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Rickettsiosis: pathogenesis, inmunidad y desarrollo de vacunas

(Rickettsioses: pathogenesis, immunity, and vaccine development)

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Resumen

Varias especies dentro del género *Rickettsia* son altamente patogénicas; por ejemplo *R. rickettsii* (el agente de la fiebre manchada de las Montañas Rocosas) y *R. prowazekii* (el agente del tifus epidemémico). Muchas de las rickettsiosis son prevalentes a lo largo de América Latina; sin embargo, estas enfermedades son desatendidas porque rara vez son consideradas en el diagnóstico diferencial de enfermedades febriles en los trópicos. Esto se explica parcialmente por el hecho de que todas las infecciones causadas por *Rickettsia* son difíciles de diagnosticar, debido a la presentación clínica no-específica inicial, sospecha clínica ausente, y la falta de pruebas diagnósticas sensibles y específicas que se pueden utilizar durante la presentación aguda. Además, la confusión diagnóstica con infecciones virales es la regla, y esto es un problema crítico ya que estas infecciones pueden tratarse con antibióticos apropiados. Con esta revisión, esperamos contribuir al conocimiento y la conciencia de estas importantes enfermedades dentro de los profesionales científicos y de salud en América Latina.

Descriptores: *Rickettsia*, patogénesis, inmunidad, vacuna

Abstract

Several species within the genus *Rickettsia* are highly pathogenic; for example, *R. rickettsii* (the agent of Rocky Mountain spotted fever) and *R. prowazekii* (the agent of epidemic typhus). Many of the rickettsioses are prevalent throughout Latin America; however these diseases are neglected because they are seldom considered in the differential diagnosis of febrile diseases in the tropics. This is partly explained by the fact that all infections caused by *Rickettsia* are difficult to diagnose due to the initial non- specific clinical presentation, absent clinical suspicion, and the lack of sensitive and specific diagnostic tests that can be deployed during the acute presentation. Furthermore, diagnostic confusion with viral infections is the rule, and this is a critical problem because these infections can be treated with appropriate antibiotics. With this review, we expect to contribute to increase knowledge and awareness of these important diseases among scientists and health care professionals in Latin America.

Keywords: *Rickettsia*, pathogenesis, immunity, vaccine

Rickettsia are the etiologic agents of two of the most lethal infections known to man, Rocky Mountain spotted fever (*Rickettsia rickettsii*) and epidemic typhus (*R. prowazekii*). Moreover, epidemic typhus has shaped History due to the massive epidemics that it produced during times of war until World War I.[1] Both agents are select agents because of their potential use as bioweapons.[2] On the other hand, several new pathogenic *Rickettsia* have been discovered in the last few decades; new rickettsioses are certainly emerging and old rickettsioses are re-emerging.[3]

Members of the genus *Rickettsia* (family *Rickettsiaceae*, order *Rickettsiales*) are α-proteobacteria that share the following general characteristics: 1) they have closely related A/T-

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rich small genomes, a consequence of evolutionary loss of genes encoding proteins that participate in various biosynthetic pathways;[4-6] 2) they can only survive in the cytoplasm of eukaryotic cells where they obtain needed metabolic substrates that they cannot synthetize themselves (they are strict obligate intracellular parasites); 3) most of the well-known rickettsiae reside within arthropods. Indeed, hematophagous insects and ticks transmit rickettsiae that are pathogenic to humans and other vertebrates (they are zoonoses); 4) in humans, rickettsiae preferentially target endothelial cells, the cells that line vascular and lymphatic vessels (except for *Rickettsia akari*, the agent of rickettsialpox, which specially targets monocytes and macrophages).[7]

The transmission of rickettsia by hematophagous arthropod vectors was established early in the 20th century. In 1906 WW King[8] and HT Ricketts[9] described their experiments with guinea pigs in which they demonstrated that ticks transmit Rocky Mountain spotted fever (RMSF). At the time, Ricketts and others recognized that the clinical presentation of Rocky Mountain spotted fever closely resembled that of epidemic typhus; however, it was not yet known that closely related organisms caused the two diseases. What was clear then was that the human body louse was the vector of typhus.[10] Charles Nicolle received the 1928 Nobel Prize for this discovery.

