Molecular structure of human GM-CSF in complex with a disease-associated anti-human GM-CSF autoantibody and its potential biological implications

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Polyclonal autoantibodies against human GM-CSF (granulocyte/macrophage colony-stimulating factor) are a hallmark of PAP (pulmonary alveolar proteinosis) and several other reported autoimmune diseases. MB007 is a high-affinity anti-(human GM-CSF) autoantibody isolated from a patient suffering from PAP which shows only modest neutralization of GM-CSF bioactivity. We describe the first crystal structure of a cytokine-directed human IgG1 autoantibody-binding fragment (Fab) at 1.9 Å (1 Å = 0.1 nm) resolution. Its CDR3-H substantially differs from all VH7 germline IgG1 structures reported previously. We derive a reliable model of the antigen–autoantibody complex by using NMR chemical shift perturbation data in combination with computational methods. Superposition of the modelled complex structure with the human GM-CSF–GM-CSF ternary receptor complex reveals only little overlap between receptor and Fab when bound to GM-CSF. Our model provides a structural basis for understanding the mode of action of the MB007 autoantibody.

Key words: autoantibody, epitope mapping, Fas fragment, granulocyte/macrophage colony-stimulating factor (GM-CSF), microscale thermophoresis (MST), pulmonary alveolar proteinosis (PAP).

INTRODUCTION

Autoantibodies are known to be implicated in many autoimmune responses such as Graves’ disease, systemic lupus erythematosus, insulin-dependent diabetes mellitus and rheumatoid arthritis. Many autoimmune disorders or diseases are associated with autoantibodies which target and damage certain organs or tissues. To date, the causes of autoantibody production are not well understood and the physiological role of their presence remains unclear [1–3]. Recently, we have isolated a human IgG1 autoantibody (MB007) from a patient suffering from PAP (pulmonary alveolar proteinosis). Idiopathic PAP is associated with high levels of autoantibodies against the cytokine GM-CSF (granulocyte/macrophage colony-stimulating factor), which regulates survival, differentiation and proliferation of macrophages. Interference with GM-CSF signalling by neutralizing autoantibodies which block GM-CSF bioactivity, thus inhibiting alveolar macrophage maturation and consequently leading to accumulation of lipoproteinaceous material within alveoli, results in pulmonary surfactant accumulation, impaired gas exchange and respiratory insufficiency. In familial PAP, mutations within the GM-CSFR (GM-CSF receptor) α or β subunits result in a similar disease presentation. Targeted disruption of the GM-CSF locus in mice also recapitulates this disorder [4–9]. Intriguingly, studies have found that GM-CSF autoantibodies are also found in healthy individuals [10,11], but at significantly lower concentrations, where it has been suggested that they might play an important role in regulation of GM-CSF bioactivity by acting as a sink or buffer [3]. It therefore appears that it is not only the presence of autoantibodies that determines the development of autoimmune disorders, but also their affinity for the antigen, potency in neutralizing cellular functions and concentration, as well as possibly the epitopes which are recognized. More studies are needed to learn about the significance and function of naturally evolved autoantibodies in healthy persons, and the critical threshold of autoantibodies leading to autoimmune responses as well as their mechanism of regulation. Investigating the structural details of antigen–autoantibody complexes are crucial for understanding the biological effects of their particular antibody. We crystallized the antigen-binding fragment (Fab) MB007 derived from an autoantibody isolated from a PAP patient. Co-crystallization trials of the GM-CSF–MB007 Fab complex were so far unsuccessful and structural determination using NMR was not attempted due to the size of the complex. We have therefore used a concerted application of experimental data and computational methods to derive a reliable GM-CSF–MB007 complex model consistent with biophysical and in vivo bioassay data. In the present study, we have solved the first crystal structure of a human IgG1 disease-associated autoantibody. Additionally, we provide structural information of this autoantibody in a simulated binary complex to its related antigen GM-CSF, thus presenting the first cytokine–autoantibody complex structure known to date. Previously published autoantibody complexes are associated with

Abbreviations used: 2D, two-dimensional; CDR, complementarity-determining region; GM-CSF, granulocyte/macrophage colony-stimulating factor; GM-CSFR, GM-CSF receptor; hGM-CSF, human GM-CSF; LED, light-emitting diode; MD, molecular dynamics; MST, microscale thermophoresis; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PAP, pulmonary alveolar proteinosis; rHM-CSF, recombinant human GM-CSF; RMSD, root mean square deviation; SPR, surface plasmon resonance; TROSY, transverse relaxation optimized spectroscopy.

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Atomic co-ordinates and structure factors of the refined model of MB007 Fab have been deposited in the PDB under accession code 4EOW.
receptors, DNA or peptides. On the basis of structural information, our results help to rationalize the observed modest neutralization of the MB007 autoantibody that reduces GM-CSF activity by exhibiting high binding affinity. It might serve as a model case describing a generally relevant regulatory role of autoantibodies in cytokine homeostasis.

EXPERIMENTAL

hGM-CSF (human GM-CSF) expression, release of properly folded soluble protein and purification

The gene encoding hGM-CSF was synthesized using the codon optimization technique for Escherichia coli by Geneart (Regensburg, Germany) and subcloned into a pET30a expression vector (Novagen). The expression vector was transformed into an E. coli chemical competent cell line BL21 CodonPlus RIL (DE3) (Stratagene) before expression of soluble rhGM-CSF (recombinant hGM-CSF) in the plasmic space. Protein expression was carried out for 16 h at 19°C after induction with 1.25 mM IPTG (isopropyl β-D-thiogalactopyranoside). Release of soluble protein was performed using an osmotic shock approach based on methods described previously [12,13]. Subsequently a combination of affinity, ion-exchange and size-exclusion chromatography was used to isolate properly folded active target protein. Full details of construct design and purification protocols are given in the Supplementary Online Data at http://www.biochemj.org/bj/447/bj4470205add.htm.

