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Characterization of SM201, an anti-hFcγRIIB antibody not interfering with ligand binding that mediates immune complex dependent inhibition of B cells

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ABSTRACT

The monoclonal antibody SM201 specifically recognizes the human inhibitory FcγRIIB without showing cross-reactivity to the related but activating FcγRIIA. The epitope recognized by SM201 is located outside the IgG-binding site of FcγRIIB. As a result, the antibody does not interfere with hIgG binding to the receptor. It was therefore hypothesized that SM201 may amplify the inhibitory signaling of FcγRIIB after coligation of B cell receptor (BCR) and FcγRIIB by immune complexes (ICs).

Mechanistic and functional studies were conducted in established B cell lines as well as in primary B cells from healthy donors to substantiate the anticipated working principle. Using an experimental setup mimicking IC binding, we were able to demonstrate that SM201 enhances the extent of ITIM phosphorylation of FcγRIIB. The antibody works synergistically with ICs and the mode of action is strictly dependent on their presence. Additionally, SM201 did not induce apoptosis, cellular depletion or NK cell activation, which indicates an advantageous safety profile. This establishes an innovative approach for the treatment of antibody-mediated autoimmune diseases.

1. Introduction

Human FcγRs are type I transmembrane proteins that are expressed on almost all immunologically active cells except T cells and recognize the Fc region of IgG [1]. Binding of immune complexed IgG by FcγRs triggers a broad range of effects regulating important downstream events of the immune response. Most FcγRs are activating receptors and contain a cytoplasmic ITAM (Immunoreceptor Tyrosine-based Activating Motif) present either within the receptors or in a receptor-associated chain (e.g. CD79a).

Crosslinking of activating FcγRs by antigen–antibody complexes results in ITAM-phosphorylation and mediates e.g. phagocytosis, secretion of inflammatory mediators or antibody dependent cellular cytotoxicity (ADCC) [2,3].

In contrast, only one inhibitory FcγR, FcγRIIB, exists that transmits inhibitory signals through an ITIM (Immunoreceptor Tyrosine-based Inhibitory Motif) within its cytoplasmic domain and thus down-modulates immune responses upon recruitment to an activating receptor. This receptor is widely expressed on most leukocyte populations that are involved in e.g. antigen presentation, antibody production and effector functions. On B cells, FcγRIIB is the sole IgG receptor, negatively regulating B cell receptor (BCR) induced activities [4,5]. During the late phases of an immune response, ICs composed of IgG and antigen bind simultaneously to B cell receptors (BCRs) and FcγRIIB. Co-aggregation of the BCR with FcγRIIB inhibits BCR signaling, in effect blocking downstream biological responses of the B cell including activation, antigen presentation, proliferation and antibody production. The initial event in inhibitory signaling is the phosphorylation of the ITIM tyrosine by the Src-family kinase Lyn [6]. This phosphorylation results in the recruitment of SH2-domain-containing phosphatases like SHIP and further downstream signaling.

In contrast to the effects transduced after coligation with the BCR, crosslinking of FcγRIIB alone has been shown to be sufficient to induce apoptosis in B cells independent of BCR co-ligation. Interestingly, this response requires only an intact FcγRIIB transmembrane domain and was shown to be independent of ITIM phosphorylation [7].

FcγRIIB is an extremely powerful regulator of the antibody-mediated immune response and additionally plays a crucial role in suppressing autoimmunity by regulating both B cell responses and effector cell functions [6]. Therefore the modulation of FcγRIIB

Abbreviations: BCR, B cell receptor; chSM201, chimeric SM201; FcγRIIB, inhibitory IgG receptor; ICs, immune complexes; ITAM, immunoreceptor tyrosine-based activating motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; h, human; rSM201, rat SM201.

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inhibitory activity constitutes an attractive approach for the development of an innovative immunotherapeutic drug. One major obstacle for the generation of a therapeutic antibody targeting this inhibitory receptor was the difficulty to generate an antibody specific for FcγRIIB, since its high sequence identity to FcγRIIA within the extracellular region exceeds 93% [8]. In this report we describe an antibody specific for FcγRIIB with the unique feature to bind to an epitope that does not interfere with IgG binding to the receptor. We demonstrate that this antibody, termed SM201, works synergistically with immune complexes and may represent a superior option for the treatment of antibody-mediated autoimmune diseases than the currently available therapeutics.

2. Materials and methods

2.1. Antibodies

The mAb mouse anti-hIgM was purchased from Southern Biotech, Birmingham, USA. Rabbit anti-mIgG(1,2a,3) and anti-hFcγRIIB/CD32 Phospho (pY292) were obtained from Epitomics (Burlingame, USA). Anti-phosphoSHIP1 (Tyr1020) was purchased from Cell Signaling Technology (Danvers, USA). Secondary anti-rabbitIgG-HRP for Western Blot analysis, PE conjugated $F(ab')_2$ goat anti-mouseIgG, goat anti-ratIgG-FITC and donkey anti-goatIgG (H&L)-HRP were purchased from Jackson ImmunoResearch (Suffolk, UK). The pan FcγRII antibody for FACS analysis and goat anti-humanFcγRII/CD32 a/b/c antibody for Western Blot analysis were obtained from R&D Systems (Minneapolis, USA). FACS antibodies anti-hCD19-APC, anti-hCD3-PerCP, anti-hCD45-FITC, anti-hCD56-APC and anti-hCD69-PE were purchased from BD Biosciences (San Jose, USA). The following control antibodies were used: Rituximab and the blocking control antibody 2B6 [9].

