycans with two or three antennae, core fucosylation, and terminal NGNA residues G2f + 2NGc and G3f + 3NGc (Fig. 2). No glycans with α-Gal epitopes were detected in the Fab.

Across tested manufactured lots of tralokinumab covering different processes and clinical stages ($n = 19$), there was some variation in Fc glycosylation, though the proportion of high-mannose and afucosylated species was consistently low at ≤ 1.7% and ≤ 2.2%, respectively. The proportion of galactosylated species were ≤ 50.8%, species with α-Gal epitopes ≤ 9.4%, and sialylated species ≤ 24.1% of the total Fc glycosylation (see Electronic Supplementary Material 2). Fc deglycosylated tralokinumab had similar bioactivity to control tralokinumab (92% vs. 100% respectively), and similar FcRn binding to control tralokinumab (87% vs. 100%, respectively).

3.2 Tralokinumab Half-Antibody Formation and Fab-Arm Exchange

A schematic diagram of the disulfide bond pattern of tralokinumab is given in Electronic Supplementary Material 3. The positions of the disulfide bonds were confirmed by comparison of Lys-C peptide maps in non-reducing and reducing liquid chromatography-mass spectrometry (LC–MS) analyses (see Electronic Supplementary Material 4). All domains of tralokinumab had low percentages of free thiols (see Electronic Supplementary Material 5). The heavy chain constant domains had the greatest proportion of free thiols (2.1–4.1%), and no free thiols were detected in the hinge region or the heavy chain variable domain. The ratio of free thiols per mAb molecule for tralokinumab was 0.3 mol/mol.

Tralokinumab samples were prepared with different degrees of reduction. Figure 3a shows non-reduced tralokinumab separated by non-reducing gel electrophoresis, and mostly comprised tralokinumab monomer (95.2%). Figure 3b shows reduced tralokinumab; the monomer peak disappeared (0%) and was reduced to heavy chains, light chains, and half IgG. In the final sample (Fig. 3c), some of the reduced disulfide bonds had reformed, so that both tralokinumab monomer (30.2%) and the reduced forms were observed. The proportion of aggregated tralokinumab did not vary a great deal among the reduced samples: 3.0% (non-reduced), 1.9% (reduced), and 1.2% (partially reduced). Reduction of tralokinumab did not appear to have a substantial impact on bioactivity measured by the reporter gene bioassay; bioactivity of the three samples was 94%

Table 1 MedDRA® Preferred Terms included in the modified MedDRA® SMQ 'anaphylactic reaction' algorithm[a]

Anaphylaxis (A)	Respiratory (B)	Dermatological (C)	Cardiovascular (D)	Gastrointestinal (E)
Anaphylactic reaction	Acute respiratory failure	Allergic edema	Blood pressure decreased	Abdominal discomfort
Anaphylactic shock	Asthma	Angioedema	Blood pressure diastolic decreased	Abdominal pain
Anaphylactic transfusion reaction	Bronchial edema	Edema	Blood pressure systolic decreased	Abdominal pain lower
Anaphylactoid reaction	Bronchospasm	Erythema	Cardiac arrest	Abdominal pain upper
Anaphylactoid shock	Cardio-respiratory distress	Eye edema	Cardio-respiratory arrest	Gastrointestinal pain
Circulatory collapse	Chest discomfort	Eye pruritus	Cardiovascular insufficiency	Vomiting
Dialysis membrane reaction	Choking	Eye swelling	Diastolic hypotension	Visceral pain
Kounis syndrome	Choking sensation	Eyelid edema	Hypotension	
Shock	Circumoral edema	Face edema		
Shock symptom	Cough	Flushing		
Type I hypersensitivity	Cyanosis	Generalized erythema		
	Dyspnea	Injection-site urticaria		
	Edema mouth	Lip edema		
	Hyperventilation	Lip swelling		
	Irregular breathing	Nodular rash		
	Laryngeal dyspnea	Ocular hyperemia		
	Laryngeal edema	Periorbital edema		
	Laryngotracheal edema	Pruritus		
	Mouth swelling	Pruritus allergic		
	Nasal obstruction	Pruritus generalized		
	Oropharyngeal spasm	Rash		
	Oropharyngeal swelling	Rash erythematous		
	Respiratory arrest	Rash generalized		
	Respiratory distress	Rash pruritic		
	Respiratory failure	Skin swelling		
	Reversible airways obstruction	Swelling		
	Sensation of foreign body	Swelling face		
	Sneezing	Urticaria		
	Stridor	Urticaria papular		
	Swollen tongue			
	Tachypnea			
	Throat tightness			
	Tongue edema			
	Tracheal obstruction			
	Tracheal edema			
	Upper airway obstruction			
	Wheezing			

MedDRA Medical Dictionary for Regulatory Activities, *SMQ* Standardized MedDRA Query

[a]A possible case of anaphylaxis according to the modified MedDRA® algorithm is: any term within category A; or at least two terms within two different categories (or more) within B, C, D, and E

(non-reduced), 91% (reduced), and 85% (partially reduced) versus tralokinumab reference standard.

The impact of half-antibody formation on tralokinumab was assessed in samples enriched with half-antibody by IEC fractionation. Analysis of bioactivity using a HUVEC assay demonstrated that the starting material had 119% bioactivity (vs. tralokinumab reference standard) and 7.5% half-antibody content. The variation in half-antibody concentration of tralokinumab samples did not impact biological activity. The IEC main peak had 124% and 0.8%, IEC post-peak 1 had 145% and 11.9%, and IEC post-peak 2 had 130% and 65.0% bioactivity and half-antibody content, respectively.

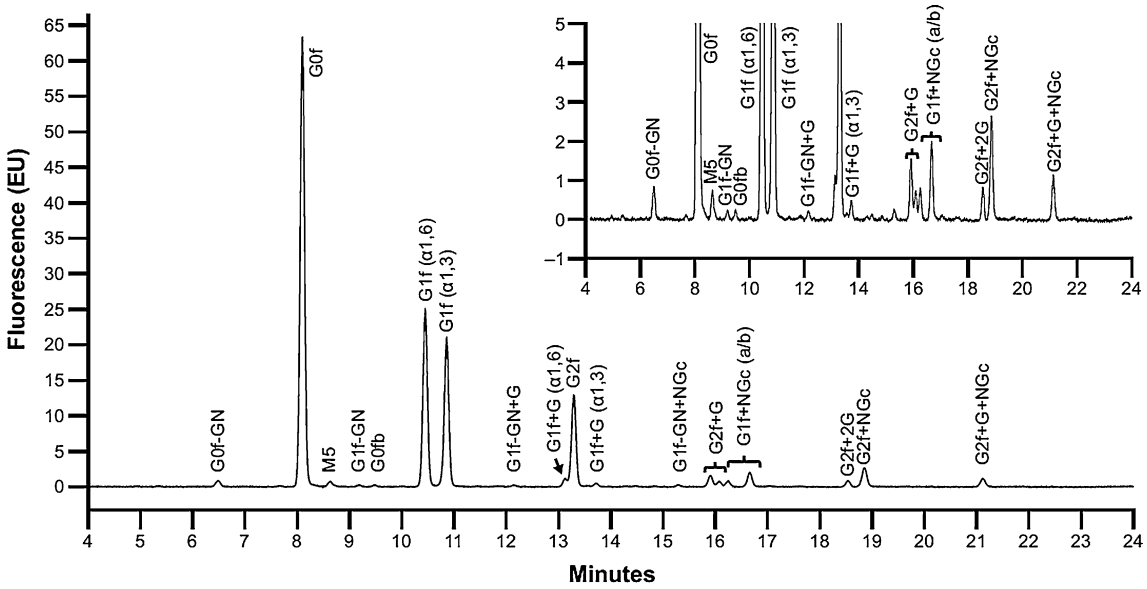


Fig. 1 Oligosaccharide profile of tralokinumab. Separation of 2-aminobenzamide labeled glycans from tralokinumab by hydrophilic interaction liquid chromatography. *f* fucose, *G* galactose, *GN* N-acetyl-glucosamine, *M* mannose, *NGc* N-glycolylneuraminic acid

Table 2 Relative percentage of identified oligosaccharides in tralokinumab

Oligosaccharide	Relative percentage (%)	Theoretical mass (Da)	Observed mass (Da)
Complex type			
G0f	43.4	1582.61	1582.65
G1f(α1,6)	17.1	1744.67	1744.71
G1f(α1,3)	14.8	1745.67	1745.70
G2f	8.8	1906.72	1906.76
G1f + NGc (a/b)	4.7	2051.71	2051.81
G2f + NGc	4.2	2213.76	2213.86
G2f + G+NGc	1.6	2375.82	2375.92
G2f + G	1.1	2068.77	2068.82
G0f-GN	0.7	1379.53	1379.57
G1f(α1,6) + G	0.7	1906.72	1906.76
G2f + 2G	0.5	2230.83	2230.88
G1f(α1,3) + G	0.4	1906.72	1906.76
G1f-GN + NGc	0.4	1848.63	1848.72
G1f-GN (a/b)	0.2	1541.59	1541.62
G1f-GN + G	0.2	1703.64	1703.68
G0fb	0.1	1785.69	1785.73
High-mannose type			
M5	0.6	1354.50	1354.53

Oligosaccharides containing the galactose-α-1,3-galactose (α-Gal) epitope are indicated in italics

f fucose, *G* galactose, *GN* N-acetyl-glucosamine, *M* mannose, *NGc* *N*-glycolylneuraminic acid

