Selective depletion of plasma cells in vivo based on the specificity of their secreted antibodies

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Antibody-mediated diseases affect more than 10% of the human population. For most, no cure is available, particularly when the pathogenic antibodies are secreted by long-lived plasma cells resistant to conventional immunosuppressive therapies. Current therapeutic approaches target not only the plasma cells that secrete pathogenic antibodies, but also those providing protective antibodies. Here, in a murine model bearing long-lived plasma cells secreting anti-OVA and -chicken gamma globulin (CGG) antibodies, we describe the first-time use of an antigen-antibody (OVA/anti-CD138 antibody) conjugate for in vivo labeling and selective ablation of plasma cells that secrete antibodies specific for the antigen OVA. The selective depletion also led to a stable reduction of the corresponding serum anti-OVA antibody levels. In contrast, CGG-specific plasma cells and circulating anti-CGG antibody levels remained unchanged. The method described here should enable the development of unique causative treatment strategies for established antibody-mediated diseases sparing humoral immunity.

Keywords: antibody-mediated disease · anti-ovalbumin antibody · ovalbumin-anti-CD138 antibody conjugate · proof-of-concept study · therapeutic plasma cell targeting

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Secreted antibodies, an essential component of the immune system, provide efficient protection against recurrent infections when specific for the infectious pathogen. If immunological tolerance fails, secreted antibodies specific for autoantigens, alloantigens, or allergens can cause chronic diseases such as autoimmune disorders, transplanted organ rejection, or allergies. This group, consisting of several hundred different clinical disease entities, affects more than 10% of the human population and is gaining increasing recognition [1, 2]. Antibodies are secreted by plasma cells, which develop from activated B lymphocytes. Most plasma cells are short-lived, with a lifespan of just a few days. Long-lived plasma cells,
however, can maintain humoral immunity by the continued secretion of antibodies and survive for several years to even decades [3–7]. The pathogenic antibodies involved in antibody-mediated diseases may be secreted by either short- or long-lived plasma cells, or both [8–10]. The survival strategy of long-lived plasma cells makes them resistant to conventional immunosuppressive drugs and therapies targeting B cells. This explains the chronicity and refractoriness of antibody-mediated diseases to remission induction [8, 11–13]. Thus, long-lived plasma cells secreting pathogenic antibodies still represent an unresolved and highly relevant therapeutic challenge.

Autologous stem cell transplantation for systemic lupus erythematosus resistant to standard immunosuppression is an experimental approach shown to induce the generic ablation of established plasma cells and the autoantibodies they secrete in patients achieving long-term therapy-free remission [14]. Likewise, experimental treatment with the proteasome inhibitor bortezomib diminished plasma cells secreting pathogenic antibodies in refractory diseases such as systemic lupus erythematosus, anti-NMDA-receptor encephalitis and kidney transplant rejection, resulting in significant clinical improvement or even the breakage of refractoriness [15–19]. While these encouraging results suggest the relevance of long-lived plasma cells secreting pathogenic antibodies as therapeutic targets, they also highlight a major inherent limitation of therapeutic strategies for efficient generic ablation of long-lived plasma cells: a loss of humoral immunity accompanied by an increase in recurrent infections. This is also to be expected for monoclonal antibodies that target CD38, which are highly expressed on plasma cells [20, 21]. Moreover, we learned that the effective depletion of long-lived plasma cells needs a targeting of their precursor B cells to prevent regeneration [19, 22–24].

Here, we describe an original approach to the in vivo ablation of plasma cells according to the specificity of their secreted antibodies. The basic principle is that after plasma cells are labeled in vivo with the antigen-of-interest, those secreting specific antibodies are targeted by their own antibodies via antibody-mediated ablative effector mechanisms, such as complement lysis and antibody-mediated cellular cytotoxicity (Fig. 1). In the present proof-of-principle study, we labeled plasma cells with a conjugate of an antibody recognizing plasma cells (anti-CD138) with the antigen, OVA. This type of “affinity matrix” was first utilized for the isolation of living cells according to their secreted molecules [25]. Its ability to achieve the effective and selective depletion of OVA-specific plasma cells from primary murine spleen cell cultures ex vivo was demonstrated by us previously [26].