2.2. Generation and expression of SM201

Rat SM201 (rSM201) was generated by immunization of LOU/C rats with the soluble extracellular domain of recombinant human FcγRIIB. A hybridoma cell line was generated by fusion of the myeloma cell line P3X63-Ag8.653 with the rat spleen cells according to standard procedures.

Chimeric SM201 (chSM201) was engineered by fusing the variable domains of the parental rat antibody to a human IgG1-Fc domain. The sequences were cloned into the mammalian expression vector pCINeo (Promega, Madison, USA). To prevent glycosylation of the Fc part and consequently the antibody's effector function mediated by the Fc part, a substitution of the asparagine residue 297 to alanine (N297A) was introduced [10]. Binding characteristics of the antibody were screened by ELISA and FACS analysis. Antibody expression was performed by transient gene expression using FreeStyle™ CHO-S cells and CHO Freestyle™ Expression Medium supplemented with GlutaMax (Gibco, Life Technologies) at 31 °C and 5% CO_2 under continuous shaking (100 rpm; HT Multitron, Infors, Bottmingen, Switzerland) for up to 10 days. chSM201 was purified using prepacked ready-to-use ProteinA columns (HiTrap rProtein A FF 1 ml column, GE Healthcare, Freiburg, Germany) and suitable buffer systems (running buffer: 10 mM Tris–HCl pH 8.0, 150 mM NaCl; elution buffer: 100 mM glycine pH 3.0, 150 mM NaCl; neutralization buffer: 2 M Tris–HCl pH 8.0). The final storage buffer of the antibody contained 150 mM NaCl and 5 mM histidine-HCl, pH 6.5. Expression and purification of larger amounts of antibody was performed in collaboration with the service provider ExcellGene (Monthey, Switzerland).

$F(ab')_2$ fragments of rSM201 and chSM201 were generated using Pierce $F(ab')_2$ preparation Kit (Pierce, Thermo Scientific, Rockford, USA) based on papain digestion according to the manufacturer's protocol.

2.3. Cell culture

Daudi cells (ACC78), Raji cells (ACC319), both human Burkitt lymphoma cell lines, and K-562 cells (ACC10), a human myeloid leukemia cell line, were purchased from DSMZ (Heidelberg, Germany) and maintained in RPMI 1640 supplemented with 10% FBS, 1 × MEM NEAA, 1 mM sodium pyruvate and 2 mM L-Glutamine (all from Gibco®, LifeTechnologies, Darmstadt) at 37 °C and 5% CO_2.

CHO-S cells were cultivated in CHO FreeStyle™ Expression Medium supplemented with GlutaMax (Gibco, Life technologies) at 37 °C and 5% CO_2 under continuous shaking (100 rpm, HT Multitron, Infors, Bottmingen, Switzerland) in flasks.

PBMCs were isolated from heparinized whole blood from healthy donors using Ficoll density gradients (Biocoll Separating Solution, Biochrom AG, Berlin) and B cells were further purified by negative magnetic isolation using Dynabeads untouched human B cells kit (Life Technologies, Darmstadt) according to the manufacturer's protocol.

2.4. Flow cytometric analyses of SM201 binding

2.4.1. Target specificity of SM201 binding

Analysis of specific binding of SM201 to its native antigen FcγRIIB was conducted via cell binding on Raji and K-562 cells. Cells were pelletized by centrifugation (400 × *g*, 5 min) and washed in FACS buffer (Hanks balanced salt solution, 1% FCS, 0.01% NaN_3). After an additional centrifugation step, cells were resuspended in FACS buffer to obtain a final concentration of 2×10^6 cells/ml and 50 μl of the cell suspension was transferred to a 96-well U-bottom plate. Serial dilutions of SM201 were prepared in FACS buffer.

Raji and K-562 cells were incubated with increasing concentrations of the antibody for 30 min. After a washing step, cell-bound antibodies were detected with goat anti-ratIgG-FITC and surface signals were measured using a BD FACSCanto™ II.

2.4.2. FACS-based competition assay

To determine whether the antibody SM201 still allows binding of IgG or immune complexes to membrane-bound FcγRIIB, a FACS based assay was conducted. Cells were harvested and washed as described above preparing a suspension of 2×10^6 cells/ml. Serial dilutions of $F(ab')_2$ fragments of rSM201 and a control pan anti-FcγRII antibody were prepared in FACS-buffer. 25 μl of diluted antibodies were mixed in a 96-well U-bottom plate with 25 μl aggregated human IgG (hIgG), isolated by size-exclusion chromatography from the commercially available pooled IgG product (Beriglobin, CSL Behring, Marburg). 50 μl of the cell suspension was added to the antibody-Beriglobin mixture and incubated for 1 h at 4 °C. After a washing step, hIgG was detected with PE-conjugated goat anti-humanIgG. After washing twice, signals were measured with BD FACSCanto™ II.

2.5. Co-immunoprecipitation and Western Blot analysis

1×10^7 Daudi cells per sample were treated with 2 μg/ml mouse anti-hIgM and 20 μg/ml rabbit anti-mouseIgG to crosslink BCR and FcγRIIB in a total volume of 100 μl assay medium (RPMI + 1% FBS). After 25 min, 5 μg/ml chSM201 or the blocking anti-hFcγRIIB antibody 2B6 were added and cells were further incubated for 30 min. Cells were harvested at 4 °C and lysed in 500 μl lysing buffer (1% TritonX-100, 150 mM NaCl, 50 mM Tris/HCl pH 7.4, Complete Mini Protease Inhibitors), incubating the cells for 30 min on ice and vortexing every 10 min. Cell debris was spun down at 13,000 rpm for 10 min at 4 °C and the supernatants were transferred to new tubes.