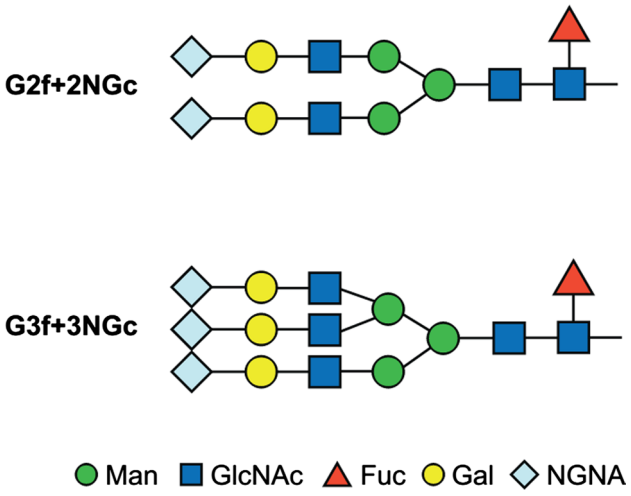


Fig. 2 Structure and nomenclature of oligosaccharides in the tralokinumab Fab region. *f* fucose, *G* galactose, *GlcNAc* N-acetylglucosamine, *Man* mannose, *NGc* N-glycolylneuraminic acid

Tralokinumab incubated in IgG-depleted human serum at 37 °C for 8 weeks had 106% bioactivity at Week 0 (vs. tralokinumab reference standard), which decreased during the incubation to 70% at Week 8. The amount of half-antibody was 5.5% at Week 0 and increasing proportions of half-antibody were detected during the incubation period, varying from 10.6 to 18.5%. The percentage of monomer as measured by SEC was 74.9% at Week 8. This indicated

that the decrease in bioactivity was probably caused by other modifications such as aggregation and fragmentation. Bioactivity results indicated that the in vitro half-antibody had similar activity to whole tralokinumab.

Tralokinumab did not readily undergo Fab-arm exchange with mavrilimumab under physiological conditions in vitro. Following incubation of non-reduced or reduced tralokinumab with non-reduced mavrilimumab in IgG-depleted human serum, no Fab-arm exchanged hybrid antibody was observed in incubates separated by IEC (Fig. 4a). In contrast, hybrid antibody was detected by IEC when both tralokinumab and mavrilimumab were reduced with glutathione before incubation (Fig. 4b). The identity of the hybrid antibody was confirmed by intact mass analysis. Bioactivity of the hybrid antibody was expected to be 50% of intact tralokinumab; the measured bioactivity (40%) of the hybrid molecule was close to the expected theoretical level.

3.3 Safety Within the STRATOS 1 and 2 Clinical Trials

3.3.1 Exposure

A total of 1227 participants received tralokinumab in the STRATOS 1 and 2 trials (Fig. 5). In STRATOS 1, 398 participants received tralokinumab Q2 W and 404 participants received tralokinumab Q4 W. In STRATOS 2, 425 participants received tralokinumab Q2 W [7]. The combined duration of exposure across both trials was 409,595 days (Q2 W); 267,698 days (Q4 W) for STRATOS 1 and 141,897 days for STRATOS 2.

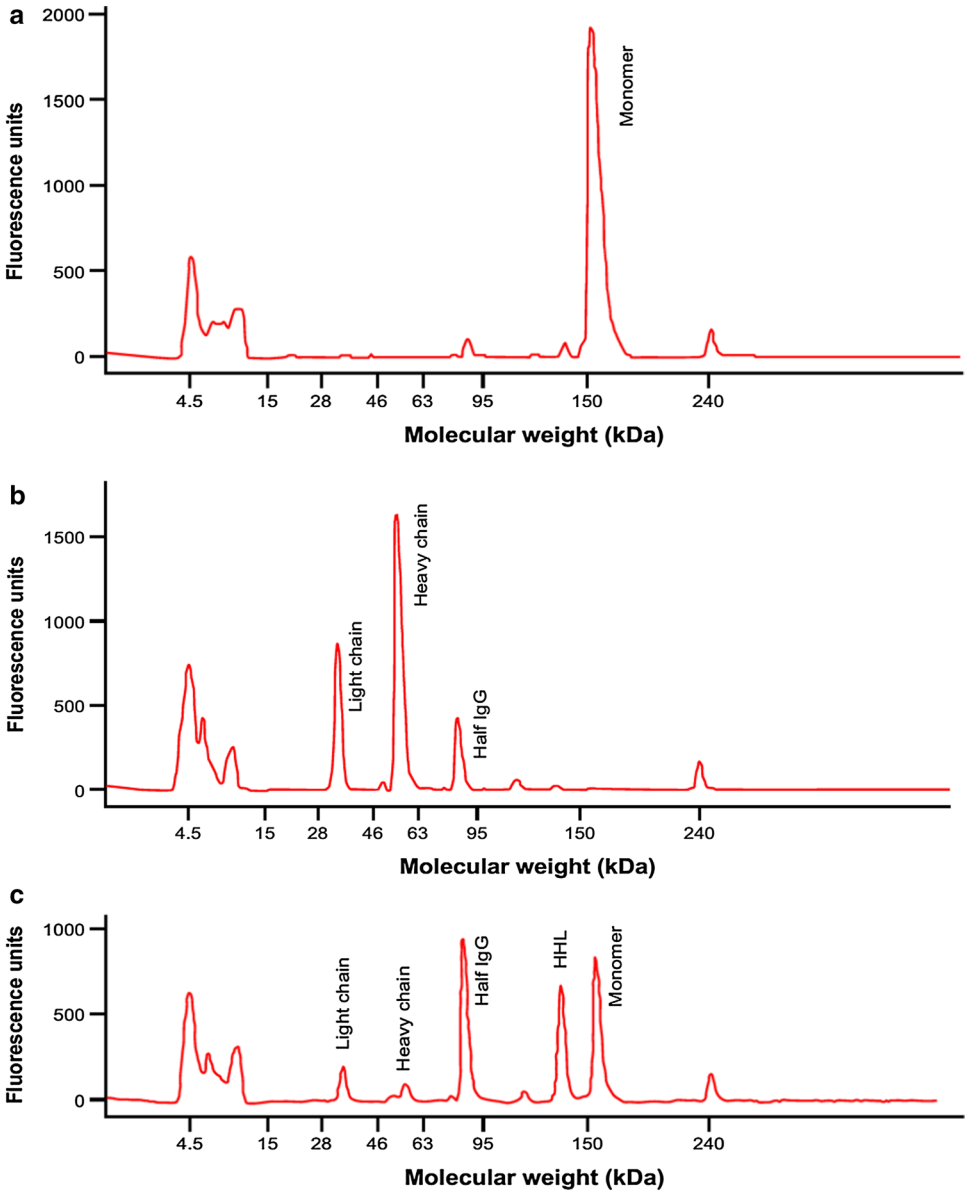


Fig. 3 Characterization of tralokinumab with different degrees of reduction by non-reducing gel electrophoresis: (**a**) non-reduced; (**b**) reduced; and (**c**) partially reduced. *HHL* two heavy chains + one light chain, *IgG* immunoglobulin G

3.3.2 Adverse Events

The most frequent AEs in STRATOS 1 and STRATOS 2 (reported by ≥ 5% of participants) were asthma, headache, and upper respiratory tract infection [7]. Review of all AEs revealed that the safety profile of tralokinumab was consistent with that observed in previous trials [7].

3.3.3 ADA Assessment

Within STRATOS 1 there was no obvious relationship between treatment group and either ADA prevalence or incidence [7]. The ADA prevalence, i.e., the proportion of the study population having drug-reactive antibodies at any point in time, was 7/398 (1.9%) in the tralokinumab Q2 W group, 7/404 (1.8%) in the tralokinumab Q4 W group, and 9/400 (2.4%) in the placebo group.

Of the seven ADA-positive participants in the tralokinumab Q2 W group, four had pre-existing ADA (ADA-positive at baseline) and three had a positive result post-baseline not detectable at baseline. Of these three, two were persistently ADA-positive (with ≥ 2 positive post-baseline assessments that had either ≥ 16 weeks between the first and last positive assessment, or were positive at the last assessment); one was transiently ADA-positive (with ≥ 1 positive post-baseline assessment, but not fulfilling the persistent definition). Of the seven ADA-positive participants in the tralokinumab Q4 W group, five had pre-existing ADA and two had a positive result post-baseline not detectable at baseline. In total, three participants were positive post-baseline (one of whom had pre-existing ADAs); of these, two were persistently ADA-positive and one was transiently ADA-positive. Of the nine ADA-positive participants in the placebo group, seven had pre-existing ADA and two had a positive result post-baseline not detectable at baseline. Eight participants were positive post-baseline (six of whom had pre-existing ADAs); of these, seven were persistently ADA-positive and one was transiently ADA-positive. No participants in either tralokinumab group had treatment-boosted ADA (defined as baseline positive ADA titer that was boosted to a four-fold or greater level following drug administration). One (0.3%) participant met this criterion in the placebo group. Thus, the ADA incidence (defined as the sum of both treatment-induced [post-baseline ADA-positive only] and treatment-boosted ADA) was 0.8% (3/373) in the tralokinumab Q2 W group, 0.5% (2/382) in the tralokinumab Q4 W group, and

