Increased macrophage phagocytic activity with TLR9 agonist conjugation of an anti-\textit{Borrelia burgdorferi} monoclonal antibody

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\textbf{ABSTRACT}

\textit{Borrelia burgdorferi} (\textit{Bb}) infection causes Lyme disease, for which there is need for more effective therapies. Here, we sequenced the antibody repertoire of plasmablasts in \textit{Bb}-infected humans. We expressed recombinant monoclonal antibodies (mAbs) representing the identified plasmablast clonal families, and identified their binding specificities. Our recombinant anti-\textit{Bb} mAbs exhibit a range of activity in mediating macrophage phagocytosis of \textit{Bb}. To determine if we could increase the macrophage phagocytosis-promoting activity of our anti-\textit{Bb} mAbs, we generated a TLR9-agonist CpG-oligo-conjugated anti-BmpA mAb. We demonstrated that our CpG-conjugated anti-BmpA mAb exhibited increased peak \textit{Bb} phagocytosis at 12–24 h, and sustained macrophage phagocytosis over 60+ hrs. Further, our CpG-conjugated anti-BmpA mAb induced macrophages to exhibit a sustained activation morphology. Our findings demonstrate the potential for TLR9-agonist CpG-oligo conjugates to enhance mAb-mediated clearance of \textit{Bb}, and this approach might also enhance the activity of other anti-microbial mAbs.

\textbf{1. Introduction}

Lyme disease results from \textit{B. burgdorferi} (\textit{Bb}) infection of humans [1], and without adequate antibiotic treatment \textit{Bb} infections can lead to long lasting and severe symptoms [2]. \textit{Bb} has evolved multiple mechanisms to evade immune clearance and establish persistent infection. The bacterium has a robust repertoire of outer surface lipoproteins that have anti-complement activity [3]. Some of these outer surface proteins, such as OspC, interfere with phagocytosis of the Lyme disease spirochete by macrophages [4]. There is great need for next-generation approaches to treat \textit{Bb} infections.

The innate immune system uses a variety of signaling pathways that can be activated by microbial infectious agents, like \textit{Bb} [5]. Toll-like receptors (TLRs) are pattern-recognition receptors (PRRs), which recognize foreign infectious agents and their pathogen-associated molecular patterns (PAMPs) to initiate inflammatory reactions [6,7]. TLRs are expressed in macrophages and dendritic cells [8]. In mammals, different TLRs can detect diverse range of bacterial ligands including lipopolysaccharide (LPS), nucleic acids, and certain proteins found in gram-positive and gram-negative bacteria [6,9]. TLRs are translated in the ER and then transferred to their designated cellular compartments, which includes the plasma membrane and endosomes. TLR9 is an endosomal TLR that recognizes unmethylated bacterial CpG DNA [10]. Among the TLRs expressed by macrophages, TLR9 represents a compelling therapeutic target, due to its ability to bind bacterial ligands, like CpG DNA, in the endosome and further activate macrophages [11–13]. Further, studies have shown that TLR9 agonists can be used to enhance the efficacy of vaccines and cancer therapies [14,15].

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Macrophages are innate immune cells that play a key role in preventing and clearing microbial infections by using a variety of mechanisms for recognition, signal transduction and phagocytosis [9]. Although macrophages have an immense ability to destroy cells by antibody-dependent phagocytosis, the literature does not specifically show the effect of opsonins on clearing of Bb. Macrophages utilize TLR expression and activation to generate robust immune responses against Bb infection [7,16]. Specifically, TLR7 and TLR9 stimulation increased phagocytosis and macrophage longevity while inducing polarization [17]. Further, recent studies showed that antibody drug conjugates can be used as an effective treatment for cancer immunotherapy by a mechanism of augmented macrophage phagocytosis [18,19]. To our knowledge, induction of macrophage phagocytic activity by patient derived, anti-Bb mAbs conjugated with a TLR9-agonist have not been developed as a therapeutic for Lyme disease.