Results

In vivo labeling of the OVA/anti-CD138-conjugate (OVA-C) on plasma cells after its administration

In vivo labeling of plasma cells with OVA was accomplished by injecting a conjugate of OVA with a recombinant anti-CD138 antibody (OVA-C) intraperitoneally. The heavy chain constant region of the recombinant anti-CD138 antibody is a human IgG1

Figure 1. Principle of antigen-specific depletion of plasma cells. All plasma cells are labeled with an antigen of interest, for example, ovalbumin conjugated to an antibody that recognizes the plasma cells (e.g. anti-CD138). OVA, ovalbumin, CDC, complement-dependent cytotoxicity, ADCC, antibody-dependent cellular cytotoxicity

Fc, which is non-functional in mice with respect to attracting ablative effector mechanisms and allowed us to perform flow-cytometric assessment of the conjugate. The use of antibodies specific for human IgG1 made the OVA-C readily detectable on the surface of bone marrow plasma cells collected from naïve BALB/c mice 3 h after injection of 50–500 μg doses of OVA-C. A significant, dose-dependent shift in the mean fluorescent intensity (MFI) of anti-human IgG1 antibody staining was observed at all dose levels. Five hundred micrograms of OVA-C resulted in CD138 saturation on the surface of the plasma cells (Fig. 2A). As expected, CD19+ B cells and CD3+ T cells were not labeled with the conjugate (Fig. 2B).

However, OVA immunized BALB/c mice did not result in the same labeling due to the capture of OVA-C by circulating OVA-specific antibodies (Fig 2C). The labeling of plasma cells was restored by neutralizing OVA-specific antibodies by injecting OVA 40 h before OVA-C administration (Fig. 2D). Hundred micrograms of OVA was sufficient to neutralize more than 80% of the circulating anti-OVA antibodies after intraperitoneal administration (Supporting Information Fig. 1). Overall, labeling of plasma cells with conjugate was strongest 3 h after injection, and that of splenic, bone marrow, and lymph node plasma cells was still substantial 24 h post-injection, but not 72 h after injection (Fig. 2D).

Single administration of OVA-C results in a selective depletion of OVA-specific plasma cells

Sixteen weeks after secondary immunization with OVA and chicken gamma globulin (CGG), we studied the effect of labeling
plasma cells with the OVA-C in vivo on the persistence of OVA- and CGG-specific plasma cells in BALB/c mice. At this time point, the majority of these plasma cells is expected to reside in the bone marrow as long-lived plasma cells, which can be confirmed by the lack of BrdU (bromodeoxyuridine) incorporation [3, 5, 27]. After 12 days of continuous BrdU feeding until the end of the observation period (Fig. 3A), ~95% of the OVA- and CGG-specific bone marrow plasma cells showed the phenotype of a non-proliferating long-lived plasma cell (Fig. 3B). Forty-eight hours after a single intraperitoneal injection of 500 μg OVA-C, there was a significant reduction by about 63% in the number of OVA-specific bone marrow plasma cells in mice treated with the conjugate compared to controls (Fig. 3C and D, Table 1). The depletion concerned both BrdU-positive proliferating and BrdU-negative long-lived OVA-specific plasma cells although the reduction of BrdU-positive OVA-specific plasma cells did not reach a statistical significance due to...
Figure 3. Effect of a single injection of the OVA-C on the depletion of bone marrow plasma cells (PCs). (A) Treatment and analysis scheme. Sixteen weeks after secondary immunization with OVA and CGG, the Balb/C mice were treated with anti-mouse CD20, and BrdU, an analogue of the nucleoside thymidine used to identify proliferating cells, was fed via drinking water until the time point of analysis. All mice received OVA and cyclophosphamide (CYC), only the ‘treated’ group received additionally OVA-C (500 μg per mouse, i.p.). (B) Representative flow-cytometric staining of OVA- and CGG-specific PCs in untreated mice (control) versus mice treated with OVA-C (treated). (C) Summary graphs of anti-OVA and anti-CGG PCs frequencies related to total PCs were shown in scatter dot plots (n = 10 mice per group). Horizontal bars represent the mean. The Mann–Whitney U-test (two-tailed) was used for statistical analysis. n.s.: not significant. (D) Scatter dot plots represent absolute numbers of bone marrow PCs (OVA- and CGG-specific PCs, total PCs) in treated mice compared to control mice analyzed by flow cytometry; n = 10 mice per group. Horizontal bars represent the mean. The Mann–Whitney U-test (two-tailed) was used for statistical analysis (n.s., not significant). One representative experiment of four independent experiments is shown.

