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43 Abstract

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Recent Findings: Structure-based epitope design approaches in the melioidosis field are preliminary and applied essentially by one research network. By combining *Burkholderia pseudomallei* antigen 3D structures and in silico epitope discovery methods, a panel of synthetic epitope peptides were designed and tested for their B and T cell stimulatory activities. Several peptides were found to be serodiagnostic for *B. pseudomallei* infection and two elicited bactericidal antibodies.
Summary: A significant amount of *B. pseudomallei* antigen structures, epitopes, and immunological data is available. Future challenges will be to test all available *B. pseudomallei* epitopes, focusing on combing multiple B/T cell epitopes onto a single scaffold to generate components, stimulating both arms of the immune system.

44	Keywords separated by ' - '	Melioidosis - Vaccines - Epitope design - Structural vaccinology - *B. pseudomallei* - Antigens
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Structural Vaccinology for Melioidosis Vaccine Design and Immunodiagnostics

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Abstract

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Recent Findings Structure-based epitope design approaches in the melioidosis field are preliminary and applied essentially by one research network. By combining *Burkholderia pseudomallei* antigen 3D structures and in silico epitope discovery methods, a panel of synthetic epitope peptides were designed and tested for their B and T cell stimulatory activities. Several peptides were found to be serodiagnostic for *B. pseudomallei* infection and two elicited bactericidal antibodies.

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Keywords Melioidosis · Vaccines · Epitope design · Structural vaccinology · *B. pseudomallei* · Antigens

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Introduction

Melioidosis is a potentially fatal disease caused by the soil-borne, intracellular Gram-negative bacillus *Burkholderia pseudomallei*, endemic in the tropical and subtropical regions of the world [1]. Proper diagnostic tests are unavailable, and accordingly, melioidosis is often misdiagnosed and/or underreported, leading to the global distribution of melioidosis being extensively underestimated [2•]. The worst affected areas are North Thailand and North Australia where mortality rates can reach 50 and 19%, respectively [3]. There are a number of risk factors that predispose individuals to *B. pseudomallei* infection, including diabetes, alcoholism, and the presence of chronic lung and/or liver pathologies. The bacterium enters the body via inhalation, ingestion, and percutaneous inoculation and causes diverse clinical outcomes including pneumonia, multiple abscesses, and fatal septicemia [2•, 4].

B. pseudomallei is resistant to all major antibiotic classes; therefore, empirically administered antibiotics given to patients in the absence of proper diagnosis are ineffective [5, 6]. Such diagnostic and therapeutic challenges, coupled to its classification as a Tier 1 select agent by the Centers for Disease Control and Prevention, have spurred research efforts directed at the discovery and development of a melioidosis vaccine and improved diagnostic tools [7].

In this context, structure-based antigen engineering is evolving as a modern-day strategy to develop improved

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61 vaccine components, and it is predicted by experts in the field
62 to deliver future vaccines targeting complicated pathogens
63 such as HIV [8••]. This report reviews the possibility of ap-
64 plying *structural vaccinology* (SV) approaches for the devel-
65 opment of a melioidosis vaccine and outlines some initial SV
66 studies made on specific *B. pseudomallei* antigens.

67 Human Immune Responses to *B. pseudomallei*

68 Due to its intracellular nature, *B. pseudomallei* invades and
69 replicates inside both phagocytic and non-phagocytic cells
70 and can do so for prolonged periods. After it is taken up by
71 the cell vacuole, it can escape and replicate in the host cytosol.
72 In fact, a key factor that renders treatment of melioidosis chal-
73 lenging is the ability of *B. pseudomallei* to persist, eventually
74 leading to chronic disease. For these reasons, a protective
75 melioidosis vaccine must contain antigenic components that
76 induce both cell-mediated and humoral responses of the hu-
77 man immune system [9].
78 The innate immune system plays a vital and primary role in
79 clearance of *Burkholderia* from the body following host inva-
80 sion. A number of key cell types are activated in response to
81 infection, e.g., macrophages and natural killer cells [10].
82 When replication is not prevented, chronic infection pro-
83 gresses, and the role of CD4^{+} T cells is critical for long-term
84 infection control [11].
85 The adaptive immune response to *B. pseudomallei* infec-
86 tion is less understood. Melioidosis patients with acute phase
87 infections exhibit high antibody titers associated to three IgG
88 isotypes, confirming the importance of the antibody response
89 [12]. A number of vaccine candidates have been tested for
90 their capacity to induce immune protection against challenge
91 with *B. pseudomallei* in vivo and will be discussed in the
92 following section.