Here, we informatically analyzed plasmablast antibody repertoires from Bb-infected humans to identify antibodies representative of the clonal families. Through recombinant expression of the encoded monoclonal antibodies (mAbs), we identified patient-derived mAbs that bind a spectrum of Bb antigens. Several of these mAbs promoted macrophage phagocytosis of Bb. To develop a more potent anti-Bb mAb, we conjugated a CpG oligo TLR9 agonist to a Bb specific clone (anti-BmpA). We found that this CpG-conjugated anti-Bb BmpA mAb was able to significantly increase Bb phagocytosis activity across multiple donors. This work provides a potential path to the development of next-generation therapeutics for Bb and other microbial infections.

2. Materials and methods

2.1. Exp293 Cell culture and recombinant monoclonal antibody production

Plasmablast antibody sequences were previously identified from patients infected with Bb [25].

Heavy chain sequences were used to produce monoclonal antibodies as previously described [20]. In short, the mAb's variable sequences of heavy chain and light chain regions were cloned into an in-house expression vector VRG01. Exp293 cells (2.5 × 10⁶ cells/ml) were transfected with Lyime mAb's cognate heavy and light chains at a final transfection concentration of 0.5μg/mL. FectoPro Transfection Reagent (VWR) was added to the plasmids at a final transfection concentration of 1.3μL/mL. Media was harvested after 7 days and antibody purification was performed with IgG elution buffer (Pierce) by AmMag Protein magnetic beads (Genscript). Antibody concentrations were measured with a nanodrop [21].

2.2. TLR9 conjugation

We conjugated anti-BmpA mAb with a TLR9-agonist CpG oligo (Human type B CPG 2006, InvivoGen, tlr7-2006). In order to do that, 350 μl of Pierce™ Immobilized TCEP Disulfide Reducing Gel slurry (ThermoFisher, 77,712) was washed and incubated with the TLR9 agonist CPG oligo ODN 2006 for 1 h, and then EDTA added to stop the reaction. The reduced oligo has thiol group available after treating with TCEP (Fig. 3A). DBCO-PEG4-Maleimide linker (ClickChemistryTools, A108P-25) was added to the reduced oligo, to result in conjugation with the thiol group of the CPG oligo (Fig. 3B). We then used click-chemistry for labeling anti-BmpA mAb to DBCO-PEG4-Maleimide-CPG oligo. Using SiteClick™ Antibody Azido Modification Kit (Thermo Fisher Scientific Logo, S20026) we added azide moiety to the carbohydrate domain in the Fc region of our mAb. The DBCO-PEG4-Maleimide-CPG oligo was thereby conjugated to the azide on sugar molecule of mAb. This site-specific conjugation leaves the mAb's Fab region intact and thereby does not interfere with antigen binding (Fig. 3C).

2.3. Human monocyte-derived macrophage

Leukocyte reduction system (LRS) chambers of different healthy donors were purchased from Stanford blood center. Peripheral blood mononuclear cells (PBMCs) and monocytes were isolated with Ficoll (Sigma-Aldrich) and Percoll (GE Healthcare) subsequently. Differentiation of Monocytes to macrophages occurred within 6–7 days using IMDM with glutamax base and 10% AB human serum (GeminiBio) [22,23].

2.4. Bb culture and Fluorescence-Activated Cell Sorting (FACS)

A GFP expressing Bb strain B31 [24], called Bb-GFP hereafter to avoid confusion with monoclonal antibody B31 (anti-BmpA), was either cultured for 3 days or a 1/20 dilution from a 4-day primary culture was subcultured for 3 days in BSK complete medium (Catalog number: BS291, Sigma-Aldrich) with 6% rabbit serum (50 mL, Sigma-Aldrich) at 37 °C. The concentration, viability of cells and percentage of GFP expression was calculated with Fluorescence-Activated Cell Sorting (FACS). FACS was conducted on a BD LSRFortessa at Stanford University School of Medicine FACS Core with BD FACS Diva software. For FACS analysis of Bb, LSRFortessa cytometer threshold levels were modified to parameter SSC 400. Voltages were set to FSC 300 and SSC 230, collected in log mode.