Table 1. Effect of a single injection of the OVA/anti-CD138-conjugate (OVA-C) on the depletion of bone marrow plasma cells (PCs)

| Plasma cell populations | Total population (×10^3) | BrdU- population (×10^3) | BrdU+ population (×10^3) |
|-------------------------|--------------------------|--------------------------|--------------------------|
|                         | Control | Treated | Depletion efficiency | Control | Treated | Depletion efficiency | Control | Treated | Depletion efficiency |
| OVA+ PCs                | 16.1 ± 8.0 | 5.96 ± 3.3 | 62.8% | 14.4 ± 7.1 | 5.53 ± 3.1 | 61.7% | 1.63 ± 1.4 | 0.55 ± 0.4 | 66.5% |
| CGG+ PCs                | 4.14 ± 1.4 | 3.44 ± 1.45 | 16.9% | 3.41 ± 1.1 | 2.85 ± 1.3 | 16.4% | 0.73 ± 0.5 | 0.59 ± 0.4 | 19.2% |
| total PCs               | 309.4 ± 94.4 | 296.0 ± 68.7 | 4.3% | 293.7 ± 95.6 | 282.4 ± 64.4 | 3.8% | 15.7 ± 2.7 | 13.6 ± 5.1 | 13.5% |

The numbers of bone marrow plasma cells (PCs) derived from Balb/C mice, harboring plasma cells secreting anti-OVA and anti-CGG antibodies which were treated with OVA-C (treated group) compared to a control group without OVA-C administration. Numbers were calculated by flow cytometry (n = 10 per group) and values represent mean with SD. OVA + PCs: OVA-specific PCs, CGG + PCs: CGG-specific PCs. The Mann–Whitney U-test (two-tailed) was used for statistical analysis (**p < 0.001).
To prevent the regeneration of OVA-specific plasma cells from their precursors induced by the OVA administration, all mice, those receiving conjugate, as well as controls, were additionally treated with a monoclonal anti-CD20 antibody and cyclophosphamide (see treatment scheme Fig. 3A), as described previously [23, 24]. After this combination treatment the plasma cell precursor B cells were depleted by about 97.5% in spleen indicating an effective prevention of plasma cell regeneration (Supporting Information Figs. 2B and 4).

The depletion of OVA-specific plasma cells leads to a sustained reduction of anti-OVA antibodies

In a next step we investigated whether the depletion of OVA-specific plasma cells leads to a selective reduction of serum anti-OVA antibody levels. After the intraperitoneal injection of OVA-C, BALB/c mice were followed-up for additional 48 days (Fig. 4A). As expected, the selective ablation of OVA-specific plasma cells resulted in a significant and sustained decrease in OVA-specific serum antibody levels, which dropped to about 40% in comparison to the control group during the observation time period while CGG-specific antibodies and total IgG levels were not affected at all (Fig. 4B). The long-term reduction of anti-OVA antibody levels is closely correlated to the degree of depletion of OVA-specific plasma cells. The drop in anti-OVA antibodies in both groups on day 2, compared to earlier and later time points, is due to the neutralizing effect of the OVA injected at -40 h. The drop seems to be even stronger in mice treated with OVA-C since parts of it may also contribute to anti-OVA neutralization (Fig. 4B). Anti-CD20 treatment alone at day -10 did not impact the anti-OVA serum titers in mice with established OVA-specific memory plasma cells, as shown in Supporting Information Fig. 5.