Antibody isolation, Fab fragment production and purification

The MB007 anti-GM-CSF antibody was initially isolated from a PAP-affected patient producing autoantibodies against GM-CSF. The gene encoding MB007 was cloned into Gateway® pDEST BIG1 (Invitrogen) and the antibody was expressed in CHO (Chinese-hamster ovary) cells. Soluble protein was secreted into the medium. The Fab fragment was prepared by papain cleavage of the intact corresponding MB007 monoclonal antibody using the Pierce® Fab preparation kit following the manufacturer’s instructions (Thermo Fisher Scientific). Fab fragments were separated by Protein A-affinity chromatography and subsequently purified by gel filtration on a Superdex 75 10/300 (GE Healthcare) column to remove remaining minor impurities and transferring the protein in appropriate buffers depending on the following experiments. For crystallization experiments, MB007 Fab was purified twice with running buffer of 10 mM Bicine and 10 mM NaCl at pH 8.3. Fractions containing homogeneous target protein were pooled and concentrated to 5 mg/ml. In order to facilitate NMR chemical shift perturbation analysis, MB007 Fab was transferred into 50 mM sodium phosphate and 150 mM NaCl buffer at pH 7.5 and 5% ²H₂O was added before the experimental approach. Protein samples containing MB007 Fab were concentrated by centrifugal ultrafiltration using Ultrafree 4 centrifugal concentrators (molecular-mass cut-off 30 kDa) (Millipore).

Crystallization and data collection

Crystals were initially screened in 96-well plates using the sitting nanodrop vapour-diffusion approach using commercially available crystallization screens (Hampton, Jena Bioscience and Qiagen) diluted 1:1 in sterile filtered distilled water before use to decrease the concentration of buffer components and thus lowering the ionic strength. The screening was done using droplets consisting of 200 nl of protein sample and 200 nl of reservoir. Prismatic crystals grew within 5 days in 15% (w/v) poly(ethylene glycol) 4000 and 0.1 M ammonium sulfate at 4°C. To optimize crystal diffraction quality, initial crystals were used for streak seeding in hanging drops. High-quality crystals grew in 15–19% (w/v) poly(ethylene glycol) 4000, 0.1 M ammonium sulfate and 0.1 M sodium acetate (pH 3.4) at 4°C. Single crystals suitable for diffraction analysis were prepared in reservoir solution supplemented with either 30% xylitol or 30% sucrose and flash frozen in liquid nitrogen directly from the hanging drop. Diffraction data were collected at the X06SA SLS (Swiss Light Source) beamline at the Paul Scherrer Institute (Villigen, Switzerland). X-ray diffraction data were recorded at cryogenic temperature (100 K) using 0.91 Å (1 Å = 0.1 nm) synchrotron radiation. A 1.9 Å resolution dataset consisting of 360 images (0.5°/frame, 0.5 s exposure) from a single crystal was collected on a PILATUS 6M detector.

Structure determination, refinement and analysis

Crystallographic intensity data were indexed, integrated, scaled and reduced with autoPROC [14], particularly XDS, SCALa and POINTLESS [15–18]. The structure of MB007 Fab was solved by molecular replacement with the program Phaser [15,19] separately using co-ordinates of the heavy chain and light chain from a recently solved in-house Fab structure (M. Blech and S. Hörer, unpublished work) as search models. The MB007 Fab heavy chain shares 68% and the light chain 55% sequence identity with its search model. Loop and CDR (complementarity-determining region) 3 residues of the heavy chain were removed from the search model. One copy of each co-ordinate set was located within the asymmetric unit. The molecular replacement solution resulted in a good and interpretable 2F o − F c map. A total of 5% of the data were randomly selected for Rfree calculation. Atomic model refinement with tight geometric weights was performed with autoBUSTER [15,20] in the resolution range 40–1.9 Å. TLS (Translation–Liberation–Screw-rotation) refinement was used in the final steps of refinement. Coot [21] was used for model building. Structure validation was carried out using Molprobity [22]. Structural figures were prepared using PyMOL (http://www.pymol.org). X-ray data collection and refinement statistics are listed in Table 1.

Chemical shift experiments and epitope mapping

A comparison of amide backbone NMR signals of free and bound states has been used earlier to identify protein–protein interaction sites [24–26]. Chemical shift Perturbation analysis of bound antigen to its Fab region was used alternatively as a rapid and reliable method to determine the antigenic epitope in solution.

Backbone resonance assignment of rhGM-CSF has been reported previously [27]. Slightly different dispersion of the cross peaks of free GM-CSF in the 2D (two-dimensional) ¹⁵N TROSY (transverse relaxation optimized spectroscopy) spectrum are probably due to the altered protein production scheme or differences in the construct and experimental conditions [28,29]. We therefore acquired a set of triple resonance experiments [HNCO, HN(CA)CO, HNCA, HN(CO)CA, HN(CA)CO and CBCA(CO)HN] using ¹³C isotope-labelled rhGM-CSF [30,31]. The comparison showed very high overall backbone resonance agreement of in-house and published resonance assignment. The binary complex of ²H,¹⁵N rhGM-CSF with MB007 Fab was preformed and purified on a Superdex 75 10/300 column allowing 1:1 complex formation and separation of unbound protein. For epitope mapping, 2D ¹⁵N TROSY values of free and Fab-bound antigen were collected at 600 MHz.
as well as 800 MHz. Samples of free antigen contained 0.4 mM 2H,15N rhGM-CSF, whereas samples of 2H,15N hGM-CSF in complex with MB007 Fab contained 0.1–0.3 mM antigen. Epitope mapping was carried out by superposition of the 2D 15N TROSY spectra of free and bound rhGM-CSF. NMR experiments were performed at 298 K on Bruker AV II + 600 MHz and Bruker Avance 800 MHz spectrometers equipped with 5 mm inverse-triple-resonance z-gradient cryogenic probes. Chemical shifts were referenced using DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid). In all experiments, 5% 2H2O was added to the sample. All spectra were processed using TopSpin 2.0 (Bruker Biospin) and FELIX2007 (Felix) respectively. Data analysis was performed using the NMRViewJ, version 8.0.rc51 (One Moon Scientific).

### hGM-CSF–MB007 complex structure prediction

Computational ab initio prediction of a protein–protein complex structure, based solely on structures of the individual binding partners, poses a substantial challenge because of the huge conformational space that needs to be sampled. However, NMR data that describe the binding epitope can be used to pre-orientate the antigen relative to the Fab for a protein–protein docking approach. This procedure leads to a reduction in rotational and translational degrees of freedom. Protein–protein docking guided by experimental data is therefore expected to be much more predictive than an ab initio approach. Docking of antibodies imposes additional challenges due to the flexible nature of the CDRs, in particular CDR3-H, which may contain a long and flexible loop. For computational efficacy, protein flexibility in docking programs is usually restricted to alternative side-chain conformations or small backbone movements that are not able to capture larger conformational adjustments frequently observed upon formation of a protein–protein complex.