2.5. Flow cytometry evaluation of anti-BmpA mAb binding to Bb, compared to IgG1

Bb cultures (20 μl/well) were plated in a 96 well v-bottom plate (Corning). After the plate was centrifuged for 10 min at 1500 xg at 4 °C and the supernatant was aspirated, the Bb were resuspended in FACS buffer (30 μL/well) (200 μL; 2% fetal bovine serum in PBS supplemented with EDTA (1 mM, Thermo Fisher Scientific)) containing anti-BmpA mAb, IgG1, or unstained (10 μg/mL). Samples were incubated on ice (30 min) and protected from light. Upon incubation completion, the plate was washed twice with PBS (150 μL). For each wash, bacteria were pelleted prior to aspiration (1500 x g, 10 min, 4 °C). After the second wash, the Bb were resuspended in PBS (30 μL) containing Alexa Fluor 647 anti-human IgG (1:200, Jackson ImmunoResearch). Samples were incubated on ice (30 min) and protected from light. Samples were again washed with PBS (2 ×) as described above. Bb were resuspended in 4% paraformaldehyde (100 μL, EMS) and incubated while protected from light (10 min, 25 °C). Samples were again washed with PBS (2 ×) as described above prior to resuspension in FACS buffer. Samples were protected from light until analysis on a BD Fortessa.

2.6. Phagocytosis assay

Macrophages differentiated from human blood monocytes were washed and released from the plate using 5 mL of TrypLE (Gibco). For clarification, antibody sequences were identified from patients infected with Bb, while monocytes were isolated from donors who were unexposed to Borrelia for all experiments conducted in this study. The pellet was confirmed and resuspended in R10 (without phenol red) at a concentration of 1.5 × 10⁵ cells/mL. Macrophages were plated at 15,000 cells per well on Imagedock plate (Essen). In order to make sure that the macrophages adhere to the plate, they were incubated at room temperature for 30 min to overnight. After three days of culture, Bb-GFP cells were centrifuged (1500 xg, 10 min, 4 °C) and pHrodo-labeled in pHrodo dye solution (1:20,000, Essen). After one hour of incubation (protected from light) at 37 °C, fetal bovine serum (FBS) was added to the pHrodo Bb-GFP solution to quench the reaction. Bb-GFP cells were resuspended at a concentration of 3 × 10⁶ cells/mL in R10 media (without phenol red) and 150,000 Bb-GFP were added to the cultured macrophages per well. Antibody stocks for both conjugated and non-conjugated clones were diluted to 160 μg/mL and 1.6 μg/well of each
antibody was added to the respective wells. 100 μl of R10 (without phenol red) was added to the control wells without macrophages and 50 μl of R10 (without phenol red) was added to the control wells without Bb-GFP. Time-lapse images of Bb-GFP and pHrodo (ThermoFisher) were taken with a Nikon TiE2 Eclipse microscope. An Incucyte System (Essen) of R10 (without phenol red) was added to the control wells without antibody was added to the respective wells. 100 μl of R10 (without phenol red) was added to the control wells without macrophages and 50 μl of R10 (without phenol red) was added to the control wells without Bb-GFP. Time-lapse images of Bb-GFP and pHrodo (ThermoFisher) were taken with a Nikon TiE2 Eclipse microscope. An Incucyte System (Essen) of R10 (without phenol red) was added to the control wells without macrophages and 50 μl of R10 (without phenol red) was added to the control wells without macrophages and 50 μl of R10 (without phenol red) was added to the control wells without macrophages.

For measuring phagocytosis activity, we use the pHrodo dye which fluoresces red in the acidic environment of the phagosome. By comparing the intensity or count of florescence of this dye we can get a measurement of actual phagocytosis of Borrelia. The Incucyte System (Essen), housed in an 37 °C incubator, was used for time lapse phagocytosis analysis at 20× magnification and 800-ms exposures per field. The incucyte images were taken in an average of 3–4 h time points. Each condition was tested in triplicate for its ability to mediate Bb phagocytosis and each technical replicate had 3 fields of view which made it possible for 9 fields of view measurement per donor. Phagocytosis events were measured with the number of pHrodo-red+ events and with the total integrated intensity (TII) of pHrodo dye per image. Threshold values to determine pHrodo-red+ events were set such that macrophages not incubated with pHrodo labeled B. burgdorferi had no pHrodo-red+ events.