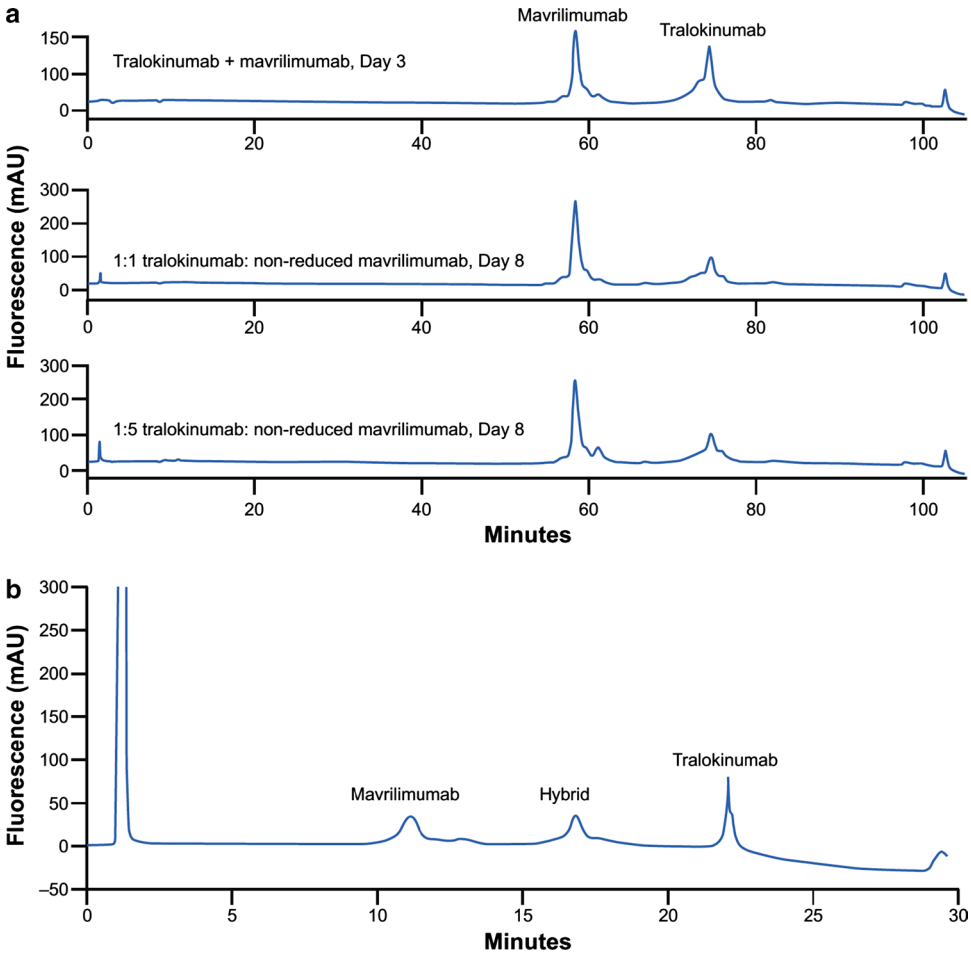
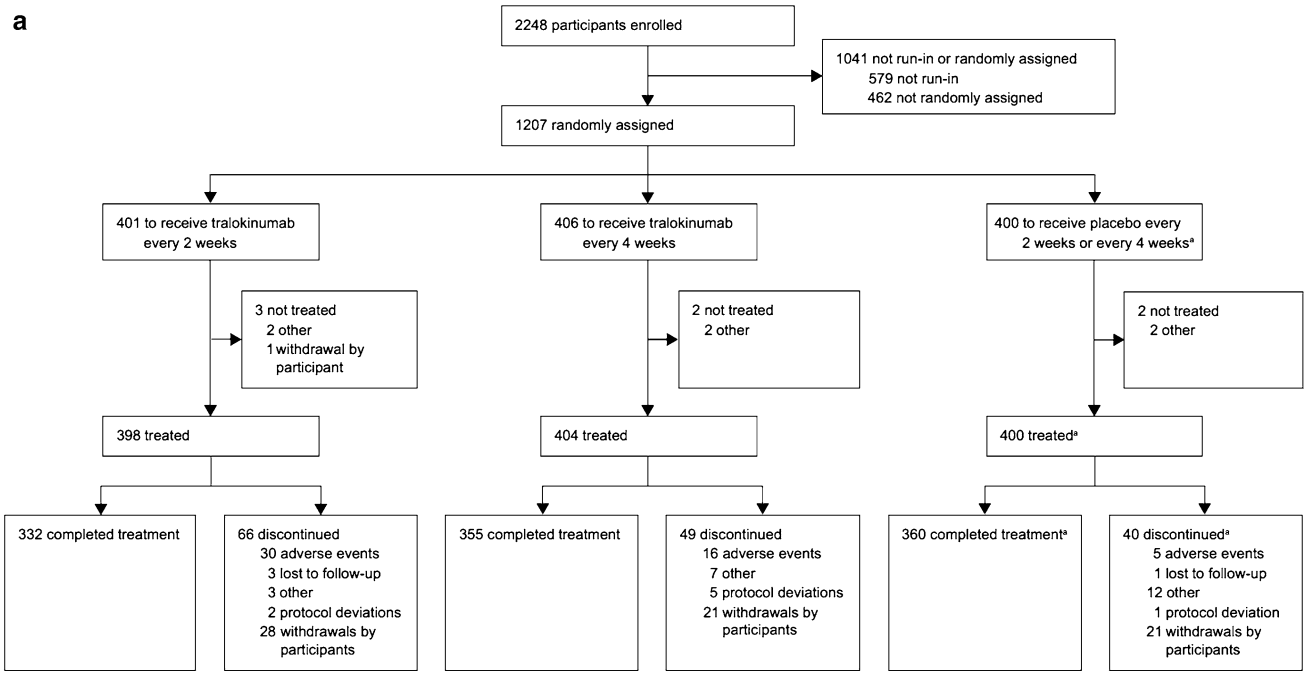


Fig. 4 Ion exchange chromatography profiles of tralokinumab and mavrilimumab Fab-arm exchange under (**a**) physiological conditions and (**b**) non-physiological conditions



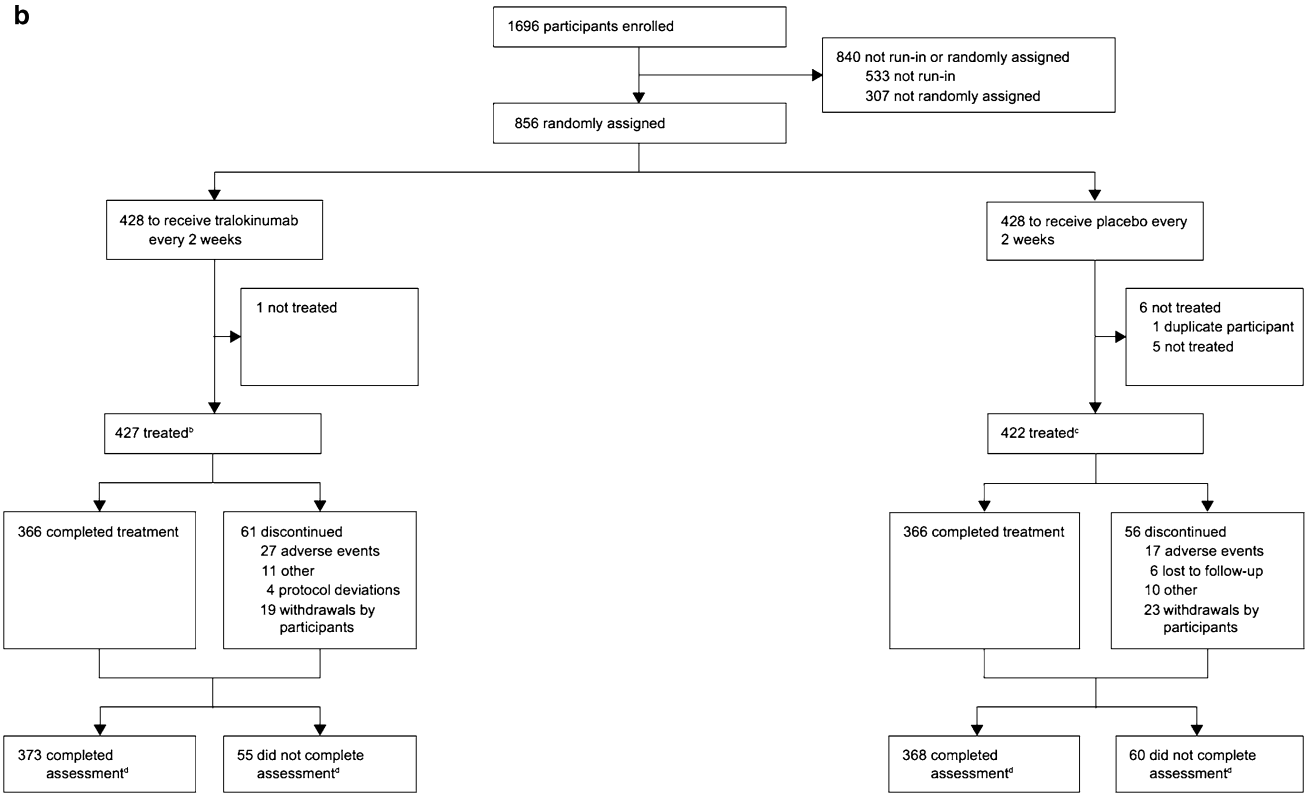


Fig. 5 Patient flow diagram for (**a**) STRATOS 1 and (**b**) STRATOS 2. [a]The STRATOS 1 placebo treatment group is a combined treatment group (placebo every 2 weeks plus placebo every 4 weeks), where the two placebo cohorts were given weights proportional to the number of participants in each cohort. For STRATOS 2, eight participants on tralokinumab every 2 weeks and 11 on placebo were excluded from the full analysis set. [b]Two participants were duplicates and were not included in the safety set; these two participants and five who did not have the potential to receive 52 weeks of treatment were not included in the full analysis set. [c]Five participants did not have the potential to receive 52 weeks of treatment, so were not included in the full analysis set. [d]Of participants randomly assigned. Republished with permission of Elsevier, from Panettieri et al. [7]; permission conveyed through the Copyright Clearance Center, Inc

0.8% (3/374) in the placebo group. ADA titers were low in nature, with median (range) titers of 26.0 (< 13–26), 26.0 (26–26), and 52.0 (13–208), respectively. See Electronic Supplementary Material 1 for titers for participants with persistent and transient positive post-baseline ADAs. Five (1.3%) participants in each of the tralokinumab groups and four (1.1%) participants in the placebo group tested positive for the presence of nAbs against tralokinumab.