Discussion

To the best of our knowledge, this is the first proof-of-principle of a selective in vivo depletion of plasma cells in an antigen specific manner resulting in a drop of the related serum antibody levels. It should be emphasized that the depletion includes long-lived bone marrow plasma cells, which are resistant to conventional immunosuppression and therapies targeting B cells as plasma cell precursors. Until now, these long-lived plasma cells can only be targeted by aggressive treatments such as proteasome inhibitors or immunoablative protocols followed by autologous stem cell transplantation, which can result in humoral immunodeficiency [11, 13, 14, 16]. In contrast, the method described here is able to spare the humoral memory due to the antigen-specificity of plasma cell depletion. It is assumed that this principle of antigen-specific plasma cell depletion works with all available antigens, including autoantigens. Preliminary data collected in a smaller group of BALB/c mice 16 weeks after secondary immunization with OVA and CGG show that a single injection of a CGG/anti-CD138 conjugate (CGG-C) also results in a significant depletion of anti-CGG secreting plasma cells in the bone marrow, while
the number of OVA-specific and total plasma cells remains nearly unchanged (Supporting Information Fig. 6A and B). Interestingly, the degree of antigen-specific plasma cell depletion was similar between the treatment with OVA-C and CGG-C.

At first, the selective ablation of antigen-specific plasma cells by in vivo labeling of all plasma cells with the antigen of interest may seem surprising. Why did antibodies of the OVA- or CGG-specific plasma cells not bind to and kill plasma cells of other specificities? The likeliest reason for this apparent weak cross-feeding is that, in established memory phases of immune responses, plasma cells are dispersed throughout the bone marrow and survive individually in niches organized by stromal cells [26, 28]. We assume that this spatial organization leads to high local concentrations of anti-OVA antibodies near the plasma cells that secreted them. In this context, it is worth mentioning that OVA-specific plasma cell depletion can also be achieved by labeling living plasma cells with a carefully titrated OVA conjugate under in vitro conditions in which the cells are not separated by distinct niches [26].

The question arises why the successful labeling of all plasma cells does not result in the depletion of 100% of the OVA- or CGG-specific plasma cells but was “only” 60–70%. It is known that the CD138 molecule on the surface of plasma cells is characterized by a high internalization rate [29, 30], which is reflected by the short labeling of OVA-C in our kinetics. Another aspect could be the different antibody secretion capacity of plasma cells [31]. Low amounts of secreted antibodies might not result in a sufficient binding to OVA-C on the surface of these plasma cells. Consequently, these cells do not engage in ‘suicide’.

Not surprisingly, the in vivo labeling of plasma cells with a conjugate of anti-CD138 antibody coupled to an antigen in individuals with serum antibodies against that antigen requires: (1) the neutralization or removal of these antibodies, and (2) efficient measures to prevent plasma cell regeneration from their precursors when activated by the antigen of the conjugate. Here, we demonstrated the effectiveness of neutralizing serum antibodies by applying antigens in a first step, in addition to using anti-CD20 and cyclophosphamide to efficiently prevent the regeneration of plasma cells in a murine model. There are also other options to reduce the serum antibody levels, such as inhibitors of the neonatal Fc receptor (FcRn) which are able to enhance IgG degradation [32], or mTOR blockade by rapamycin [33]. In humans, however, it may be preferable to remove circulating antibodies by extracorporeal apheresis and to use alternative immunosuppression strategies (including B-cell targeting approaches) for the prevention of plasma cell regeneration. Recently, strategies of an antigen-specific B-cell targeting in autoimmune models have been described [34, 35]. The combination of an (auto)antigen-specific B-cell depletion strategy, which is not able to affect the related long-lived plasma cells, with an antigen-specific plasma cell depletion technology described here could be an exciting prospect for treating autoimmune diseases in the future that keeps the humoral immunity alive.

In conclusion, this study describes for the first time a method for the antigen-specific depletion of plasma cells in vivo in which a conjugate is used to label plasma cells with the antigen of interest, resulting in the elimination of the plasma cells by their own secreted antibodies. This technology offers unique and entirely new options for assessing the pathogenic role of a specific antibody in a given disease, and for the development of causative therapies for antibody-mediated diseases.

Materials and methods

Mice and immunization

Female BALB/c mice were obtained from Charles River Laboratories and kept at the animal facility of Charité University Hospital (Charité – Universitätsmedizin Berlin) under defined, pathogen-free conditions. Eight- to ten-week-old BALB/c mice were immunized intraperitoneally with 100 μg ovalbumin (OVA, Sigma-Aldrich) and 100 μg chicken gamma globulin (CGG, Cedarlane) emulsified in the same volume of alum (Thermo Scientific), and were boosted using the same protocol 3 weeks after primary immunization.