2.7. Statistical analysis

The statistical difference between the two different conditions of CpG-oligo-conjugated anti-BmpA mAb as compared to unconjugated anti-BmpA mAb at serial time points was calculated from 4 donors using the Multiple Mann-Whitney test (Prism 9). Error bars were calculated as standard error to the mean (SEM) in GraphPad Prism.

3. Results

3.1. Recombinant expression and characterization of the binding specificity of human anti-Bb plasmablast mAbs

We generated a panel of anti-Bb mAbs by recombinantly expressing plasmablast mAbs derived from Bb-infected humans. First, we informatically analyzed our plasmablast antibody repertoires from Bb-infected humans [25] to identify antibodies representative of clonal families for recombinant expression. Clonal families were defined based on shared V and J gene usage, identical CDR3 lengths, and >75% CDR3 homology. Using antibody repertoire sequence datasets from 7 Bb infected humans (Supp Fig. 1.), we selected 31 plasmablast antibodies for recombinant expression. The heavy and light chain variable regions were gene synthesized, and the mAbs were expressed using our previously described methods [21].

To characterize the binding specificity of the expressed 31 anti-Bb mAbs, we used a Bb antigen bead array on the Luminex platform. Bb antigen array analysis identified mAbs with specific reactivity to Bb proteins and peptides, including anti-BmpA mAb to BmpA, mAb B11 to OspC, and mAb B153 to BmpA (Fig. 1). We also identified a Bb patient mAb, mAb B155, that exhibited reactivity to BmpA derived from both Bb and the closely related Bafz strain (Fig. 1), demonstrating the ability of certain mAbs to bind shared epitopes across different strains of Borrelia.

3.2. Recombinant Bb plasmablast mAbs differentially mediate macrophage phagocytosis of Bb

We characterized the ability of our panel of recombinant mAbs to mediate Bb phagocytosis, by comparing macrophage phagocytosis of Bb-GFP in vitro. Macrophages were generated by differentiation of human blood monocytes in the presence of human serum over 7 days. We used the Incucyte system to analyze macrophage phagocytosis of a GFP-engineered strain of Bb, called Bb-GFP [24,26]. Fluorescence enabled tracking of Bb-GFP and the relative macrophage phagocytic activity mediated by our panel of Bb mAbs are presented (Fig. 2). A range of phagocytic activity was detected, for example, Bb mAb B158 and B149, which exhibited robust binding to Bb NapA and p45, respectively, by antigen bead array, did not result in significant Bb phagocytic activity (Fig. 2). In contrast, mAb anti-BmpA and B11, bound surface antigens BmpA and OspC, and mediated robust Bb phagocytic activity (Fig. 2). FACS analysis of anti-BmpA and B11 revealed a high level of BmpA expression on the surface of Bb (Supp Fig. 2). Colocalization of Bb-GFP with macrophage phago-lysosomes was confirmed by time-lapse
epifluorescence imaging of Bb-GFP and the pHrodo pH-sensitive dye (Supp Fig. 3., and Supp Movie).

3.3. Generation of TLR9-agonist CpG oligo-conjugated anti-BmpA mAb

To determine if conjugation of anti-Bb mAbs with a TLR9-agonist would increase macrophage phagocytic activity, we selected an anti-Bb mAb with significant macrophage promoting activity in its native format (Fig. 2). Additionally, this mAb bound Bb BmpA, which is known to be expressed on the outer surface of Bb. Next, we conjugated this anti-BmpA mAb with a TLR9-agonist CpG oligo (Fig. 3A). We treated the CpG oligo with tris(2-carboxyethyl) phosphine (TCEP) to provide a free thiol group at the 5′ end of the CpG oligo, and then added a DBCO-PEG4-maleimide linker to form a DBCO-PEG4-maleimide-conjugated CpG oligo (Fig. 3B). The DBCO-PEG4-maleimide-CPG oligo conjugate was then linked to anti-BmpA mAb using click chemistry, resulting in addition of an azide moiety to the carbohydrate domain of the IgG antibody (Fig. 3C). The oligo added approximate 17 kDa to the anti-BmpA heavy chain (~15 kDa for the linker-CpG oligo plus ~1–3 kDa for the azide moiety), resulting in a ~66–68 kDa polypeptide band. Western Blot analysis confirmed successful conjugation of anti-BmpA mAb to the DBCO-PEG4-maleimide-CPG oligo (Fig. 3D).