Within STRATOS 2, again there was no obvious relationship between either ADA prevalence or incidence and treatment group. The ADA prevalence was 7/405 (1.7%) in the tralokinumab Q2 W group and 8/394 (2.0%) among placebo recipients.

Of the seven ADA-positive participants in the tralokinumab Q2 W group, five had a positive result post-baseline not detectable at baseline (three were persistently ADA-positive and two were transiently ADA-positive) and two had pre-existing ADA. For the eight ADA-positive participants in the placebo group, six had a positive result post-baseline (all six were persistently ADA-positive; three also had pre-existing ADAs and three had a positive result post-baseline not detectable at baseline), and two who had pre-existing ADA only. No participants in the tralokinumab or the placebo group had treatment-boosted ADA. Thus, the ADA incidence was 1.2% (5/405 participants) for the tralokinumab Q2 W group compared with 0.8% (3/394) for placebo. ADA titers were low in nature, with median (range) titers of 39.0 (< 13–208) and 13.0 (13–26), respectively. See Electronic Supplementary Material 1 for titers for participants with persistent and transient positive post-baseline ADAs. All five of the ADA-positive participants in the tralokinumab Q2 W group tested positive for the presence of nAb against tralokinumab. However, it should be noted that the number of ADA-positive participants in each of the treatment groups was small (less than ten), and thus it is not possible to draw definitive conclusions.

A review of AEs in the ADA-positive participants indicated that the safety profile of tralokinumab was consistent with that observed in previous trials; however, the numbers were too small for any meaningful interpretation of patterns of AEs. See Electronic Supplementary Material 6 for the most frequently reported AEs in ADA-positive participants.

3.3.4 Anaphylaxis

Analysis of MedDRA® SMQs during the treatment period identified AEs of hypersensitivity, anaphylactoid shock, and anaphylaxis. In STRATOS 1, hypersensitivity AEs were reported by 103/398 participants (25.9%) in the tralokinumab Q2 W group, 101/404 (25.0%) in the tralokinumab Q4 W group, and 102/400 (25.5%) in the placebo group. In STRATOS 2, hypersensitivity AEs were reported by 56/425 participants (13.2%) in the tralokinumab Q2 W group and 38/422 (9.0%) in the placebo group (see Electronic Supplementary Material 7). No cases of hypersensitivity type III reactions were reported in either STRATOS 1 or 2.

No cases of potential hypersensitivity reactions related to tralokinumab administration met the Sampson criteria for anaphylaxis in either STRATOS 1 or STRATOS 2 (see Electronic Supplementary Material 8). In STRATOS 1, one patient reported pharyngeal edema and a swollen tongue, which were considered to be related to tralokinumab administration, but did not meet Sampson criteria for anaphylaxis (see Electronic Supplementary Material 8). Conversely, the criteria were met by two AEs (urticaria and wheezing) in a participant receiving placebo in STRATOS 2. One AE (urticaria) in a participant in the tralokinumab Q2 W group of STRATOS 2 met the Sampson criteria for non-anaphylactic hypersensitivity.

4 Discussion

In this study, characterization of the glycans of tralokinumab and its ability to undergo Fab-arm exchange did not suggest that there would be an increased risk for anaphylaxis or hypersensitivity reactions with tralokinumab treatment. The clinical safety data from the STRATOS 1 and 2 trials reported here were in line with these in vitro data, with low reported incidence of ADAs and nAbs, and no anaphylaxis or severe hypersensitivity reactions according to Sampson criteria.

In vitro analyses determined that small amounts of tralokinumab contained glycoforms with the α-Gal epitope, which were only detected in the Fc region and not expected to impact tralokinumab immunogenicity. Although therapeutic mAbs often contain detectable concentrations of α-Gal epitopes, studies have demonstrated that binding by IgE antibodies appears to be restricted to α-Gal epitopes in the Fab domains only [10, 27]. It is thought this is the result of steric hindrance in the Fc domain that prevents IgE binding to the α-Gal epitopes [28]. In contrast, cetuximab (chimeric IgG_1 epidermal growth factor receptor [EGF-R] mAb) is N-glycosylated in both the Fab and Fc regions; in the V_H and C_H1 domain of the Fab region, 89% of glycans contain α-Gal epitopes while α-Gal epitopes are undetectable in the Fc region [29]. While cetuximab and tralokinumab were both produced in murine cell lines that are known to introduce α-Gal epitopes (Sp2/0 and NS0, respectively), the amount and distribution of α-Gal epitopes were different in the two mAbs.

Tralokinumab was found to contain glycans with the sialylated species N-glycolylneuraminic acid (NGNA). Although anti-NGNA antibodies have been reported in humans [30, 31], no ADAs associated with NGNA have

been reported in therapeutic antibodies to date, so the presence of NGNA is not thought to pose a concern for the immunogenicity of tralokinumab. The presence of high-mannose glycans in antibodies is associated with enhanced antibody-dependent cell-mediated cytotoxicity (ADCC), decreased complement-dependent cytotoxicity (CDC), and may lead to more rapid clearance in serum [32–36], which could affect the antibodies' bioactivity. Moreover, several studies in mice have shown that the immunogenicity of recombinant proteins with different glycosylation patterns is impacted by the presence of mannose-containing glycans [37–39]. However, ADCC and CDC are not thought to form part of the mechanism of action of tralokinumab, and we have demonstrated that the deglycosylated Fc had similar relative bioactivity and FcRn binding as the glycosylated control. In addition, we observed low concentrations of high-mannose glycans in the samples in this study. Overall, this suggests that high-mannose glycans are unlikely to influence tralokinumab bioactivity or immunogenicity, and that variations in Fc glycoforms would be unlikely to impact the pharmacokinetics or pharmacodynamics of tralokinumab.

Using non-reducing Lys-C peptide mapping, we confirmed that the disulfide bond structure of tralokinumab is consistent with the typical structure for native and recombinant IgG_4 antibodies: four inter-chain disulfide bonds, of which two were at the hinge region connecting the heavy chains and 12 intra-chain disulfide bonds [21]. Reduction of tralokinumab's disulfide bonds did not appear to have a substantial impact on its bioactivity. The percentage of free thiol groups detected in tralokinumab was low (0.3 mol/mol free thiol to mAb), suggesting the likelihood of intermolecular disulfide bond formation using free thiol groups would be low. IgG antibodies with more free thiols are also more likely to have exposed hydrophobic regions, which can form aggregates through hydrophobic interactions between molecules [21]. Phagocytic cell removal of these antibody aggregates can subsequently elicit an immune response [40]. However, partially reduced tralokinumab samples did not show increased aggregation (as measured by SEC compared with non-reduced tralokinumab), suggesting that the impact of tralokinumab's partial reduction on immunogenicity is low.

In vitro analyses of samples in which half-antibody was enriched by IEC fractionation showed that the concentration of half-antibody did not affect the biological activity of tralokinumab. Further, in this study, tralokinumab did not readily undergo Fab-arm exchange in vitro under physiological, non-reducing conditions when tested with mavrilimumab. This was consistent with previous observations in vitro, in which IgG_4 was reported to have strong non-covalent interactions between C_H3 domains and formed a stable four-chain molecule [18]. In contrast, when both tralokinumab and mavrilimumab were fully reduced, we observed that a hybrid antibody was formed whose bioactivity was close to predicted levels.

Fab-arm exchange is an inherent characteristic of wild-type IgG_4 antibodies [19, 20, 41], and has been observed in vivo with therapeutic mAbs [18–20] and in pathogenic autoantibodies [42]. It has been observed, both in mice and humans, that Fab-arm exchange in IgG_4 antibodies is dependent on the amino acid sequence in the core-hinge area [19]. Half-antibody formation and Fab-arm exchange with endogenous IgG_4 is therefore not expected to impact on the safety profile of tralokinumab. It has been hypothesized that the formation of bispecific antibodies with endogenous IgG_4 antibodies may lead to undesired cross-linking resulting in severe AEs [14, 19, 20], but thus far there have been no reports directly linking Fab-arm exchange in IgG_4 therapeutic mAbs with AEs in treated individuals [43]. In a simple pharmacokinetic model developed from experimental data, Labrijn et al. [19] showed that under normal serum conditions, the majority of therapeutic IgG_4 is expected to be monovalent bispecific antibody, which serves as a natural buffer to substantially reduce undesired cross-linking by bispecific antibodies.

The ADA incidence, prevalence and titers in STRATOS 1 and 2 were similar between the tralokinumab and placebo groups. Compared with placebo, there was no evidence of treatment-boosted ADA titers in patients who received repeated dosages of tralokinumab. The number of participants with post-baseline ADAs and nAbs was small (less than ten). Given the low reported rate of ADAs and difficulty in predicting individual patients' immune responses, identification of specific properties that contributed to the immunogenicity of tralokinumab is difficult, and would be purely speculative without further investigation. Indeed, it is possible that the development of ADAs was not related to the therapeutic effects of tralokinumab. However, the participants with ADA-positive samples did not appear to have reduced tralokinumab exposure. Potentially, therefore, these samples categorized as positive were false-positive results, consistent with the fixed 5% false-positive rate of the ADA screening assay and 1% false-positive rate of the confirmatory assay. In support of this, no pattern of AEs was detected in ADA-positive participants in the current analysis.

In the STRATOS 1 and 2 trials, the safety profile of tralokinumab was consistent with that observed in previous trials. Hypersensitivity AEs were reported by similar numbers of participants in the tralokinumab and placebo treatment groups in both studies. None of the reported hypersensitivity reactions were type III, which are known to result from the presence of ADAs [22]. While asthma is a known risk factor for anaphylaxis [44, 45], no hypersensitivity or anaphylactic reactions were identified according to the blinded, external assessment using the Sampson criteria in tralokinumab-treated participants in

either trial. A recent trial of lebrikizumab, another IgG_4 mAb administered subcutaneously, also reported small numbers of hypersensitivity reactions in clinical use in asthma [46].