In 1914, H. Plotz reported the identification of a gram-positive bacillus in the blood of patients with typhus as well as their lice.[11] H. da Rocha-Lima confirmed these findings in 1916;[12] he named the organism *Rickettsia prowazekii* in honor of Ricketts and Stanislaus von Prowazek, both of whom died of typhus acquired in the course of their investigations. In 1916, SB Wolbach studied samples from guinea pigs with Rocky Mountain spotted fever and identified very small gram-negative organisms in vascular vessels.[13,14] Subsequently, in 1917, he confirmed this finding as well as the vascular nature of the infection in autopsies of human patients with Rocky Mountain spotted fever.[15] The integration into a single genus, *Rickettsia*, would not be proposed until 1943.[16] By the late 1960s and early 1970s a more modern conception began to be synthetized.[17,18]

At the present moment, there are 22 entries for *Rickettsia* genomes in the database of NCBI. They are *R. rickettsii*, *R. prowazekii,*[19] *R. conorii,*[20] *R. typhi*, *R. massiliae*, *R. canadensis*, *R. slovaca*, *R. bellii*, *R. africae*, *R. sibirica*, *R. peacockii*, *R. akari*, *R. felis*, *R. montanensis*, *R. rhipicephali*, *R. australis*, *R. parkeri*, *R. philipii*, *R. japonica*, *R. heilongjiangensis*, *Candidatus Rickettsia amblyommii,* and *Rickettsia endosymbiont of Ixodes scapularis.* Based on the analysis of a subset of these data,[21,22] new phylogenetic relationships were proposed. Accordingly, there are four groups: 1) the non-pathogenic ancestral group (*R. bellii* and *R. canadensis*), which diverged earlier; 2) typhus group (*R. typhi* and *R. prowazekii*); 3) spotted fever group (*R. rickettsii*, *R. parkeri, R. conorii*, and several others); and 4) transitional group (*R. akari*, *R. australis,* and *R. felis*). A more recent analysis proposes to split the ancestral group in two with one *Rickettsia* in each group (i.e., *R. bellii* and *R. canadensis*) and to include the transitional group within the spotted fever group (SFG).[23] According to this new scheme, the SFG group is divided in four subgropus: 1) the *R. rickettsii* subgroup (*R. rickettsii*, *R. conorii*, *R. africae*, *R. parkeri*, *R. sibirica*, *R. slovaca, R. honei, R. japonica, R. heilongjiangensis*, and a few others); 2) *R. massiliae* subgroup (*R. massiliae* , *R. montanensis*, *R. aeschlimannii* and *R. rhipicephali*, *R. raoultii* and others); 3) *R. helvetica* subgroup (*R. helvetica, R. asiatica, R. tamurae, R. monacensis*); 4) *R. akari* subgroup (*R. akari, R. australis, and R. felis*). A phenotypic characteristic of the *R. rickettsii* subgroup is its susceptibility to rifampin, while the *R. massiliae* subgroup is resistant to this antibiotic.[24] For a long time, the serological response was the main criterion used to classify rickettsiae in only two groups,[25,26] spotted fever and typhus; using those criteria, *R. canadensis* was included in the typhus group at that time. Also, until 1995,[27] *Orientia tsutsugamushi*, the etiologic agent of scrub typhus, was included in the genus *Rickettsia* (i.e., *Rickettsia tsutsugamushi*) and considered a third group.

In temperate regions of the globe, the seasonality of SFG rickettsioses is explained by the activity of the tick vectors, particularly the adults, which are more active during the spring and early summer. There is also a periodicity in a timeframe of decades that has not been appropriately explained yet. It is possible that climate change may affect the behavior of tick vectors.[28] One of the recent peaks of reporting of Rocky Mountain spotted fever (RMSF) occurred during the early 2000s.[29] This may be related to increased disease activity but also to renewed interest not only in the United States but also throughout the Americas (RMSF occurs only in the Americas). The disease has now been documented in almost all countries of Latin America.[30-40] Even more importantly, new SFG rickettsioses have been discovered. For instance, *R. parkerii*, which was considered a non-pathogenic *Rickettsia* for a very long time, was recently shown to produce a mild spotted fever with an eschar and local lymphadenopathy.[41-44] Other recently described *Rickettsia* associated with eschars and relatively mild disease include *Rickettsia* 364D[45] and *R. massiliae.*[46,47]

One of the consequences of the non-specific initial febrile syndrome and the lack of commercially available diagnostic methods that are sensitive and specific during the acute presentation of the rickettsioses is that the disease is frequently underreported and diagnosed as a viral illness.[48] In Latin America, the umbrella diagnosis of dengue is frequently applied to cases of rickettsiosis.[49]

Pathogenesis

Rickettsioses are systemic febrile diseases that affect individuals of any age independently of their immune status.[48,50-53] Although the pathogenetic mechanisms are shared, not all rickettsioses are equally severe, which is explained by differences in virulence of the individual species and vector-related factors.

The entry of *Rickettsia* into host cells is an active process that requires energy from both the host and the rickettsiae.[54] There is evidence that rickettsiae use surface cell antigen 0 (sca0 or rOmpA)[55] and sca 1[56] to attach to target cells (these and the other rickettsial sca proteins are autotransporters). Subsequent to attachment, which is mostly a passive process, endocytosis of rickettsia is actively triggered when the rickettsial outer

membrane protein B (rOmpB or sca5) binds to the host cell membrane form of Ku70.[57] Since blocking of this interaction only inhibits about 50% of rickettsial entry, other ligands and receptors must be present; sca2[58] and adr2[59] appear to be some of those bacterial ligands.