In a first attempt to predict the MB007–GM-CSF complex structure, we used a protein–protein docking approach with RosettaDock (Rosetta version 3.1) [32]. hGM-CSF (PDB code 2GFM) was placed at a distance of 15 Å above the CDRs of MB007 with helix 6 facing towards the antibody. Before each docking run, GM-CSF was rotated randomly around the axis connecting the centre of mass of the Fv fragment to the centre of mass of the ligand. Both were subsequently subjected to a random translational perturbation of 3 Å and a random rotational perturbation of 8°. Hence, instead of a blind docking search, a local docking was performed accounting for the information obtained from the NMR chemical shift perturbations. Default weights and the default scoring scheme for the docking protocol was used and 10 000 poses in total were generated. As a post-filtering stage, we used MD (molecular dynamics) simulations starting from the ten lowest scoring docking poses. The complex structures were energy-minimized and 100 ps MD runs with position restraints on the protein heavy atoms were carried out to relax the solvent around the complex model. Subsequently, free MD runs of 200 ns length were performed for each system. For seven models, partial or complete dissociation of the complex was observed during the simulation, whereas three models, two of which had very similar initial conformations, yielded trajectories of stable complexes. Since both complex models are consistent with the NMR data, we used both as input for an additional two sets of simulations in which we addressed the question whether the complex would form spontaneously during a simulation when the partners are separated by 10 Å in the initial configuration. The solvent was again relaxed with a 100 ps position restraint run, then for both complex models 20 simulations of 200 ns length each were performed with different initial velocities. In the simulations started from the perturbed conformation 1, no trajectory was obtained that resulted in a conformation close to the docked complex structure from which it derived. MD experimental details are specified in the Supplementary Online Data.

### Optical laser-induced thermophoresis

MST (microscale thermophoresis) is a fluorescence-based technique using IR lasers to locally induce a temperature gradient within thin glass capillaries, which typically leads to a depletion of solvated molecules in the region of elevated temperature. The thermophoretic movement of a fluorescently labelled molecule is measured time-dependently during diffusion and back-diffusion [33–37]. Assuming a local thermodynamic equilibrium, the thermodiffusive drift velocity depends linearly on the induced temperature gradient \( \nabla T \) with a proportionality constant equally to the thermal diffusion coefficient (thermophoretic mobility, \( D_T \)) according to \( v = \frac{D_T}{\kappa} \nabla T \). The directed motion of solvated molecules along a localized microscopic temperature gradient is attributed to the Ludwig–Soret effect, which can be described by the Soret coefficient (\( S_S = D_S/D_T \), with \( D_S \) as the diffusion coefficient). Under these conditions, molecules are subjected to local thermal fluctuations following the Boltzmann distribution \( c_{\text{hot}}/c_{\text{cold}} = \exp\left(-\frac{D_T}{D_S}(T_{\text{hot}} - T_{\text{cold}})\right) \). Further implementation of thermodynamic aspects such as ionic shielding, temperature-sensitive hydration entropy of a single molecule with its Gibbs free enthalpy, \( G \), and the influence of the complex effects on thermophoretic movement can be mathematically expressed as follows:

\[
S_T = \frac{A}{kT} \left(-\Delta S_{\text{hyd}} + \frac{\beta \sigma_{\text{eff}}}{4\kappa \varepsilon_{\varepsilon_T}} \cdot \lambda_{\text{OH}}\right)
\]

with \( A \) being the particle surface area, \( \sigma_{\text{eff}} \) the effective surface charge density, \( \Delta S_{\text{hyd}} \) the hydration entropy of the...
molecule–solution interface, $\lambda_{D\mu}$ the Debye–Hückel screening length, $\varepsilon$ the dielectric constant, and $\beta$ its temperature derivative. For a complete derivation see [35,36].

For the setup-expectant, GM-CSF was fluorescently labelled with the Monolith NT$^{TM}$ Protein Labeling Kit NT-647 (NanoTemper Technologies) according to the manufacturer’s protocol. In salt-dependence experiments, labelled hGM-CSF was used at a concentration of 5 nM, whereas MB007 Fab was titrated in a 1:1 serial dilution. The measurements were performed at 30% LED (light-emitting diode) power and 25% IR laser power which induces a temperature increase of approximately 2 K. Laser-on and -off times were adjusted to 30 and 5 s respectively. Samples were incubated for 20 min before measurement. For competition experiments, MB007 Fab and hGM-CSFRα (R&D Systems) (expressed in E. coli) were each initially titrated in a 1:1 serial dilution to 5 nM labelled hGM-CSF. Afterwards, the hGM-CSF–hGM-CSFRα complex was pre-formed by combining 5 nM labelled hGM-CSF with a 3-fold higher binding affinity concentration of hGM-CSFRα as determined in binary complexation. The samples were incubated for 20 min before adding MB007 Fab in a 1:1 serial dilution. The MST signal was measured again after 20 min of incubation. The competition measurements were performed at 40% LED power and 30% IR laser power ($\Delta T = 3$ K) adjusting the laser-on time to 30 s and laser-off time to 5 s. All measurements were carried out in 50 mM sodium phosphate buffer at pH 7.4 containing 150 mM NaCl and 0.05% Tween 20 at 298 K. Salt-dependence was determined using increasing NaCl concentrations. The measurements were performed on NanoTemper Monolith NT.015 and NT.115 instruments in standard treated capillaries and analysed using NanoTemper Analysis version 1.2.205.