Our clinical observations with tralokinumab can be contrasted with cetuximab, with which a high prevalence of hypersensitivity reactions (bronchospasm, stridor, hoarseness, urticaria, and/or hypotension) was noted during clinical trials of colorectal cancer and squamous cell carcinoma of the head and neck in the USA. In Tennessee and North Carolina, 22% of patients treated with cetuximab had severe hypersensitivity reactions, while rates were lower (< 1%) in most centers in the Northeast of the country [47, 48]. Among those treated with cetuximab in Southeast USA, most had IgE antibodies specific for α-Gal. It was suggested that the regional difference observed might be due to exposure to regional pests and diseases, such as ticks [10]. As a result, cetuximab has a boxed warning for infusion-related, including anaphylactic, reactions in the US label [12]. Mild or moderate infusion-related reactions are listed as very common (≥ 1/10) and severe infusion-related reactions, sometimes with fatal outcomes, are listed as common (≥ 1/100 to < 1/10) in the European Union (EU) Summary of Product Characteristics (SmPC). Anaphylactic reactions may occur in the first infusion or with subsequent infusions and the risk increases with a history of allergy to red meat or tick bites, or presence of anti-α-Gal IgE antibodies [49]. The US label notes that severe infusion reactions, sometimes fatal, may occur in about 3% of patients [12].

With other therapeutic mAbs produced in murine cell lines that are administered subcutaneously, rates of hypersensitivity reactions and anaphylaxis were generally low, and it is important to note that manufacture of a mAb in a murine cell line is not a predictor of increased risk of severe hypersensitivity reactions in humans. For example, golimumab (human $IgG_1\kappa$ anti-tumor necrosis factor [TNF]-α mAb), ustekinumab (human $IgG_1\kappa$ mAb targeting the p-40 subunit of IL-12 and IL-23), and canakinumab (human $IgG_1\kappa$ anti-IL-1β mAb) are all produced in the same murine cell line as cetuximab, Sp2/0, and daclizumab (humanized IgG_1 anti-IL-2Rα mAb) is produced in murine cell line NS0, but none are associated with hypersensitivity reactions or anaphylaxis at the same frequency [50–53].

Of 1659 golimumab-treated participants from five phase II and III trials in rheumatoid arthritis (RA), psoriatic arthritis (PsA), and ankylosing spondylitis, none developed anaphylactic reactions [50, 54]. However, in post-marketing experience, serious systemic hypersensitivity reactions, including anaphylaxis, have been reported, of which some reactions occurred after the first administration of golimumab. The golimumab SmPC lists allergic reactions (bronchospasm, hypersensitivity, urticaria) as common (≥ 1/100 to < 1/10), and serious systemic hypersensitivity reactions (including anaphylactic reactions) as rare (≥ 1/10,000 to < 1/1000) [50]. Similarly, clinical trials (involving 4135 participants with psoriasis and/or PsA who received active drug) and post-marketing studies of ustekinumab have reported hypersensitivity reactions as uncommon (≥ 1/1000 to < 1/100) and serious hypersensitivity reactions, including anaphylaxis and angioedema, as rare (≥ 1/10,000 to < 1/1000) [52]. For canakinumab, no anaphylactic or anaphylactoid reactions were reported in clinical trials in gouty arthritis (n = 691) and RA (n = 441) [55]. Hypersensitivity reactions associated with canakinumab treatment have been reported, though the symptoms of cryopyrin-associated periodic syndromes for which canakinumab is approved are similar to symptoms of hypersensitivity [53]. The daclizumab US label has a warning for anaphylaxis and angioedema and notes that anaphylaxis, angioedema, and urticaria can occur after the first dose or at any time during treatment. Multiorgan hypersensitivity was observed in two or more patients in trials treating relapsing multiple sclerosis [51].

The low ADA incidence, similar frequency of hypersensitivity reactions in tralokinumab- and placebo-treated participants, and lack of Sampson criteria-defined anaphylaxis in the STRATOS 1 and 2 clinical trials support our assessment that tralokinumab is unlikely to be associated with an increased risk of severe hypersensitivity reactions based on in vitro characterization of the antibody structure. This favorable immunological safety profile is expected to extend to people with atopic dermatitis treated with tralokinumab, for which phase III trials are currently ongoing (NCT03131648; NCT03160885).

4.1 Limitations

The statistical methods used in the determination of ADA titers, as mandated in the US FDA and EMA guidance documents [24, 25], may have resulted in biased estimates of the screening, confirmatory, and neutralizing cut-points established from the study populations. This could cause the false-positive rate to be greater than expected [56]. Further, some of the analyses were undertaken in the in vitro setting. As a result, the findings may not be truly representative of the complex in vivo situation and, thus, have limited applicability in the clinical setting [57].

5 Conclusions

In vitro analyses and safety data from two pivotal phase III clinical trials of people with severe, uncontrolled asthma have not identified an increased risk of severe hypersensitivity or anaphylactic reactions with

tralokinumab. In vitro characterization of tralokinumab's glycans found that the α-Gal epitope was present in small amounts in the Fc region only. The half-antibody content of tralokinumab did not appear to affect bioactivity and tralokinumab did not undergo Fab-arm exchange easily in vitro. The rates of ADAs in the STRATOS 1 and 2 trials were low. No pattern of other AEs was found in ADA-positive participants. Together these results suggest that tralokinumab treatment would not lead to an increased risk for severe hypersensitivity or anaphylactic reactions.

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Author Contributions Mats Carlsson contributed to the design of the STRATOS trials, and collection, analysis, and interpretation of clinical safety data from the STRATOS trials. Martin Braddock contributed to the analysis and interpretation of the in vitro and preclinical studies and building the scientific link with the clinical safety data from the STRATOS trials. Yuling Li led team efforts on developing the production process and analytical methods for tralokinumab. Jihong Wang contributed to the design, execution, data analysis, and interpretation of the in vitro experiments. Weichen Xu contributed to the design, execution, data analysis, and interpretation of the in vitro experiments. Nicholas White developed, validated, and oversaw the ADA bioanalysis of the ATMOSPHERE program, including the STRATOS trials; contributed to protocol design, for the ADA strategy; and was an author of the Clinical Study Report for both STRATOS trials. Ayman Megally contributed to the design, execution, and data analysis of the reporting system for hypersensitivity and anaphylaxis reactions to the external evaluator, and performed the role of study physician during both STRATOS trials. Gillian Hunter contributed to the analysis and interpretation of clinical data from the STRATOS trials. Gene Colice contributed to the design of the STRATOS trials, and collection, analysis, and interpretation of clinical safety data from the STRATOS trials.

Compliance with Ethical Standards

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Conflicts of interest Yuling Li was an employee of MedImmune, the biologics division of AstraZeneca, at the time the study took place; her current affiliation is CBT Pharmaceuticals. Jihong Wang and Weichen Xu are employees of MedImmune, the biologics division of AstraZeneca. Nicholas White is an employee of MedImmune and an AstraZeneca shareholder. Mats Carlsson, Martin Braddock, and Ayman Megally are employees of AstraZeneca, the sponsor of STRATOS 1 and STRATOS 2. Gene Colice is an employee of and holds stock options in AstraZeneca. Gillian Hunter is a consultant employed by AstraZeneca.

Ethical approval and informed consent STRATOS 1 and STRATOS 2 were performed in accordance with the ethical principles that have their origin in the Declaration of Helsinki and that are consistent with International Conference on Harmonisation (ICH)/Good Clinical Practice (GCP), applicable regulatory requirements, and the AstraZeneca policy on Bioethics. All participating centers obtained approval from an independent ethics committee and all participants provided written informed consent.

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Drug Safety

Mats Carlsson,[1] Martin Braddock,[2] Yuling Li,[3] Jihong Wang,[3] Weichen Xu,[3] Nicholas White,[4] Ayman Megally,[5] Gillian Hunter,[6] Gene Colice[5]

[1]Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden

[2]Global Medicines Development, AstraZeneca, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

[3]Biopharmaceutical Development, MedImmune, One MedImmune Way, Gaithersburg, Maryland 20878, United States

[4]Clinical Pharmacology, Pharmacometrics and DMPK, MedImmune, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

[5]Global Medicines Development, AstraZeneca, One MedImmune Way, Gaithersburg, Maryland 20878, United States

[6]Biometrics and Information Sciences, AstraZeneca, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

Corresponding author: Mats Carlsson, Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden.

Mats.Carlsson@astrazeneca.com

Electronic Supplementary Material 1

1 Supplementary Methods

1.1 Determination of Sialic Acid Content

Sialic acid content determination was performed to measure the amount of two forms of sialic acid, N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA). Sialic residues were released from tralokinumab by acid hydrolysis using 2 M acetic acid at 80°C for 2 h. The released NANA and NGNA molecules were fluorescently labeled with 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) at 60°C for 1.5 h. Separation was conducted using reverse phase high-performance liquid chromatography (RP-HPLC) with a C-18 3 µm, 4.6 mm x 7.5 cm CapCell Pak column (Shiseido Co, Ltd, Japan), followed by elution with a gradient of acetonitrile in methanol and water, and fluorescence detection. Concentrations of labeled NANA and NGNA were measured relative to an external standard curve, and were reported as a molar ratio to total protein.

1.2 Characterization of Fab Glycosylation

The Fab glycosylated species was prepared from tralokinumab using preparative size exclusion chromatography (SEC) with a TSK-gel G3000SWxL column, 7.8 mm x 30 cm (Tosoh Bioscience LLC, King of Prussia, PA, USA). Fab glycosylated mAb was eluted isocratically using 0.1 M sodium phosphate, 0.1 M sodium sulfate and 0.05% sodium azide, pH 6.8 at a flow rate of 1 ml/min with UV absorbance detection. The fraction enriched in Fab glycosylated mAb was eluted at ~8.4 min as the monomer pre-peak. The presence of Fab glycosylation in the enriched fraction was confirmed by intact mass analysis with a Synapt quadrupole time of flight (Q-Tof) mass spectrometer (Waters, Elstree, UK). Mass spectrometric data were collected in positive ion mode in m/z range 800–4500 and deconvoluted using MaxEnt 1 software (Waters, Elstree, UK). The starting material and the

monomer pre-peak were subsequently characterized by physicochemical and biological analyses.