93 Current *Burkholderia* Vaccine Research

94 Tested vaccine candidates range from live-attenuated forms of
95 the bacterium, to recombinant protein antigens and to DNA
96 and polysaccharide subunits; however, a neutralizing vaccine
97 has yet to be formulated [13••].
98 *Mycobacterium tuberculosis* and *B. pseudomallei* have
99 shared characteristics e.g., both are intracellular, they can per-
100 sist for years and they have similar histological and clinical
101 profiles. Immunization with chronic and acute phase *M.*
102 *tuberculosis* antigens expressed on the same polypeptide led
103 to increased immune protection in mice challenged with in-
104 fection, and human clinical trials are in progress [14–16]. This
105 led Champion et al. to conduct a similar study on *B.*
106 *pseudomallei*. Three chronic phase *B. pseudomallei* antigens
107 (BPSL3369, BPSL1897, and BPSL2287) and BPSL2765,
108 due to its link to non-recurrent incidences of melioidosis, were
109 used to immunize mice achieving significant immune protec-
110 tion in comparison with the most protective vaccine candi-
111 dates tested to date, namely the LolC recombinant protein
112 and capsular polysaccharides [17••, 18, 19].

113 Structural Vaccinology

114 The 3D architecture of antigens can reveal the structures, the
115 (a)polar and electrostatic surfaces recognized by neutralizing
116 antibodies, and can guide the design of improved immuno-
117 gens [20, 21]. Introducing structural modifications can ad-
118 dress both practical and immunological challenges. For exam-
119 ple, as only epitope containing portions of an antigen are re-
120 quired to induce an immune response, 3D structure informa-
121 tion can be used to focus on smaller immunogenic substruc-
122 tures of an antigen, thus facilitating its production (e.g., when
123 the production of large multi-domain antigens is problematic).
124 With respect to substructure size, the smallest example is the
125 translation of antigen portions into relatively small peptides, a
126 strategy that potentially brings a considerable potential over
127 traditional vaccination approaches [22]. Also, by identifying
128 antigenic domains that specifically elicit protective immunity,
129 rational antigen engineering based on structural consider-
130 ations, can be driven. For example, when neutralizing epitope
131 conformations are transient, or hidden by more
132 immunodominant yet non-protective epitopes, attempts may
133 be made to block or improve the presentation of neutralizing
134 conformations. Epitope grafting, peptide cyclization, and sta-
135 pling techniques are some useful and successful approaches
136 that have been applied in this context [23–28].

137 Computationally Assisted Antigen (re)Design

138 The prerequisites for structure-based antigen engineering, also
139 termed structural vaccinology, is the 3D antigen structure,
140 obtained via experimental methods (X-ray crystallography or
141 nuclear magnetic resonance [NMR]), or through in silico
142 methods (homology modeling) when suitable structural ho-
143 mologs are available in the Protein Data Bank (PDB; www.
144 rcsb.org), coupled to knowledge of epitope region locations.
145 There have been significant advances in computational
146 biology and the development of depositories hoarding large
147 amounts of epitope data. Consequently, in addition to
148 experimental determination, epitope sequence information
149 may be accurately predicted using more rapid and
150 economical sequence-based and (to a lesser extent) struc-
151 ture-based, in silico epitope prediction methods [29–32].
152 When knowledge of both epitope sequence and 3D conforma-
153 tion is known, structure-based antigen engineering may thus
154 proceed to further vaccine design.