TF-1 in vivo neutralization assay

The neutralizing effect of anti-GM-CSF autoantibodies was determined using the TF-1 human cell line as described previously [11]. Briefly, the proliferation of TF-1 cells is dependent on growth factors such as GM-CSF. In the absence or inactivity of GM-CSF, cells will not proliferate and undergo apoptosis within 3–4 days. We used a calorimetric method in the CellTiter 96 Aqueous One Solution Assay (Promega) to determine the number of viable cells. Since the assay solution contains MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], colour changes during proliferation owing to reduction of MTS to formazan can be monitored. The quantity of formazan is directly proportional to the number of living cells in culture and can be measured at 490 nm. The TF-1 cells were suspended in assay medium and the cell number was adjusted to 10$^5$ cells/ml. Cell suspension was seeded at 100 µl/well into a sterile 96-well plate (Nunclon$^\text{TM}$ Surface). The anti-hGM-CSF antibodies and hGM-CSF were pre-diluted into medium and cell plates were incubated for 72 h at 37°C supplemented with 5% CO2. Then, 20 µl of MTS was added before incubation for 150 min at 37°C and 5% CO2. The number of living cells was quantified at 490 nm using the Multiskan RC. Data were analysed with GraphPad Prism Software package (version 5.04).

RESULTS

MB007 Fab structure characterization

The MB007 light chain is derived from a Vκ1 germline gene and shares 92% sequence identity with its precursor (eight amino acids difference in the V region). The heavy chain originates from a VH7 gene but differs in 19 positions in the variable region, resulting in only 86% sequence identity with its VH7 germline precursor. Sequence differences from the most closely related germline genes mainly occur at solvent-exposed residue side chains. Prismatic crystals of MB007 Fab grow within 4 days in 15–19% poly(ethylene glycol) 4000, 0.1 M ammonium sulfate and 0.1 M sodium acetate (pH 3.4) at 4°C using hanging-drop vapour-diffusion to approximate dimensions of 400 $\mu$m × 100 $\mu$m × 30 $\mu$m. Diffraction data of 1.9 Å resolution were collected from a single crystal. The orthorhombic crystals contain one copy of MB007 Fab within the asymmetric unit [Matthew’s coefficient ($V_m$) of 2.72 corresponding to a solvent content of 54.7%]. MB007 Fab subunits consisting of Vκ and CL(λ) domains of the light chain and VH and CH1 domains of the heavy chain adopt the typical Ig fold characterized by an antiparallel β-sheet sandwich architecture (Figure 1A). Its most striking structural feature is the unusual seven amino acid α-helical segment (TEYWEDG, residues 107–113 according to the Kabat numbering convention [38]) within the hypervariable loop of CD3H-H (Figures 1A and 1C), which presumably constitutes a part of the GM-CSF-binding paratope (see below). The CD3H-H loop contains 18 residues, which is longer than the average length of 11.6 residues of human antibodies [39]. A relatively large positively charged groove spans the CDR platform in which CD3H-H protrudes from the surface by approximately 11 Å (Figure 1A). The electron-density map for the Fab is generally of high quality and no main-chain breaks were observed, except for the 18-amino-acid-long CD3H-H loop, in which a lower-quality electron density is observed for H:Pro103, H:Arg102, H:Asp105, H:Pro101, H:Arg102, H:Asp105, and NT.115 instruments in standard treated capillaries and analysed using NanoTemper Analysis version 1.2.205.

CDR3-H is flexible and moves in part as a rigid body

The key structural element of MB007 is its 18-residue-long CD3H-H loop that, to the best of our knowledge, substantially differs from all previously reported VH7 germline IgG1 CD3H-H structures (Figure 1B). This loop is a prominent structural feature, by projecting two aromatic amino acids (H:Phe103 and H:Tyr104) [38] from the base of the CDR surface (Figure 1A). The electron-density map for the Fab is generally of high quality and no main-chain breaks were observed, except for the 18-amino-acid-long CD3H-H loop, in which a lower-quality electron density is observed for H:Pro103, H:Arg102, H:Asp105, H:Glu111, and H:Asp116 (Figure 2A). The other CDRs are well defined. Stereochemical analysis reveals good quality of the structure: 95.6% of all residues reside in preferred regions and only 4.1% are located in allowed regions of the Ramachandran plot. Crystallographic refinement and model statistics are given in Table 1.
Figure 1  Structural features of the MB007 Fab

(A) Crystal structure of MB007 Fab in a combined cartoon surface representation. The heavy chain is highlighted in orange (variable domain in dark orange; constant domain in pale orange), and the light chain is highlighted in green (variable domain in dark green; constant domain in pale green). Labels correspond either to domain names or CDRs. The most remarkable structural element is the 18-residue-long CDR3-H loop (red), which extends approximately 11 Å from the base of the CDR surface. (B) Superposition of all published VH7 antibodies in which the CDR3-H loops are highlighted in red except for MB007 (orange). MB007 features a unique α-helical secondary-structure element in its CDR3-H region and thereby differs substantially from germline VH7 structures reported previously. (C) A close-up view of the CDR3-H loop with its annotated residues shows that the C-terminal seven-residue-long α-helical segment fits snugly into a surface groove of the Ig domain and that the N-terminal part of the loop exposes Phe103 and Tyr104.

NMR epitope mapping of the Fab-binding site on GM-CSF reveals a discontinuous three-dimensional epitope

We acquired well-dispersed 2D 15N TROSY spectra of free and bound 2H,15N-labelled recombinant hGM-CSF (Figure 3A, shown in black and red respectively) under identical conditions of 298 K and buffer at pH 7.4. The spectra obtained confirm the presence of properly folded protein and quantitative complex formation with MB007 Fab. On the basis of cross-peak assignment of the free hGM-CSF [27], a semi-quantitative residue-specific chemical shift perturbation analysis was performed. Superposition of the spectra of the free and bound hGM-CSF (Figure 3A) revealed a significant overall line broadening due to the increase in molecular mass upon complex formation. Most resonances exhibit no chemical shift perturbations (Δδ<0.05 p.p.m.). The corresponding residues are non-interacting residues (e.g. Ile101 and Phe103 in Figure 3A) and could be readily assigned in the spectrum of the complex. Residues experiencing small chemical shift changes (0.05 p.p.m. < Δδ<0.15 p.p.m.) (e.g. Val40 and Gln99 in Figure 3A) are probably either located remote of the binding site or undergo small conformational changes due to minor changes in the structural environment upon complex formation. In contrast, cross-peaks of residues interacting directly with Fab display significant chemical shift changes in their resonance frequencies (0.2 p.p.m. ≤ Δδ≤0.4 p.p.m.) (e.g. Ser44 in Figure 3A). These cross peaks were supposed to be involved directly in antibody binding and their Δδ arbitrarily set to 0.45. Figures 3(C) and 3(D) show a relative broadly mapped epitope of MB007, in which residues identified by chemical shift perturbation analysis are scattered around a prominent binding patch. The prominent binding patch composed of helices 1 and 6 as well as loop 2 which is connecting β-strand 1 with helix 3, were sequentially apart, but in close proximity on the surface of hGM-CSF, and thus forming a sequentially discontinuous three-dimensional epitope.