Preparation of OVA/anti-CD138 conjugate (OVA-C)

The conjugate, consisting of a recombinant anti-mouse CD138 antibody with a heavy chain constant region of a human IgG1 (clone: REA104; Miltenyi Biotec) and OVA, was manufactured in-house. For conjugation, the anti-mouse CD138 antibody was first reduced with dithiothreitol (Sigma-Aldrich), and OVA (Sigma-Aldrich) was activated by succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (ThermoFisher Scientific). Afterwards, both reactants were incubated overnight, and the conjugate was then purified using a Superdex column (GE Healthcare Life Sciences).

The labeling detection of OVA-C

A single intraperitoneal injection of the OVA-C at different doses of 50–500 μg was given to analyze the labeling of plasma cells from the bone marrow of unimmunized BALB/c mice 3 h after injection. For OVA immunized BALB/c mice, 100 μg OVA was injected intraperitoneally 40 h before 500 μg OVA-C administration, spleen, bone marrow, and lymph node plasma cells were analyzed 3, 24, and 72 h after OVA-C administration.

Selective depletion of OVA-specific plasma cells

The treatments were performed in BALB/c mice 16 weeks after secondary immunization with OVA and CGG. At this time point, the majority of OVA- and CGG-specific plasma cells in the bone
morrow are of the long-lived phenotype (BrdU-negative). A single intraperitoneal injection of 500 μg of the OVA-C was administered to deplete OVA-specific plasma cells. Serum anti-OVA antibodies were neutralized by a single intraperitoneal injection of 100 μg of OVA 4 h before intraperitoneal administration of the OVA conjugate. To prevent plasma cells regenerating from their precursors, mice were treated with monoclonal anti-CD20 antibody (Biogen; 10 mg/kg, i.p.) on day −10 and with cyclophosphamide (20 mg/kg, i.p.) at −24, +8, and +24 h. Right after anti-CD20 treatment till 48 h after OVA-C administration, the mice received bromodeoxyuridine, an analogue of the nucleoside thymidine, (BrdU, Sigma-Aldrich, 1 mg/mL) via drinking water with 1% glucose to enable distinction between proliferating short-lived and non-proliferating long-lived plasma cells. In the experiment aimed to follow-up the serum antibody levels over 48 days after OVA-C administration, cyclophosphamide was additionally injected at +48 and +72 h, and serum was collected at the different time points.

Flow cytometry

Flow cytometric analysis was conducted according to the recently published guidelines [27]. Briefly, single-cell suspensions were prepared from spleen, bone marrow (femur and tibia) and mesenteric lymph node samples. These suspensions were then stained with anti-human-IgG1-PE (clone: IS11-12E4.23.20; Miltenyi Biotec), anti-mouse CD138-PE-Vio770 (clone: REA104; Miltenyi Biotec), and anti-mouse CD267 (TACI)-biotin antibodies (clone: 8F10; Miltenyi Biotec), anti-mouse CD19-APC/Cy7 (clone: 6D5; BioLegend), and anti-mouse CD267 (TACI)-biotin antibodies (clone: 145-2C11; BioLegend). In preparation for intracellular staining, the cells were treated with BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences). Intracellular immunoglobulin kappa light chain was detected with anti-kappa-Pacific Orange (clone 187.1; DRFZ), and intracellular OVA specific and CGG specific antibodies were identified using OVA conjugated with FITC and CGG conjugated with PerCP, respectively. BrdU incorporation was analyzed using the BrdU Flow Kit (BD Biosciences) according to the manufacturer’s protocol, and staining was performed with anti-BrdU-Alexa647 (clone: 3D4; BD Biosciences). Flow cytometric analysis was performed with a FACScanto II cytometer (BD Biosciences), and the data were analyzed with FlowJo software (Tree Star, Inc.). Absolute cell numbers were calculated based on population frequencies and total cell numbers per organ.

ELISA

Total IgG, anti-OVA, and anti-CGG antibody levels were determined as follows: 96-well plates (Corning) were coated with Goat Anti-Mouse IgG (SouthernBiotech), OVA, and CGG, respectively, and blocked by adding 3% BSA/PBS. After adding an appropri-
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