3.4. CpG-oligo conjugation of an anti-BmpA mAb increased macrophage phagocytosis of Bb

Next, we used the Incucyte System to test the ability of TLR9-agonist conjugation to enhance macrophage-mediated Bb phagocytosis. Human
monocyte-derived macrophages were generated from healthy donors PBMCs by differentiation of blood monocytes over 7 days. We compared the ability of our CpG-conjugated anti-BmpA mAb vs. non-conjugated anti-BmpA mAb to mediate macrophage phagocytosis of Bb-GFP over a 96-h time course. Flow cytometry confirmed expression of BmpA on the outer surfaces of in vitro cultured Bb-GFP (Fig. 4A). Incucyte analysis of mAb-facilitated macrophage phagocytosis showed that the CpG-conjugated anti-BmpA mAb increased macrophage phagocytosis of Bb-GFP as compared to the unconjugated mAb (Fig. 4B). Phagocytosis data from 4 individual donors showed heterogeneous macrophage activity with a range of 294 to 817 total integrated intensity (TII) (Fig. 4B). The mean baseline activity for all macrophages + Bb (without antibody) had a pHrodo TII of 484 as indicated in light gray (Fig. 4C). A significant increase in macrophage phagocytosis in the CpG-conjugated anti-BmpA mAb, when compared to non-conjugated anti-BmpA mAb, was confirmed by use of the Multiple Mann-Whitney test with a P value of <0.01 (Fig. 4B). We further analyzed the phagocytosis data for all four donors and found a 1.5× higher area ratio for CpG-conjugated anti-BmpA mAb compare to non-conjugated anti-BmpA mAb (Fig. 4D).

We confirmed the ability of our CpG-conjugated anti-BmpA mAb to enhance macrophage clearance of Bb-GFP in two independent experiments, using macrophage generated from 6 independent donors (Supp. Fig. 4, and Supp Fig. 5.). In 3 of these donors, we also tested a similar molar amount of the CPG ODN in combination with unconjugated anti-BmpA to control for thier ability to activate macrophages to phagocytose Bb. The averaged data across these 3 donors suggests that CpG-conjugated anti-BmpA mAb showed higher and sustained phagocytosis activity compared to the unconjugated anti-BmpA mAb alone or together with CPG ODN during the time course of the assay (Supp Fig. 5.).

3.5. Analysis of peak macrophage phagocytosis activity

We analyzed peak macrophage phagocytosis activity mediated by CpG-conjugated anti-BmpA mAb vs. non-conjugated anti-BmpA mAb, by further analyzing our Incucyte datasets. Based on the total integrated intensity (TII) of the pHrodo-positive macrophages (Fig. 5). These representative images show extensive macrophage mediated phagocytosis and internalization of pHrodo-labeled Bb-GFP in samples to which CpG-conjugated anti-BmpA mAb was added, when compared to the non-conjugated anti-BmpA mAb. Colour changes, representing macrophage activity, are seen after phagocytosis of Bb (Fig. 5). The macrophages incubated with CpG-conjugated anti-BmpA mAb exhibited histologic features of activation, including elongation, that was most prominent at later timepoints (including peak time points), as compared to incubation with the non-conjugated mAb (Supp. Movie 2 and Supp. Movie 3).

4. Discussion

New treatment approaches are needed for Bb infection, to reduce the complications associated with Lyme disease. Here, we sequenced the antibody repertoire of plasmablasts in Bb-infected humans and characterized encoded monoclonal antibodies (mAbs). We demonstrated that our recombinant anti-Bb mAbs exhibit a range of Bb phagocytosis activity, and that conjugation with a TLR9-agonist CpG-oligo enhanced macrophage phagocytosis of Bb, including both peak phagocytosis at 12–24 h, as well as sustained macrophage phagocytosis over 60+ hours. Our findings demonstrate the potential for TLR9-agonist CpG-oligo conjugates to enhance macrophage-mediated macrophage phagocytosis of Bb. Borrelia membrane protein A (BmpA) is a 39 kilodalton laminin-binding protein that is expressed on the outer surface of Bb. Antibodies against BmpA are often detected in humans during early infection.