Predicted MB007–GM-CSF complex structure

Despite substantial efforts we were unable to obtain hGM-CSF–MB007 Fab co-crystals with acceptable diffraction properties.
We therefore sought to determine the complex structure using an *in silico* docking approach, based on the NMR epitope mapping data. We obtained a number of distinct complex models with favourable docking scores (Supplementary Figure S2 at http://www.biochemj.org/bj/447/bj4470205add.htm). With the exception of one model of the complex in which the orientation of hGM-CSF is rotated 180° about the horizontal axes, the models cluster around a single arrangement which is discussed in detail below. The binding interface is formed by the face of hGM-CSF comprising helices 1 and 6, CDR3-H, CDR1-L and CDR3-L of MB007 form the paratope. Figure 4 shows a representative complex of GM-CSF–MB007 after 500 ns of MD simulation. Putative ‘hotspot’ residues Asn27, Arg30 and Asp31 (helix 1) and Glu104, Lys107, Glu108, Lys111 and Asp112 (helix 6) of the epitope are mostly charged amino acids. A positively charged canyon built by Tyr33 (CDR1-L), Asp94 and Arg99 (CDR3-L), Arg54 (CDR2-H), and Arg99, Arg102, Phe103 and Tyr104 (CDR3-H) [38] spans the MB007 CDR platform. This suggests that the formation of the hGM-CSF–MB007 complex is mainly driven by complementary electrostatic potential surfaces in which the predominantly negatively charged epitope on hGM-CSF faces a predominantly positively charged paratope surface area on MB007 (Figure 4, right-hand panel). The shape of electrostatic potential surfaces (Supplementary Figure S3 at http://www.biochemj.org/bj/447/bj4470205add.htm) derived from adaptive Poisson–Boltzmann calculations using PyMOL’s APBS plugin tool shows two prominent bulges at the centre of the interfaces. A negatively charged convex-formed electrostatic potential at the GM-CSF-binding interface is located opposite a positively charged convex-formed electrostatic potential on MB007. In order to confirm that charges play a prominent role in complex formation, we analysed the dependence of the binding affinity on ionic strength using MST analysis. The formation of the hGM-CSF–MB007 complex is characterized by high binding affinity (Kₐ 1 nM). We observed an approximately 400-fold reduction in binding affinity of MB007 Fab to hGM-CSF by increasing the salt concentration of the buffer from 10 to 1000 mM (Figure 5), thereby confirming the role of charged interactions in complex formation. Finally, the binding interface region of the best scoring complex covers approximately 1590 Å² of buried accessible surface area. This is consistent with previously published antigen–antibody complex structures that typically cover 1400–2300 Å² of surface area [40].

Superposition of modelled hGM-CSF–MB007 complex with the hGM-CSFR ternary complex

Superposition of the isolated hGM-CSF structure (PDB code 2GMF) to hGM-CSF as found in the ternary receptor complex as well as in the modelled MB007 complex shows that the secondary-structural elements adopt remarkably similar conformations (Cα RMSD 0.73 Å, Figure 6C). The N- and C-terminal regions of hGM-CSF exhibit major conformational changes, probably due to their flexibility. In contrast, bound and unbound MB007 show significant conformational changes in CDR3-H upon complex formation (Figure 6B). Superposition of the hGM-CSFR ternary complex (PDB code 3CXE) with the GM-CSF–MB007 simulated complex structure reveals a fairly small sterical overlap with GM-CSFRα (Figure 7). A restricted area in CDR2-H of MB007 consisting of residues 63–67 clashes with a small region...
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Figure 3  Mapping the GM-CSF epitope by backbone amide chemical shift analysis

(A) Superposition of the 2D $^{15}$N TROSY NMR spectra of free (black) $^{2}$H,$^{15}$N-labelled hGM-CSF and of hGM-CSF in complex with the unlabelled MB007 Fab (red) with resonance assignments indicated. Two small regions of the spectra show NMRs undergoing either no chemical shifts (e.g. Ile101 and Phe103), undergoing minor chemical shifts (e.g. Val40 and Gln99), exhibiting line broadening (e.g. Gin19), or disappearing or lacking reassignments in the complex spectrum (e.g. Trp13 and Gin126). (B) Semi-quantitative backbone amide chemical shift analysis. Regions of secondary-structural features are indicated above by grey barrels for helices, grey arrows for sheets and half-rings for loops. (C and D) The chemical shift perturbation (csp) data are mapped on to a surface and cartoon representation of hGM-CSF (PDB code 2GMF). Significantly perturbed residues are coloured on a gradient (yellow to dark red). The mean $^{1}$H,$^{15}$N-weighted chemical shift perturbation was calculated using the formula

$$
\Delta \delta = \frac{1}{N} \sum_{i=1}^{N} \left( \delta_{HN}^i - \delta_{HN}^0 \right)
$$

in which ($\delta_{HN}^i$) corresponds to $^{1}$H and ($\delta_{HN}^0$) to $^{15}$N chemical shift change [42].

comprising residues 304–308 of domain 3 of GM-CSFRα. In that, the sterical overlap accounts for approximately 7% with respect to the overall buried surface area of the hGM-CSF–MB007-binding interface.