The Fab glycosylated–enriched fraction was analyzed by non-reducing and reducing gel electrophoresis. Peptide mapping was used to identify N-glycosylation sites.

The bioactivity and Fab glycosylation level from tralokinumab drug substance and the enriched fraction were measured by a reporter gene assay and gel electrophoresis, respectively.

1.3 Characterization of Fc Glycosylation

The deglycosylated Fc species was prepared by treating tralokinumab with PNGase F, as in section 2.1 of the main manuscript, to remove all Fc oligosaccharides, and then purified using Protein A chromatography with a gradient of phosphate-buffered saline in 20 mM sodium phosphate, pH 3.0, followed by elution with 20 mM sodium phosphate, pH 3.0. Deglycosylation was confirmed by reduction by dithiothreitol (DTT) and gel electrophoresis (Protein LabChip electrophoresis system, PerkinElmer, Inc, Waltham, MA, USA) and intact mass analysis. Non-reduced deglycosylated Fc and deglycosylated Fc reduced by DTT at 37°C were separated by RP-HPLC with a PLRP-S column, 8 µm, 4000 Å, 2.0 x 150 mm (Agilent, Santa Clara, CA, USA) with a linear gradient of acetonitrile in 0.1% trifluoracetic acid in H_2O and analyzed with a Synapt Q-Tof mass spectrometer (Waters, Elstree, UK). Mass spectrometric data were collected in positive ion mode in m/z range 800–4500 and deconvoluted using MaxEnt 1 software (Waters, Elstree, UK). The deglycosylated material was tested by the reporter gene bioactivity assay and the FcRn binding assay.

1.4 Reporter Gene Bioactivity Assay

Biological activity of tralokinumab was assessed using a reporter gene assay. Serial dilutions of tralokinumab were prepared in cell culture medium; these were mixed 1:1 with 0.8 ng/ml interleukin (IL)-13 and aliquots were added to three flat-bottomed 96-well plates. Activity of tralokinumab was measured using HEK-Blue cells expressing the human *STAT6* gene and a

secreted alkaline phosphatase reporter gene induced by STAT-6 (InvivoGen, Toulouse, France). The concentration of STAT-6–induced secreted embryonic alkaline phosphatase in the supernatant was measured with QUANTI-Blue detection medium (InvivoGen, Toulouse, France). Absorbance was measured with a Molecular Devices Spectramax2 plate reader (Molecular Devices, San Jose, CA, USA).

1.5 Neonatal Fc Receptor Binding Assay

Recombinant human neonatal Fc receptor (FcRn) was amine coupled to a Biacore CM5 sensor chip (GE Healthcare Life Sciences, Chicago, IL, USA). A reference flow cell was created by amine coupling in the absence of FcRn. Samples of tralokinumab were prepared at 1000 nM in assay running buffer (50 mM sodium phosphate, 100 mM sodium chloride, 0.005% polysorbate 20, pH 6.0) and serial dilutions were prepared 1:2 from 1000–0.98 nM. Tralokinumab dilutions were injected across the FcRn and reference flow cells and binding was measured by surface plasmon resonance. After binding, the FcRn surface was regenerated with PBS, pH 7.4. Sensorgram data were processed using the Biacore evaluation software. Reference cell data and buffer data were subtracted from tralokinumab data and fitted to a heterogeneous ligand model that assumes that FcRn has a high and a low affinity binding site.

1.6 Determination of Disulphide Bonds and Site-Specific Free Thiol Concentration Using Lys-C Peptide Mapping

Tralokinumab samples were denatured by incubation in 100 mM phosphate buffer, 1% N-ethylmaleimide, 7 M guanidine HCl, pH 7.0 at 37°C for 30 min. The denatured mixture was diluted about four-fold with 100 mM phosphate buffer, 0.1 mM EDTA, and endoproteinase Lys-C was added at 1:10 enzyme:protein ratio. The reaction mixtures were incubated at 37°C overnight. Then the same amount of Lys-C was added and the samples were incubated again at 37°C for 4–6 h.

Following Lys-C digestion, half of each reaction mixture was reduced by adding 500 mM DTT. The reduced and non-reduced digests were separated by RP-HPLC using a C18 column, the mobile phase A was 0.02% TFA in H_2O and the mobile phase B was 0.02% TFA in acetonitrile. The peptides were eluted at a flow rate of 0.2 ml/min with a gradient of mobile phase B from 0% to 95% over 90 min, and were detected by an UV detector and an on-line mass spectrometer. Disulphide-bond linked peptides were those that could be seen in non-reducing runs and that disappeared under reducing conditions.

Site-specific free thiol concentration was determined from non-reduced Lys-C peptide mapping; free thiols were capped by N-ethylmaleimide (NEM). The percentage of NEM-capped cysteine represented the free thiol percentage of that cysteine residue under non-reducing conditions. The sum of free thiols for each cysteine residue multiplied by two equaled the percentage of total free thiols.

1.7 Generation and Characterization of Partially-Reduced Tralokinumab

Reduced and non-reduced control samples were prepared from tralokinumab. Reduced samples were prepared by diluting tralokinumab with Tris pH 8.0 buffer, adding 5 mM DTT and incubating at 37°C for 30 min. Control non-reduced samples were prepared by replacing DTT with water in the incubations. All samples were transferred by buffer exchange into formulation buffer (50 mM sodium acetate, 85 mM sodium chloride, 0.01% [w/v] Polysorbate 80, pH 5.5). Sample aliquots of 100 µl were placed in 1.5 ml Eppendorf tubes and stored either at –80°C (initial time point) or at room temperature for 1 month. At the end of the incubation period, all samples were analyzed by high-pressure SEC, non-reduced gel electrophoresis, and the reporter gene bioassay.

1.8 In-Vitro Fab-Arm Exchange

Tralokinumab and another IgG_4 therapeutic mAb, mavrilimumab (anti–granulocyte macrophage colony-stimulating factor receptor), were used to generate a Fab-arm

exchanged hybrid molecule under reducing conditions and non-reducing physiological conditions.

Glutathione-reduced tralokinumab and mavrilimumab were mixed together 1:1 and incubated at 37°C for 1 day. Non-reduced tralokinumab and mavrilimumab were mixed 1:1 or 1:5 at 25°C for 3 days or 8 days. Incubates were then separated by IEC with a Dionex ProPac WCX-10 4 mm x 250 mm column. Elution was carried out with 20 mM sodium acetate, pH 5.0 with a 0–90% gradient of 200 mM NaCl in elution buffer, followed by UV absorbance detection. Intact mass analysis, conducted as above, was used to confirm which eluted fractions contained tralokinumab, mavrilimumab and the hybrid antibody.

2 Supplementary Results

2.1 Disulphide-Bond Determination

Tralokinumab comprises two heavy chains and two light chains covalently linked with four inter-chain disulphide bonds, two in the hinge region and two in the Fab region, and 12 intra-chain disulphide bonds (see Electronic Supplementary Material 3). The identification of these disulphide bonds was confirmed by the disappearance of disulphide-containing peptides in the reducing Lys-C peptide map, compared with the non-reducing Lys-C peptide map (see Electronic Supplementary Material 4). The theoretical masses of disulphide bonded peptides were compared with observed masses (data not shown). Together these results confirmed that all the expected disulphide bonded peptides were present.

2.2 Free Thiol Concentration

Percentages of free thiols were greatest in the heavy chain constant domains of tralokinumab, being between 2.1% and 4.1% (see Electronic Supplementary Material 5). No free thiols were detected on the cysteines in the heavy chain variable domains and in the hinge region.

2.3 Titers for Persistent and Transient ADAs

In STRATOS 1, median (range) titers for participants with persistent positive post-baseline ADAs were 26.0 (<13–26) in the tralokinumab Q2W group, 19.5 (13–26) in the tralokinumab Q4W group, and 52.0 (<13–208) in the placebo group. Median (range) titers for those with transient post-baseline ADAs were 26.0 (26–26) in the tralokinumab Q2W group, 26.0 (26–26) in the tralokinumab Q4W group, and 13.0 (13–13) in the placebo group.

In STRATOS 2, median (range) titers for participants with persistent positive post-baseline ADAs were 26.0 (13–104) in the tralokinumab Q2W group and 13.0 (13–208) in the placebo group. Median (range) titers for those with transient post-baseline ADAs were 26.0 (<13–26) in the tralokinumab Q2W group and no participants had transient ADAs in the placebo group.

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[1]Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden

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[3]Biopharmaceutical Development, MedImmune, One MedImmune Way, Gaithersburg, Maryland 20878, United States

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[6]Biometrics and Information Sciences, AstraZeneca, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

Corresponding author: Mats Carlsson, Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden.