Fig. 4. CpG oligo-conjugated anti-BmpA mAb increases macrophage clearing activity of Borrelia burgdorferi. (A) Flow cytometry evaluation of anti-BmpA mAb binding compared to IgG1 isotype control and unstained Bb-GFP. (B) incucyte phagocytosis assay of 4 individual donor monocyte derived macrophages (donor 1–4) incubated with Bb-GFP and treated with unconjugated BmpA mAb (light blue line), CpG oligo-conjugated anti-BmpA mAb (dark blue line) or vehicle control (gray line) as analyzed by pHrodo fluorescence intensity which represents phagocytic events. The baseline pHrodo intensity control in pink represents pHrodo-labeled Bb-GFP on their own in the absence of donor driven-macrophages that could phagocytose them. The incucyte determent values for Total integrated intensity (TII) are shown on the Y axis. (C) Display of mean values for the results of donors 1–4 from B. ** P < 0.01 comparing CpG oligo-conjugated anti-BmpA mAb with anti-BmpA. (D) Area Ratio of CpG oligo-conjugated anti-BmpA mAb / anti-BmpA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
and are used for the diagnosis of Lyme disease [27,28]. Analysis of Bb bacteria found in mammalian skin and joint tissues, revealed that BmpA was expressed on bacterial cell surface, where it is accessible to host antibodies [29,30]. In addition, our flow cytometry results showed that BmpA was expressed on Bb surfaces across all independent cultures that were tested, supporting our choice of an anti-BmpA mAb (B31) for this study.

Macrophages play a major role in the innate immune response against pathogens including Bb through phagocytosis activity. Macrophages express pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), that bind to microbe-associated surface proteins. Our goal was to increase mAb-facilitated macrophage phagocytosis activity. For this reason, we chose an anti-BmpA antibody with high binding activity, that also targeted an antigen that was expressed on the outer surfaces of Bb.

Our findings support the observations from other groups that linking TLR-agonists to mAbs can enhance their ability to mediate immune responses against cancer and microbes. This linkage utilizes binding specificity of mAbs, while leveraging the immune activation of TLR agonists. In our experiments we demonstrated the ability of mAb-CpG-oligo conjugates to activate macrophages and enhance macrophage phagocytosis. Further, TLR-agonist conjugates have the potential to link innate and adaptive immunity, by capturing the antigen and thereby facilitating the loading and activation of macrophage and dendritic cells that in turn activate T cells in a highly specific manner.

In the future we hope to extend this work to a larger number of Bb antigens and other effector cell types. Future studies are also needed to investigate the potency of mAbs against additional Bb antigens, as mAb targeting of different antigens as well as different epitopes on these antigens could differentially impact macrophage activation and phagocytosis. In addition, different effector cells express different TLRs, and examination of TLR agonists that activate other TLRs and other cell types has the potential to identify other TLR agonists with significant anti-Bb phagocytosis activity. In addition, it would be good to examine effects of our TLR agonist CpG-oligo anti-Bb BMPA mAb on different cell types, including their impact on the activation and antigen loading of dendritic cells, their impact on T cell activation, and their effect on neutrophils and other cell types. The potential use of anti-Bb mAbs conjugated with CpG-oligos could be further explored for their therapeutic potential using mouse models of Bb infection.

In conclusion, there is great need to develop additional effective treatments for Bb infections that are resistant to antibiotic treatment or cause long-lasting post Bb infection symptoms. Here we show that anti-Bb mAbs can mediate macrophage phagocytic clearance of Bb, and that conjugation of anti-Bb mAbs with CpG-oligo enhances macrophage activation and phagocytic clearance. Such an approach could be used to develop new therapeutic approaches for Bb and other microbial infections.

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Author contributions

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Robinson had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design: Jahanbani, Nemati, Tal, Robinson.

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Analysis and interpretation of data: Jahanbani, Hansen, Blum, Ramadoss, Bastounis, Kirschmann, Blacker, Love, Weissman, Nemati, Tal, Robinson.

Data availability

Data will be made available on request.

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