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SV Approaches Applied to *B. pseudomallei* Antigens

Examples of SV applied to *Burkholderia* antigens are limited and focus on the discovery and design of antigenic epitopes as an alternative to the full antigen, using structure-based in silico epitope predictions and design. Target antigens belong to a list of 49 proteins found to be serodiagnostic for *B. pseudomallei* infection, based on the results of a protein microarray study presenting over 1000 in silico-predicted surface antigens, and on a subsequent study involving convalescent sera that reported 27 proteins that are specifically recognized by recovery IgGs [18, 33••].

B. pseudomallei SV studies involved the use of two 3D structure-based in silico epitope prediction methods called matrix of local coupling energies (MLCE) and electrostatic desolvation profiles (EDP) [34, 35]. These two methods detect different physico-chemical properties that are characteristic of epitopes; therefore, epitope prediction precision is improved by selecting consensus sequences.

MLCE specifically pinpoints antigenic residues that are located in dynamic and conformationally flexible regions of the antigen by identifying solvent-accessible residues that are less energetically coupled with the rest of the protein, e.g., that are not involved in stabilizing interactions within the protein fold. In other words, these regions can adapt to bind a partner, specifically an antibody, with minimal energetic expense and can tolerate well-conformational changes determined by antibody recognition [35]. In contrast, EDP identifies generic protein-protein interaction interfaces by looking at surface cavities where desolvation would be energetically favored upon antibody binding [34]. Both methods were successfully tested on a *Chlamydia* antigen and led to the design of a cross-species immunogenic domain [36].

Peptidoglycan-Associated Lipoprotein

Peptidoglycan-associated lipoprotein (Pal) (BPSL2765) is a seroreactive recovery antigen, recognized by IgGs from patients who have had one episode of melioidosis in comparison with those with recurrent melioidosis, suggesting a role in conferring immune protection [18]. Accordingly, Pal was shown to offer limited protection in a mouse immunization study [17••, 18, 37], and together with two other antigens (FliC and the N-terminal domain of seroreactive antigen BPSS1599) was found to stimulate human memory T and B cells in a humanized melioidosis mouse model [38•].

MLCE and EDP were combined and applied to the Pal crystal structure (3D structure coordinates are available from the PDB under entry code 4B5C) and led to the identification of a highly immunogenic epitope (Pal3) that, when administered to rabbits in peptide form, elicited antibodies that were bactericidal in vitro against *B. pseudomallei* [39••]. In addition, Pal3 clearly discriminated between melioidosis healthy seronegative, healthy seropositive, and convalescent patient subgroups. The full-length recombinant Pal antigen did not exhibit such properties, underlining the successful outcome of this SV approach and the potential of generating a better immunogen by "extracting" epitope regions from the initial antigen and producing them separately (Fig. 1). Future applications of the Pal3 epitope may be for both vaccine and diagnostic purposes [39••].

Oligopeptide Binding Protein

Oligopeptide-binding protein (OppA) (BPSS2141) is a member of the ATP-binding cassette (ABC) transporter family, whose members are known immunogens in Gram-negative bacteria in general. OppA, together with two other ATP-binding cassette system proteins, PotF and LolC, were shown to induce both humoral and cell-mediated immune responses in BALB/c mice challenged with *B. pseudomallei* [40]. A SV study analogous to that carried out on Pal was applied to the OppA crystal structure (PDB entry 3ZS6) [41]. Given the structural organization of the OppA fold into two lobes, a computational fragmentation into sub-domains prior to predictions using MLCE resulted in improved prediction accuracy. All three OppA epitopes were confirmed to be B cell epitopes, as judged by their immune sera reactivity when synthesized in peptide form [41]. One peptide, in particular, clearly distinguished between healthy seronegative, healthy seropositive, and convalescent melioidosis patient groups, implying a possible role in diagnosis.