Mats.Carlsson@astrazeneca.com

Electronic Supplementary Material 2

Fc glycosylation variants in tralokinumab across different manufacturing lots

Lot number	Afucosyl-ation (%)	Galactosyl-ation (%)	α-Gal (%)	Sialylation (%)	High-mannose (%)	Aglycosyl-ation (%)
1	2.2	30.4	3.7	13.7	1.7	1.0
2	1.9	36.1	4.2	17.3	1.5	1.3
3	1.7	43.8	5.7	21.3	1.3	2.0
4	1.7	50.2	9.0	22.8	1.3	2.1
5	1.7	44.2	6.3	20.8	1.4	1.7
6	1.5	42.5	5.7	24.1	1.1	1.4
7	1.6	41.8	3.8	12.4	1.2	1.2
8	1.7	33.6	1.4	5.5	1.1	1.0
9	0.7	32.3	2.4	5.4	0.7	1.3
10	1.1	38.2	3.3	3.9	0.7	1.8
11	0.9	45.7	5.1	12.6	0.7	0.9
12	0.7	46.7	7.7	14.1	0.5	0.7
13	0.5	50.6	8.5	15.3	0.4	0.5
14	0.4	50.8	9.4	15.2	0.4	0.4
15	0.5	45.4	4.2	10.9	0.5	0.5
16	0.6	46.1	4.5	11	0.6	0.6
17	1.0	37.1	2.3	11.4	0.8	1.0
18	1.2	37.4	3.2	10.8	0.8	1.2
19	1.2	40.1	3.1	11.6	0.9	1.2

α-Gal, galactose-α-1,3-galactose

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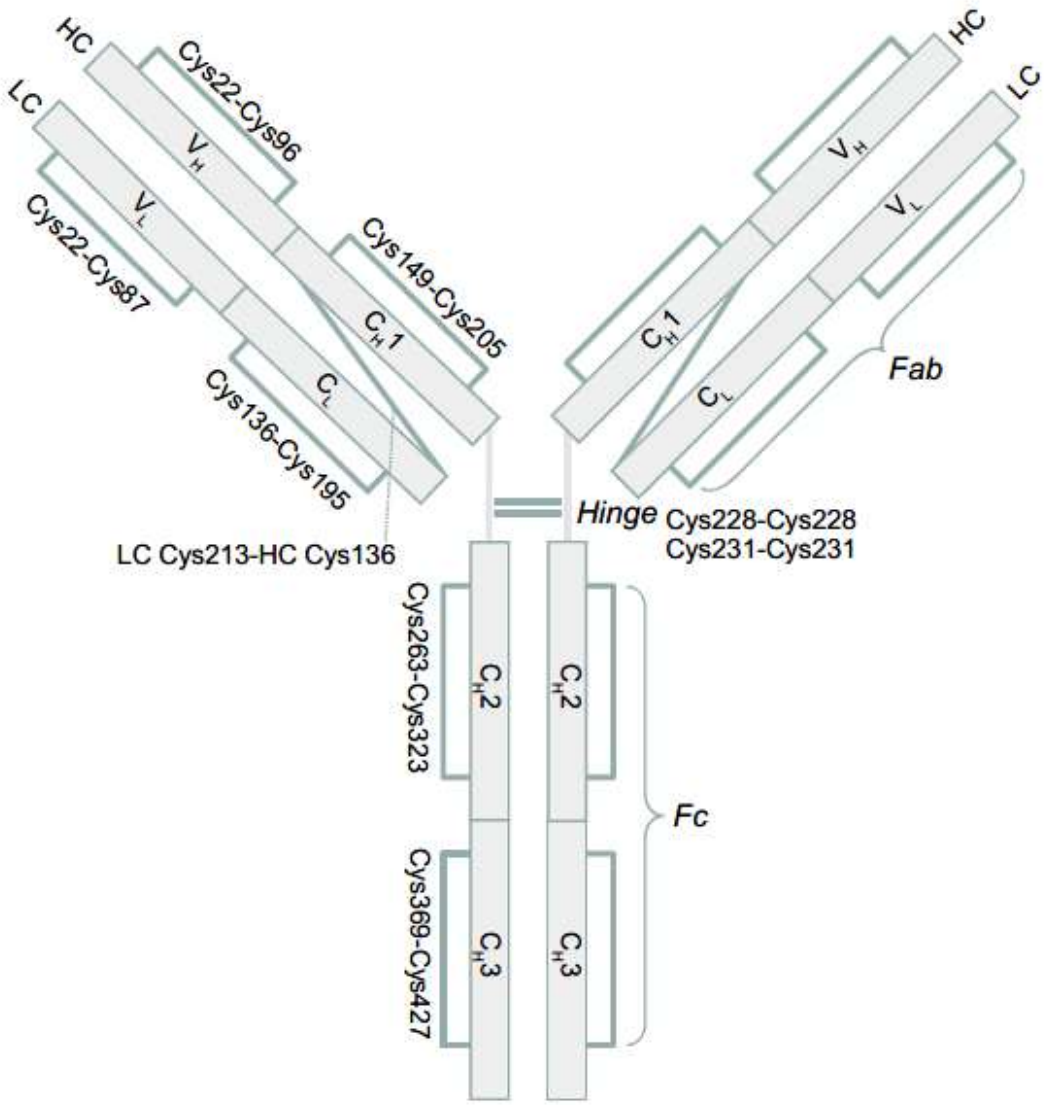
[6]Biometrics and Information Sciences, AstraZeneca, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

Corresponding author: Mats Carlsson, Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden.

Mats.Carlsson@astrazeneca.com

Electronic Supplementary Material 3

Disulphide bond pattern of tralokinumab. C_H1 Heavy Chain Constant Domain 1, C_H2 Heavy Chain Constant Domain 2, C_H3 Heavy Chain Constant Domain 3, C_L Light Chain Constant Domain, *Cys* cysteine, *Fab* fragment antigen-binding, *Fc* fragment crystallizable, *HC* heavy chain, *LC* light chain, V_H Heavy Chain Variable Domain, V_L Light Chain Variable Domain



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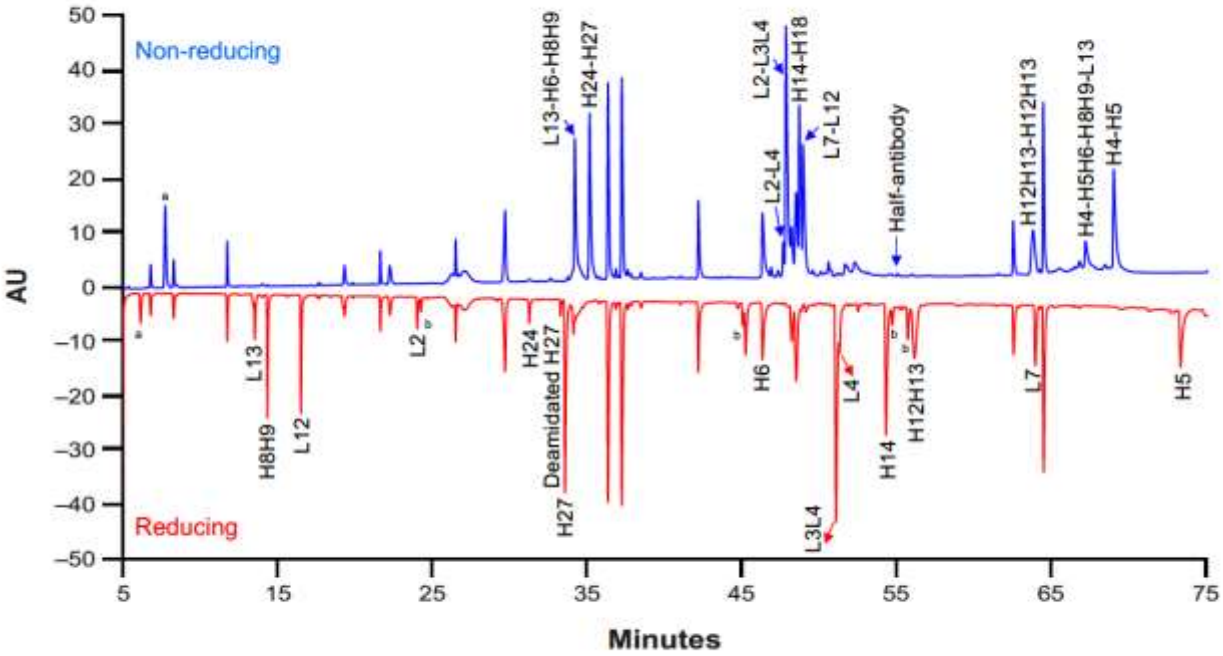
Corresponding author: Mats Carlsson, Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden.

Mats.Carlsson@astrazeneca.com

Electronic Supplementary Material 4

Lys-C disulphide bond mapping to confirm disulphide bonds of tralokinumab. *H* heavy chain peptide, *L* light chain peptide. [a]Reagent peak. [b]peptide from Lys-C enzyme autolysis



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Mats.Carlsson@astrazeneca.com

Electronic Supplementary Material 5

Free thiol percentages by tralokinumab domain

Location of cysteine bond	Identity of cysteine residue	Free thiol (%)
V_L; intra-chain	LC Cys22+NEM	0.2%
	LC Cys87+NEM	ND
C_L; intra-chain	LC Cys136+NEM	0.3%
	LC Cys195+NEM	0.7%
LC-HC; inter-chain	LC Cys213+NEM	0.4%
	HC Cys136+NEM	0.3%
V_H; intra-chain	HC Cys22+NEM	ND
	HC Cys96+NEM	ND
C_H1; intra-chain	HC Cys149+NEM	2.6%
	HC Cys205+NEM	4.1%
Hinge HC-HC; inter-chain	HC Cys228+NEM	ND
	HC Cys231+NEM	ND
C_H2; intra-chain	HC Cys263+NEM	2.3%
	HC Cys323+NEM	Peptide not detected
C_H3; intra-chain	HC Cys369+NEM	2.1%
	HC Cys427+NEM	2.6%

Cys: cysteine, C_H1 Heavy Chain Constant Domain 1, C_H2 Heavy Chain Constant Domain 2, C_H3 Heavy Chain Constant Domain 3, C_L Light Chain Constant Domain, *HC* Heavy Chain, *LC* Light Chain, *ND* not detected, *NEM* N-ethylmaleimide, V_H Heavy Chain Variable Domain, V_L Light Chain Variable Domain

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Electronic Supplementary Material 6

Adverse events reported in ADA-positive participants during the treatment period of STRATOS 1 and STRATOS 2 (safety analysis set)