**MB007 autoantibody interference in hGM-CSFR signalling**

GM-CSF initiates signalling by first binding to GM-CSFRα, which then associates with homodimers of the affinity-enhancing GM-CSFRβc (GM-CSFR common β-chain). The proliferation of the TF-1 human cell line is dependent on growth factors such as GM-CSF. In its absence, cells will not proliferate. We determined the neutralizing activity of MB007 in a GM-CSF-dependent TF-1 in vivo proliferation assay performing three independent dose-response measurements (Figure 7D). Applying 1 ng/ml GM-CSF, MB007 inhibited proliferation of the cells with an IC$_{50}$ of 14.9 ± 0.8 nM. Compared with the commercially available reference rat anti-hGM-CSF antibody BVD2–23B6 (BD Biosciences) (IC$_{50}$ 19.7 ± 3.4 pM), we observed only modest affinity and only partial neutralization. Thus BVD2–23B6 neutralizes GM-CSF activity with approximately 750-fold higher efficacy (Figure 7D). In vitro binding experiments using MST showed that MB007 binds with high affinity for hGM-CSF (K$_{d}$ 1 ± 0.26 nM). Competition experiments in the absence and presence of soluble extracellular GM-CSFRα showed a 10-fold reduction in MB007 affinity for hGM-CSF (K$_{d}$ 10 ± 0.66 nM) (Figure 7B). Moreover, a 2-fold increase in fluorescence signal due to a reduction in thermophoretic mobility was detected, suggesting that a ternary complex was formed. This finding is supported by SPR (surface plasmon resonance) competition measurements showing that hGM-CSFRα is capable of binding the pre-formed hGM-CSF–MB007 Fab complex, and consequently forming a functional ternary complex (Figure 7C).

Thus, in summary, the cellular assays demonstrated that MB007
Cartoon representation of the simulated GM-CSF–MB007 complex structure. hGM-CSF is shown in grey. The MB007 variable domains are depicted as in other Figures. CDRs are labelled as follows: CDR1 in yellow, CDR2 in brown and CDR3 in red. Helices 1 and 6 of GM-CSF are involved in MB007 binding. Close-up views of the binding interface show details of the interaction sites. The electrostatic potential surface representation of the binding interface was generated with PyMOL (http://www.pymol.org). hGM-CSF shows a predominantly negatively charged epitope, whereas MB007 shows a predominantly positively charged paratope. The model suggests that the main drivers of the strong interaction between MB007 and hGM-CSF are complementary surface electrostatics and shape complementarity.

**Figure 5 Influence of ionic strength on binding of MB007 to GM-CSF**

(A) hGM-CSF was fluorescently labelled with Alexa Fluor® 647, and binding to MB007 Fab was analysed by MST. Binding isotherms show high salt-dependence of complex formation. Results are the mean fluorescence differences calculated from three independent titration series acquired at 298 K by locally applying a temperature gradient of approximately 2 K to each individual measurement. Curves were fitted using a non-linear single-site equation. Binding curves are coloured blue (10 mM NaCl), green (100 mM NaCl), grey (500 mM NaCl), orange (750 mM NaCl) and red (1 M NaCl). a.u., arbitrary units. (B) Dependence of $K_d$ on influence of increasing salt concentration is linear on a semi-logarithmic scale.

DISCUSSION

In the present study, we solved the crystal structure of an anti-GM-CSF autoantibody isolated from a donor suffering from PAP. It is the first crystal structure of a germline VH7 IgG1 human Fab fragment of the \( \lambda \) isotype and the first crystal structure of a disease-associated cytokine-related human IgG1\( \lambda \) autoantibody Fab fragment.

The CDR3-H loop region exhibits remarkable structural features such as a seven-residue-long helical extension protruding from the paratope surface (Figure 1), which is exceptional compared with VH7 germline IgG1 structures reported previously. Its structure suggests that it functions as a tentacle providing a large interaction surface for antigen binding. Mobility of the loop in the crystal structure as well as observed during MD simulations suggest that it is relatively highly flexible and moves as a small rigid protein domain preserving the helical conformation (Figure 2). This loop most obviously forms the major interaction site which mediates antigen binding (Figure 6A). The autoantibody heavy chain sequence differs in 19 positions from the human germline gene. These remarkable
Molecular structure of an anti-GM-CSF autoantibody in complex with its antigen

Figure 6  Influence of conformational changes upon complex formation

Detailed structural analysis showing effects of hGM-CSF and MB007 complex formation. (A) In the simulated hGM-CSF–MB007 complex structure, CDR3-H forms the major interaction site on the Fab. The heavy chain engages a relatively flat binding surface with the protruding CDR3-H most likely acting as a ‘tentacle’. The colour scheme is as in other Figures. (B) Superposition of the MB007 Fab experimental structure (blue) and the Fab in the simulated complex structure (orange and green). CDR3-H and CDR1-H experience large conformational changes with the α-helix of the latter moving as a rigid body. (C) Superposition of the hGM-CSF experimental structure (blue; PDB code 2GMF) and hGM-CSF in the simulated complex structure (grey). Merely insignificant conformational changes occur upon binding (RMSD 0.88 Å) and are mainly located to chain termini.

Figure 7  Therapeutic relevance of MB007 in GM-CSFR signalling

Superposition of the GM-CSFR ternary complex (receptor α chain and domains D1 and D4 as part of the common β-chain, and hGM-CSF) in surface representation with the GM-CSF–MB007 simulated complex in cartoon representation. Top view of the ternary complex corresponds to the view down the membrane surface, whereas the front view illustrates the orthogonal view in relative orientation to the membrane surface. (A) One monomer of the β-chain is shown in dark blue (domain 4 of chain βc) and the other in pale blue (domain 1 of chain βc) (PDB code 3CXE). Receptor domain numbering is indicated as in [41]. Labels denote domain names. There is a relatively small overlap region between the heavy chain of MB007 and GM-CSFRα. The overlap region is highlighted in semi-transparent surface area and indicated with an arrow. MB007 does not prevent, but merely weakens, GM-CSF binding to its receptor and thereby modulates GM-CSF signalling. (B) MTS competition experiment with Alexa Fluor® 647 fluorescently labelled hGM-CSF carried out at 298 K using a locally applied temperature increase of 3 K. The binding curve of hGM-CSF with MB007 forming a binary complex is shown in red, whereas the competition measurement in the presence of GM-CSFRα is shown in grey. Results are normalized mean fluorescence changes calculated from three independent measurements. Curves were fitted using a non-linear single-site equation. (C) Competition binding of MB007 Fab and GM-CSFRα to hGM-CSF using SPR (Biacore T100). GM-CSFRα is capable of binding hGM-CSF after hGM-CSF–MB007 complex formation, thus associating with a ternary complex. (D) Neutralization properties of MB007 against 1 ng/ml hGM-CSF in a cytokine-dependent TF-1 cellular proliferation assay. Results are means ± S.D. of triplicate measurements. Curves are non-linear fits to a double-site equation corresponding to full-length antibody. Binding curves are reference rat anti-human-IgG1 antibody BVD2–23B6 (green) and MB007 (red).

features and its high affinity for GM-CSF make MB007 an attractive autoantibody model system for structural and mechanistic studies.