Flagellin and the Flagellar Hook-Associated Protein

The flagellar hook-associated protein flagellar hook-associated protein (FlgK) (BPSL0280) and the flagellar subunit flagellin (FliC) (BPSL3319) are two *B. pseudomallei* antigens that displayed the highest seroreactivity toward immune sera from melioidosis recovery patients [18]. Antibodies raised against FliC proteins from diverse *B. pseudomallei* species were shown to offer passive protection in vivo [42]. Based on these findings, both antigens were deemed good SV targets. In this case and considering recent improvement in predictions made by MLCE alone, EDP was excluded from epitope predictions applied to the crystal structures of FliC (PDB entry 4CFI) and FlgK (PDB entry 4UT1); for FliC, sequence-based epitope predictions were also carried out [43, 44]. Relevant to melioidosis, two out of three FliC epitope peptides were found to be joint good T and B cell epitopes [44]. With regard to FlgK, epitopes were found to be clustered to discrete domains that may represent good starting points for the design of immunogenic domains [43].

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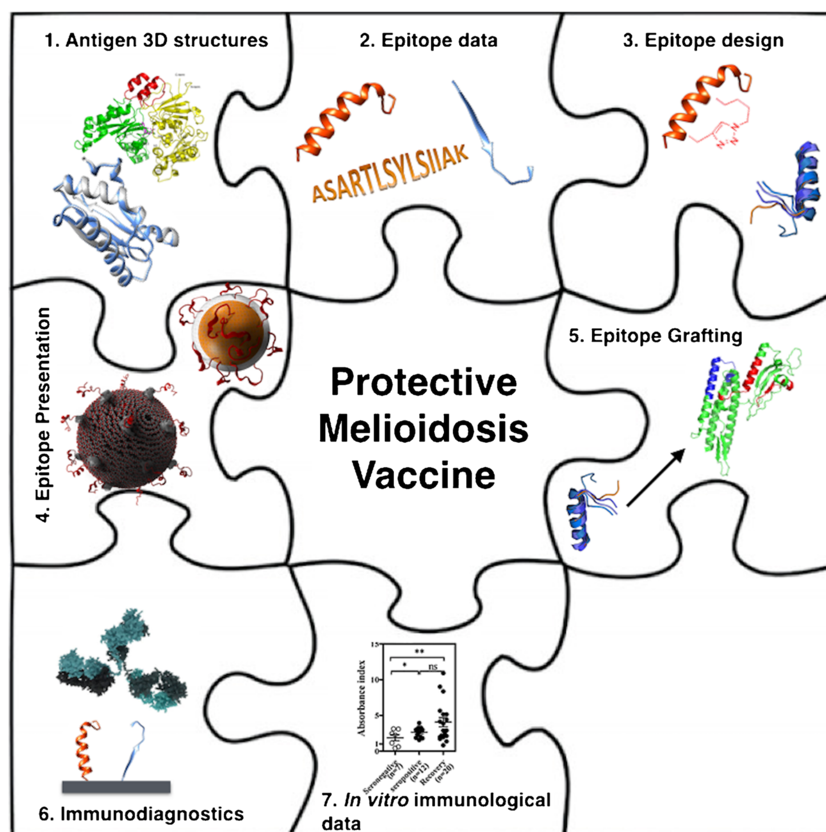

Q3 **Fig. 1** The melioidosis vaccine development puzzle. Pictorial representation of the state-of-the-art for structure-based antigen/epitope discovery and engineering, targeting *B. pseudomallei* (*Bp*) seroreactive antigens of known 3D structure. The figure illustrates how diverse advances in diverse scientific areas (structural biology, computational biology, chemistry, and immunology), must come together toward a common aim—melioidosis vaccine development—and summarizes the progress made to date. *1*. 3D *Bp* antigen structures are used for epitope discovery; *2*. Epitope sequence and structure data are obtained via in silico and in vitro methods; *3*. Chemistry is used to block epitopes in the desired conformation; *4*. Epitopes may be combined and presented on vaccine delivery vessels, such as outer membrane vesicles (OMVs) and nanoparticles (e.g., gold); *5*. Structure-based epitope grafting may be used as an alternative to combine multiple antigens, generating "super" antigens; *6*. B cell epitopes may be presented in microarray format for immunodiagnostic purposes to detect *B. pseudomallei* infection and infection stage; *7*. In vitro immunological data is essential to determine effective B cell and T cell stimulatory activities. The missing piece of the puzzle regards in vivo protection studies with *B. pseudomallei* epitopes, which are still lagging behind

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251 BPSL1050

252 The NMR structure of seroreactive antigen of unknown func-
253 tion BPSL1050 (PDB entry 2MPE) was used for the applica-
254 tion of MLCE and EDP methods, and the two designed epi-
255 tope peptides successfully induced antibodies with *B.*
256 *pseudomallei* agglutination activities that were superior to
257 those induced by antibodies raised against recombinant
258 BPSL1050 [45].