STRATOS 1	Tralo Q2W (*N* = 398)	Tralo Q4W (*N* = 404)	Placebo (*N* = 400)
Participants with any relevant AE, *n* (%)	6 (1.5)	6 (1.5)	8 (2.0)
Most frequently reported relevant AEs (≥2 participants on tralokinumab), *n* (%)			
Injection site erythema	3 (0.8)	0	0
Rhinitis allergic	2 (0.5)	1 (0.2)	1 (0.3)
Erythema	1 (0.3)	1 (0.2)	0
Influenza	1 (0.3)	1 (0.2)	0
Injection site reaction	1 (0.3)	1 (0.2)	0
Upper respiratory tract infection	1 (0.3)	1 (0.2)	3 (0.8)

STRATOS 2	Tralo Q2W (*N* = 425)	Placebo (*N* = 422)
Participants with any relevant AE, *n* (%)	4 (0.9)	6 (1.4)
Most frequently reported relevant AEs (≥2 participants on tralokinumab), *n* (%)		
Injection site reaction	2 (0.5)	0

ADA anti-drug antibody, *AE* adverse event, *Q2W* every 2 weeks, *Q4W* every 4 weeks, *tralo* tralokinumab

AEs were included with an onset date on or after the first day of treatment and up to the date of the last day of study treatment plus the dosing frequency

Participants were defined as ADA positive if they had an ADA positive blood sample at any time during the study

Evaluation of Antibody Properties and Clinically Relevant Immunogenicity, Anaphylaxis, and Hypersensitivity Reactions in Two Phase III Trials of Tralokinumab in Severe, Uncontrolled Asthma

Drug Safety

Mats Carlsson,[1] Martin Braddock,[2] Yuling Li,[3] Jihong Wang,[3] Weichen Xu,[3] Nicholas White,[4] Ayman Megally,[5] Gillian Hunter,[6] Gene Colice[5]

[1]Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden

[2]Global Medicines Development, AstraZeneca, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

[3]Biopharmaceutical Development, MedImmune, One MedImmune Way, Gaithersburg, Maryland 20878, United States

[4]Clinical Pharmacology, Pharmacometrics and DMPK, MedImmune, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

[5]Global Medicines Development, AstraZeneca, One MedImmune Way, Gaithersburg, Maryland 20878, United States

[6]Biometrics and Information Sciences, AstraZeneca, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

Corresponding author: Mats Carlsson, Patient Safety, Global Medicines Development, AstraZeneca, Pepparedsleden 1, Mölndal, SE-431 83, Sweden.

Mats.Carlsson@astrazeneca.com

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Adverse events of hypersensitivity, anaphylactic reaction, and anaphylactic shock condition reported during the treatment period of STRATOS 1 and STRATOS 2 (safety analysis set)

STRATOS 1	Tralo Q2W (*N* = 398)	Tralo Q4W (*N* = 404)	Placebo (*N* = 400)
Participants with any relevant AE, *n* (%)	103 (25.9)	101 (25.0)	102 (25.5)
Most frequently reported relevant AEs (≥3 participants on tralokinumab), *n* (%)			
Asthma	47 (11.8)	48 (11.9)	51 (12.8)
Cough	12 (3.0)	13 (3.2)	12 (3.0)
Rhinitis allergic	10 (2.5)	15 (3.7)	14 (3.5)
Dyspnea	5 (1.3)	9 (2.2)	7 (1.8)
Rash	8 (2.0)	4 (1.0)	5 (1.3)
Injection site hypersensitivity	5 (1.3)	4 (1.0)	0
Pruritus	5 (1.3)	3 (0.7)	3 (0.8)
Urticaria	3 (0.8)	4 (1.0)	4 (1.0)
Erythema	5 (1.3)	1 (0.2)	0
Injection site rash	4 (1.0)	1 (0.2)	0
Conjunctivitis	2 (0.5)	2 (0.5)	3 (0.8)
Dermatitis contact	1 (0.3)	3 (0.7)	0
Injection site urticaria	3 (0.8)	1 (0.2)	1 (0.3)
Conjunctivitis allergic	2 (0.5)	1 (0.2)	1 (0.3)
Seasonal allergy	2 (0.5)	1 (0.2)	5 (1.3)
Wheezing	2 (0.5)	1 (0.2)	5 (1.3)
STRATOS 2	Tralo Q2W (*N* = 425)		Placebo (*N* = 422)
Participants with any relevant AE, *n* (%)	56 (13.2)		38 (9.0)
Most frequently reported relevant AEs (≥3 participants on tralokinumab), *n* (%)			
Asthma	14 (3.3)		16 (3.8)
Pruritus	6 (1.4)		2 (0.5)
Rash	5 (1.2)		0
Skin reaction	5 (1.2)		0
Erythema	3 (0.7)		0

AE adverse event, *Q2W* every 2 weeks, *Q4W* every 4 weeks, *tralo* tralokinumab

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[6]Biometrics and Information Sciences, AstraZeneca, Granta Park, Great Abington, Cambridge, CB21 6GH, United Kingdom

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Mats.Carlsson@astrazeneca.com

Electronic Supplementary Material 8

Independent evaluation of anaphylaxis and hypersensitivity reactions

AE identified as possible anaphylaxis	Number of events			Presence of the following, consistent with Sampson criteria					AE considered by external evaluator as	
	Tralo Q2W	Tralo Q4W	Placebo	Shock	Respiratory component	Dermatology component	Cardiovascular component	Gastrointestinal component	Anaphylaxis	Non-anaphylactic hypersensitivity
STRATOS 1										
Angioedema	1	0	0	No	No	No	No	No	No	Undetermined
Asthma	1	0	0	No	No	No	No	No	No	No
Blood pressure decreased	0	0	1	No	No	No	No	No	No	No
Circulatory collapse	1	0	0	Undetermined	No	No	No	No	No	No
Cough	1	0	1	No	No	No	No	No	No	No
Dizziness	0	0	2	No	No	No	No	No	No	No
Drug hypersensitivity	0	3	0	No	No	No	No	No	No	No
Dyspnea	1	0	0	No	No	No	No	No	No	No
Erythema	1	0	0	No	No	No	No	No	No	No
Injection site reaction	1	0	0	No	No	No	No	No	No	No
Injection site urticaria	1	0	0	No	No	No	No	No	No	No
Lip swelling	0	1	0	No	No	No	No	No	No	No
Pharyngeal edema	1	0	0	No	No	No	No	No	No	Undetermined*
Pruritus	1	0	0	No	No	No	No	No	No	No

Rash	0	0	1	No	No	No	No	No	No	No
Somnolence	0	2	0	No	No	No	No	No	No	No
Swollen tongue	1	0	0	No	No	No	No	No	No	Undetermined*
Syncope	1	2	2	No	No	No	No	No	No	No
Throat irritation	1	0	0	No	No	No	No	No	No	No
Vomiting	0	0	2	No	No	No	No	No	No	No
Wheezing	1	0	1	No	No	No	No	No	No	No
STRATOS 2										
Blood pressure decreased	1	–	0	No	No	No	Yes	No	No	No
Eyelid edema	1	–	1	No	No	No (*n* = 1, placebo); Yes (*n* = 1, tralo Q2W)	No	No	No	No (*n* = 1, placebo); Undetermined (*n* = 1, tralo Q2W)
Hypotension	0	–	1	No	No	No	Yes	No	No	No
Pruritus	2	–	1	No	No	Yes	No	No	No	Undetermined
Rash	4	–	0	No	No	No (*n* = 1); Yes (*n* = 3)	No	No	No	No (*n* = 1); Undetermined (*n* = 3)
Rash generalized	0	–	1	No	No	Yes	No	No	No	No
Rash macular	0	–	1	No	No	Yes	No	No	No	Undetermined
Urticaria	2	–	3	No	No (*n* = 4: *n* = 2, placebo; *n* = 2, Q2W); Yes (*n* = 1, placebo)	Yes	No	No	No (*n* = 4; *n* = 2, placebo; *n* = 2, tralo Q2W); Yes (*n* = 1, placebo)	Undetermined (*n* = 3; *n* = 2, placebo; *n* = 1, tralo Q2W); Yes (*n* = 1, tralo Q2W); [a](*n* = 1, placebo)

Wheezing	0	–	1	No	Yes	Yes	No	No	Yes	[a]

AE, adverse event; *Q2W*, every 2 weeks; *Q4W*, every 4 weeks; *tralo*, tralokinumab

[a]No anaphylactic hypersensitivity judgment made by external evaluator, due to lack of narrative information available

– indicates Q4W treatment arm not used in STRATOS 2 trial

***Case narrative for subject who discontinued treatment due to pharyngeal oedema and swollen tongue, reported as serious AEs**

This subject's current medical conditions included asthma triggered by allergens and iodine, allergic rhinitis, chronic sinusitis, and eczema. The subject was randomized to receive tralokinumab with a dosing regimen of Q2W (total expected doses 26). On the day of first dose (of one dose received) of tralokinumab, the subject developed serious adverse events of swollen tongue and pharyngeal oedema. At the time of discharge, two hours after receiving the investigational product, no complications had been reported. However, 5 minutes after discharge, the subject experienced slight symptoms of tingling on the tongue and in the throat. After 10 more minutes, the subject had difficulty swallowing water, following which the subject took diphenhydramine 25 mg daily orally. At 30 minutes from discharge, the subject had difficulty forming words due to swelling of the tongue. The subject did not seek any medical help, but decided to self-medicate with cetirizine 10 mg daily orally for swollen tongue. The symptoms dissipated gradually on the same day but did not resolve completely. However, the subject's condition improved by next morning and had recovered from the events two days after receiving the dose.

Both the events were considered by the Investigator to be important medical events of moderate intensity, related to the investigational product. As a result of the events, the investigational product was permanently discontinued on the first day of administration, which was also the last dose prior to discontinuation.