2D TROSY NMR experiments resulted in a well-resolved and dispersed NMR spectrum (Figure 3), a prerequisite for semi-quantitative residue-specific epitope mapping. Analysis of GM-CSF residues undergoing chemical shift perturbations affected by MB007 Fab complex formation determined a sequentially discontinuous epitope with key contact residues contained within helices 1 and 6, and composed of several other peptide stretches which are spatially proximal and thus characterize a conformational GM-CSF epitope (Figures 3C and 3D). This
finding explains the failure of our earlier attempts to identify short linear peptides of hGM-CSF with affinity for MB007 (results not shown).

If all residues experiencing chemical shift perturbations are considered as being involved in binding, a relatively broad epitope on hGM-CSF is mapped. However, conformational changes associated with the binding event indirectly influence residues on the periphery of a binding site. Hence the area described by chemical shift perturbation analysis is generally larger than the region directly in contact. Using merely the more drastic chemical shift perturbations for epitope mapping, the defined prominent binding patch described above is revealed. This example shows that NMR spectroscopy can be used efficiently to classify therapeutically relevant antibodies by clustering the chemical shift patterns employing 15N-labelled antigen.

The concerted use of NMR experimental information on the hGM-CSF epitope utilized by MB007 and computational ab initio protein–protein docking procedures resulted in a reliable model structure of the MB007–hGM-CSF complex. The model exhibits the hallmark features of antibody–antigen complexes such as shape and charge complementarity (Figure 4). The experimentally observed dependence of binding affinity on ionic strength (Figure 5) is consistent with the model. The size of the interaction surface (1590 Å²) is reasonable and within the limits found for other antigen–Fab complexes. Assuming a fairly rigid hGM-CSF structure, the uncertainties in the derived model reside in the prediction of the exact conformation of the MB007 hypervariable region, most importantly CDR3-H. In conclusion, we need to regard the model as a rather low-resolution view of the actual structure which needs to be determined experimentally.

In a cellular assay, MB007 modulates hGM-CSF signalling by partially inhibiting cell proliferation at relatively high concentrations. This finding is consistent with biophysical data showing a 10-fold reduction in affinity of MB007 for hGM-CSF in the presence of hGM-CSFRα, but no full blockade of receptor binding (Figure 7).

Therefore autoantibodies such as MB007 could simply act in synergy with other distinct high-affinity autoantibodies in the same patient by an avidity effect, creating an inactive higher-order complex. However, the bioactivity data and our structural model of the MB007–hGM-CSF complex suggest a further novel potential mode of action for MB007 in PAP. MB007 binding to hGM-CSF blocks, to a fairly small extent, the binding interface of hGM-CSFR in proximity to the elbow region of the GM-CSFRα chain. A restricted area in CDR2-H of MB007 consisting of residues 63–67 clashes with a small region comprising residues 304–308 of GM-CSFRα chain domain 3. We suggest that the functional ternary complex hGM-CSF–GM-CSFRα–GM-CSFRβ is not disrupted by just blocking one part of the receptor assembly. Noticeably, GM-CSF signalling is strongly associated with the GM-CSFRβ chain [39], which remains unaffected by MB007 binding. More specifically, GM-CSF initiates signalling by first binding to the specific GM-CSFRα chain (Kᵦ 0.2–100 nM) following association with the affinity-enhancing GM-CSFRβ chain (Kᵦ 100 pM) [41]. Moreover, PAP is a polyclonal response to GM-CSF, and therefore MB007 is only one of many anti-GM-CSF antibodies present in the plasma of these patients. Simultaneous binding of another antibody recognizing a separate blocking epitope to the same GM-CSF molecule would prevent the association of MB007 with GM-CSFRs. The pharmacokinetics and biodistribution of the MB007–hGM-CSF complex and the other anti-GM-CSF antibodies will determine their relative abundance. Tissue penetration could limit this mechanism to only certain tissues.

These findings may be relevant for therapeutic antibody drug design. Instead of directly addressing receptor-binding sites of cytokines, it may be advantageous to target epitopes that are proximal to the receptor-binding site with a merely small steric overlap. This can result in partial neutralization of the target cytokine and might be an alternative therapeutic strategy that avoids complete abolishment of antigen-binding activity.

**AUTHOR CONTRIBUTION**

Michaela Blech wrote the manuscript, performed NMR epitope mapping and analysis, performed biophysical characterization of the individual constituents, performed and analysed crystallography, protein expression and purification, and contributed to ideas related to numerous aspects of the project and to the discussion and interpretation of experimental results. Daniel Seelig performed MD simulations and contributed to the manuscript and to discussion about protein docking. Barbara Kistler performed cellular assays, contributed to the manuscript and performed the initial experiments to characterize the antibody. Margit M.T. Bauer contributed to the manuscript and contributed ideas related to numerous aspects of the project and to discussion related to the project. Mathias Halfer contributed to the manuscript and to discussion of cellular aspects. Stefan Hoerer contributed to the manuscript and to crystallographic interpretation. Markus Zieb contributed to the manuscript, performed NMR experimental setup and data acquisition and contributed to the interpretation of experimental results. Herbert Nar contributed to and reviewed the manuscript and contributed to the discussion and interpretation of experimental results. John E. Park contributed to the manuscript and to its discussion, and performed the initial experiments to characterize the antibody.