259 Future Therapeutic Applications for Discovered *B.* 260 *pseudomallei* Epitopes

261 *B. pseudomallei* epitope peptides may serve as vaccine com-
262 ponents (Fig. 1). Although, peptides are poorly immunogenic
263 per se, they can be easily conjugated to other chemically
264 diverse immunogens, e.g., individual proteins or carbohy-
265 drates, and their stability in plasma can be improved via di-
266 verse engineering/chemical modification strategies [46].
267 Examples of protein carriers include tetanus and diptheria tox-
268 oids, known per se to induce an immune response [47••, 48].
269 One means of improving their immunogenicity is to present
270 them in vessels that prime the immune response. One such
271 delivery vessel that is gaining attention in the field is outer
272 membrane vesicles (OMVs) derived from the pinching off of
273 portions of the outer membrane (OM) of Gram-negative bac-
274 teria. OMVs contain molecules that encounter the key players
275 of the host immune response, e.g., membrane and periplasmic
276 proteins and membrane polysaccharides, which may act as
277 adjuvants of the immune response. OMVs isolated from *B.*
278 *pseudomallei* 1026b have been shown to provide good pro-
279 tection in non-human primates, although they did not
280 completely neutralize the bacteria [49]. Decoration of OMVs

with *B. pseudomallei* antigens or epitopes has not yet been exploited; however, the availability of several *B. pseudomallei* epitopes, with both B and T cell stimulatory activities, and the intrinsic immunogenicity of *B. pseudomallei* OMVs suggest this to be a vital avenue to pursue in the immediate future.

Multiple-Epitope Presentation

A subunit melioidosis vaccine represents a safer alternative to a live-attenuated vaccine and efforts should focus on formulating a multivalent vaccine containing several immunogenic epitopes that lead to improved immune responses [50•]. In this context, once the sequence and 3D structure of epitopes are known, SV methods can be used to engineer multiple epitopes for presentation on a single protein scaffold. Structure-based antigen engineering, such as epitope grafting, can be used to transplant neutralizing epitopes from one antigen onto a structurally homologous region of a diverse protein scaffold [24–27]. When the protein scaffold itself is a full-length protein antigen, this can result in multiple-epitope presentation that can lead to accentuated immune responses. With regard to *B. pseudomallei*, in light of promising subunit protection studies with three chronic phase antigens and BPSL2765, combining T and B cell *B. pseudomallei* epitopes on the same scaffold could prove an ideal strategy to contemporarily induce both cell and antibody immune responses; however, no studies of this type have been reported to date.

Future Diagnostic Applications for Discovered *B. pseudomallei* Epitopes

Current *B. pseudomallei* diagnostic tests are based on lengthy bacterial culture procedures that exhibit poor sensitivity and specificity, leading to many melioidosis cases being unreported and to disease progression. Potential biomarkers include O-polysaccharide (OPS) and hemolysin co-regulated protein 1 (Hcp1) [51]. A recent report cites preliminary data on the use of a monoclonal antibody-based immunofluorescent assay (IFA) that recognizes a *B. pseudomallei* exopolysaccharide [52].

A more rapid method is the indirect hemagglutination assay (IHA) used to diagnose infections in Australia by measuring antibody titers to three lipopolysaccharide (LPS) types; however, its sensitivity is poor and is as low as 25% in North Eastern Thai populations, where complications are encountered due to high antibody titers in the local population, resulting from natural exposure to the non-pathogenic *B. thailandensis* species that co-habits with *B. pseudomallei* [53, 54].