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SUPPLEMENTARY ONLINE DATA

Molecular structure of human GM-CSF in complex with a disease-associated anti-human GM-CSF autoantibody and its potential biological implications

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EXPERIMENTAL

hGM-CSF construct design, expression in the periplasm and release of properly folded soluble protein

A custom signal sequence for transport into the periplasm and a C-terminal His6 affinity purification tag with a thrombin cleavage site were included before the starting amino acid of hGM-CSF. DNA constructs were subsequently transformed into BL21 competent cells. A single colony was inoculated into 50 ml of LB (Luria–Bertani) medium containing 30 μg/ml kanamycin and grown at 37°C overnight. This culture was used to inoculate pre-warmed LB medium (1:20) and cells were grown at 37°C until a D600 of 0.8–1.0 was reached. After induction with a final concentration of 1.25 mM IPTG (isopropyl β-D-thiogalactopyranoside), cells were grown further at 19°C for 24 h. In the first step, cells were harvested in HarvestLine System liners (Beckman Coulter) at 3350 rev./min for 90 min at 4°C overnight. This culture was used to inoculate an ice-cold hypotonic solution containing 0.05 M Tris/HCl (pH 7.5), EDTA-free protease inhibitor and DNase relative to the wet weight of the pellet. After centrifugation at 4°C for at least 30 min under constant shaking, the outer membrane of a fraction of the harvested cells was disrupted, leading to a partial release of soluble native folded protein. After centrifugation at 3350 rev./min for 60 min using a JA-10 rotor (Beckman Coulter), the supernatant was removed for further purification. In the second step, cells underwent an osmotic shock procedure by incubation of the cell pellet with twice the volume of 20% (w/v) sucrose, 0.05 M Tris/HCl (pH 7.5), EDTA-free protease inhibitor and DNase for 30 min at room temperature under constant shaking to completely open the outer membranes of previously unaffected cells. Again, the supernatant containing soluble protein was removed for further purification. Release of bulk target protein occurred in the last step where the hypotonic cells were treated with an ice-cold hypotonic solution containing 0.05 M Tris/HCl (pH 7.5) at 4°C upon constant shaking. The periplasmic extract was clarified by a final centrifugation step at 10000 rev./min for 60 min at 4°C using a JA-10 rotor.

Three-step purification of recombinant hGM-CSF

All supernatants containing soluble protein were pooled and NaCl and imidazole were subsequently added to a final concentration of 350 mM and 20 mM respectively, before applying to Protino® Ni-NTA (Ni2+-nitrilotriacetate)–agarose beads (Macherey-Nagel) for metal-affinity purification using the constructs’ C-terminal His6 tag. Non-specifically bound proteins were eluted by washing the beads with 50 mM imidazole. Recombinant hGM-CSF was eluted by an imidazole gradient of 100–750 mM. Fractions containing a protein concentration greater than 0.3 mg/ml target protein (NanoDrop) were pooled and dialysed against 50 mM sodium phosphate buffer (pH 7.5) and 20 mM NaCl at 4°C overnight. The protein solution was loaded on to a HiTrap™ Q HP anion-exchange column (GE Healthcare) attached to an AKTA Explorer™ system (GE Healthcare), pre-equilibrated with dialysis buffer. The target protein was eluted with a linear gradient of 0.02–1 M NaCl in 50 mM sodium phosphate buffer (pH 7.5). A major peak eluted at approximately 17–20% elution buffer, resulting in a final concentration of 166–196 mM NaCl. The sample was concentrated with an Ultrafree-15 centrifugal concentrator (molecular-mass cut-off 10 000 Da) (Millipore) before passing through a Superdex 75 10/300 column (GE Healthcare) for a final polishing step upon removal of minor remaining impurities. Sample purity and identity were assessed with SDS/PAGE, MS (obtained mass, 15 512 Da; theoretical mass, 15 514 Da) and static light scattering (obtained mass, 15 210 Da).

MD simulations

MD simulations were carried out using Gromacs version 4.5 [1,2] and the amber99sb force field [3,4]. For all simulations of the antibody Fv and the complex models, the solute was put into a simulation box and solvated with TIP3P water [5]. NaCl was added to achieve a 150 mM concentration. Electrostatic interactions were calculated at every step with the particle mesh Ewald method [6]. Short-range repulsive and attractive dispersion interactions were described together by a Lennard–Jones potential, which was cut off at 1.0 nm. The Settle algorithm [7] was used to constrain bond lengths and angles of water molecules, and LINCS [1] was used to constrain all other bond lengths. In addition, the fastest angular degrees of freedom involving hydrogen atoms were removed by using virtual interaction sites [VSITE], allowing for a time step of 4 fs. The temperature was kept at 300 K through velocity rescaling [8] (τ = 2.5 ps) and the pressure was controlled at 100 kPa using the Parrinello–Rahman coupling scheme (τ = 5 ps) [9].

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Atomic co-ordinates and structure factors of the refined model of MB007 Fab have been deposited in the PDB under accession code 4EOW.
**Figure S1 MD simulation of CDR flexibility**

MD simulations were used to investigate loop dynamics in MB007 variable domains. RMSDs were calculated for the variable domains excluding CDR3-H (black, top panel), for CDR3-H alone (blue, middle panel) and for CDR3-H after fitting to the variable domains excluding CDR3-H (red, bottom panel). The Fv fragment excluding CDR3-H shows constant RMSD values of approximately 2 Å from the X-ray structure, indicating normal thermal motion. The intrinsic flexibility of CDR3-H is also very low with RMSD values of approximately 1.5 Å (blue). However, the motion of CDR3-H relative to the rest of the variable domains (red) is very distinct.

**Figure S3 Shape of the electrostatic isosurface potential surfaces derived from non-linear adaptive Poisson–Boltzmann equation**

Isopotential contour surfaces depicting the spatial distributions of electrostatic potentials of the GM-CSF-binding interface (top) and MB007 Fab (bottom) respectively. Blue and red surface areas represent positive and negative electrostatic potentials respectively at ±2 k_B T/e units (k_B, Boltzmann constant; T, temperature; e, electron charge). Protein surfaces yield equally electrostatic potential values indicated by equal surface elevation. Electrostatic potential calculations were performed using the APBS implementation within PyMOL (http://www.pymol.org).

**Figure S2 Favourable GM-CSF positioning after antigen–antibody docking using MD simulation**

Representation of superposition of the ten best scoring docking complex models with favourable docking scoring. MB007 light chain is depicted in green, and the heavy chain is in orange. GM-CSF is indicated in grey, except for one model featuring a different orientation compared with the generality shown in red. Structural analysis revealed that GM-CSF is generally oriented such that helix 6 faces a groove between the heavy and light chain surface of MB007.
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