The key to developing a serological-based test is the identification of specific biomarkers that do not lead to ambiguous diagnosis. In this context, the antigens identified in the protein microarray studies carried out by Felgner et al. and Suwannasaen et al. represent serodiagnostic antigens for further evaluation as biomarkers [18].

There are over 40 highly conserved species pertaining to the *Burkholderia* genus. Other members that are pathogenic include *B. mallei*, responsible for glanders in horses and other solipeds, and *B. cenocepacia*, which causes opportunistic infections in cystic fibrosis (CF) patients. Peptide-based immunodiagnostic tests are advantageous, as peptides are easy to produce and chemical modifications may be easily introduced to constrain peptide conformation, thus presenting peptides in microarrays in conformations that are optimally recognized by serum IgGs (Fig. 1).

The possibility of using *B. pseudomallei* synthetic peptide epitopes to diagnose *B. cenocepacia* infections in CF patients was confirmed by a recent study [55••]. Silica chips were used to present the synthetic epitope peptides from Pal, FliC, OppA, and BPSL1050 [55••]. All *B. pseudomallei* peptides were seroreactive against immune sera form CF patients harboring *B. cenocepacia* infections. Moreover, the sensitivity of this microarray was found to be excellent, and the same peptides were not recognized by IgGs from healthy controls or CF patients with different bacterial infections, such as *Pseudomonas aeruginosa* [55••]. For peptide-based immunodiagnostics, the extensive natural variability of *Burkholderia* species must be taken into consideration when selecting cross-reactive antigens. Genome sequence information for diverse *Burkholderia* species should be screened, and selected candidates should belong to the conserved core genome.

In a separate study, the effect of peptide conformational flexibility was evaluated relative to immune sera recognition and the elicitation of bactericidal antibodies. The more rigid α-helical conformation of Pal3, constrained by introducing a 1,4-disubstituted-1,2,3-triazole chemical staple could discriminate better between diverse melioidosis patient serotypes than the linear peptide [56•]. In contrast, the constrained peptide elicited a more limited repertoire of antibodies with reduced bactericidal properties in comparison to the linear epitope peptide, suggesting that peptide epitope design strategies should evaluate peptide conformation and dynamics, relative to its desired application [56•].

Conclusion

Based on the results, data, and recent developments that we review in this paper, we conclude that the full integration of computer-based approaches with structural biology is coming of age, and that the next few years will witness a huge increase in the use of designed biomolecular agents

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for immunological applications. The increasing availability of 3D *B. pseudomallei* antigen structures solved by X-ray crystallography or NMR, and the increasing accuracy of in silico homology models, together with computational-based epitope prediction tools raises the possibility of rapidly generating large libraries of immunoreactive peptides for immunological testing and hence, diagnostic and therapeutic potential. There are 11 structures of known seroreactive antigens (identified by Felgner et al. [33••]) deposited in the PDB; structural homologs with higher than 30% sequence identity to 18 *B. pseudomallei* antigens are available, together with two additional structure homologs for chronic phase antigens BPSL2287 and BPSL3369 tested in the most successful protection study carried out to date [17••]. We can also consider 3D structures pertaining to other *Burkholderia* species, such as *B. mallei* and *B. cenocepacia*. Such wealth of structural data can be valuable for designing improved immunogens when complemented with in vitro and in vivo immunological studies.

With regard to melioidosis diagnostics, initial findings render the notion of using a single peptide-based immunodiagnostic test to diagnose diverse *Burkholderia* infections a reality. Incorporating multiple epitopes from diverse bacteria raises the possibility of screening multiple bacterial infections in one shot.

SV applications to vaccine design and production have been so far limited, despite initial promising results reported for protein antigens from diverse pathogens such as the respiratory syncytial virus, *Neisseria meningitidis* serotype B, and *Haemophilus influenzae* [57]. There is no reason to suggest that a melioidosis vaccine may not be achieved using analogous approaches; however, we believe that a large multidisciplinary research effort is required; in vivo studies currently represent one of the missing pieces of the puzzle and are essential to understand whether any of the *B. pseudomallei* epitopes identified to date display effective protection in a vaccine formulation (Fig. 1).

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Papers of particular interest, published recently, have been highlighted as:
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