The necessary cytoskeletal rearrangements that produce the zipper-like entry mechanism of *Rickettsia* spp. involve multiple host pathways that activate the Arp2/3 complex[60] with the participation of Cdc42, cofilin, c-Cbl, clathrin, and caveolin 2.[61] *Rickettsia* may also enter phagocytic cells such as monocytes and macrophages (which are a secondary target of most *Rickettsia*) by antibody-mediated opsonization.[62] Within a short period of time after endocytosis, rickettsia escapes into the cytosol. The rickettsial genes *pld*, which encodes an enzyme with phospholipase D activity,[63] and *tlyc*, which encodes a hemolysin[64] are believed to be effectors of this function. This conclusion is based on the ability of the normally vacuolar *Salmonella enterica* to escape into the cytosol when it expresses rickettsial *tlyc* or *pld*.[65] In addition, rickettsial proteins with phospholipase A activity were confirmed[66, 67] but only in the typhus group *Rickettsia*. That activity underlies the phenomenon of hemolysis produced by these rickettsiae in vitro.[68,69]

Once *Rickettsia* escapes the phagocytic vacuole, it acquires multiple metabolic substrates from the host cytoplasm. The availability of those substrates allowed genome reduction through loss of many genes including, among many others, those for nucleotide synthesis and enzymes for sugar metabolism.[70] Multiple transporters of substrates from the host cytoplasm, including ATP,[71] compensated for these gene losses.[72] The mechanisms of transport are active and include the use of the transmembrane electrical potential.[73]

Typhus group *Rickettsia* grow until they burst the host cell[74] while spotted fever group *Rickettsia* rapidly spread from cell to cell[75] due to their actin propulsion. Of course, host cells are damaged in the process;[76] the mechanisms may involve the production of free radicals[77,78] and phospholipase activity.[79] On the other hand, there is experimental evidence that rickettsiae can maintain their cellular niche through inhibition of apoptosis,[80] and that pathogenic *Rickettsia* can inhibit autophagy.[81]

The main target cells of most *Rickettsia*, with the exception of *R. akari* are endothelial cells, the cells that line all vascular vessels in the body. These cells have important regulatory functions in angiogenesis, hemostasis, permeability and solute exchange, vascular tone, and inflammation.[82-84] Thus, their targeting by rickettsiae explains many of the clinical features of the diseases including systemic involvement and leakage of intravascular fluid. Rickettsial infection of endothelial cells induces cellular damage leading to detachment. Those infected endothelial cells circulate in the blood[85,86] and are likely to be the source of new foci of infection once they lodge in distal capillaries.

Several mechanisms are likely to contribute to the increased vascular permeability observed in clinical cases. They include production of vasoactive prostaglandins as a consequence of increased expression of COX-2,[87] endothelial production of nitric oxide,[88] effects of inflammatory cells and their mediators,[89] and endothelial detachment and denudation of vessels. Such damage may be caused by phospholipase activity,[79] mechanical damage to the membrane caused by exiting rickettsiae under actin propulsion,[90] or lipid peroxidation of the cell membrane.[76,77,91,92] The most severe clinical presentations are a consequence of endothelial damage in the lungs and brain and include noncardiogenic pulmonary edema, interstitial pneumonia, adult respiratory distress syndrome, meningoencephalitis, seizures, and coma;[93-97] involvement of these organs explains the majority of the mortality, which is observed particularly with Rocky Mountain spotted fever and epidemic typhus (the reported mortality without antibiotics ranges from 10 to 60%). However, it should be emphasized that reliance on serological methods for diagnostic confirmation may lead to underestimation the actual case-fatality rate. This was well illustrated in a recent report of nine fatal cases with negative serological results that were confirmed by immunohistochemical demonstration of the antigen in tissues.[51] At the other end of the clinical spectrum are several rickettsioses; murine typhus, with a mortality of less than 2%, is the most important of them because of its global distribution.[98]

Although multiple coagulation abnormalities have been described during the course of clinical and experimental rickettsiosis,[99] disseminated intravascular coagulation occurs only rarely in lethal cases and is not a common feature of rickettsiosis.[100]

The cells that are infected immediately after inoculation have not been identified. Many of the rickettsiae that result in less severe disease also produce an eschar (area of necrosis with a rich inflammatory infiltrate and local rickettsial proliferation) at the bite site.[101] When an eschar is present, another frequent clinical finding is local lymphadenitis, suggesting initial spread through lymphatics. Rocky Mountain spotted fever, the most severe of the spotted fever rickettsioses, does not manifest with an eschar or local lymphadenitis. This could be due to a more rapid hematogenous dissemination.

The recommended antibiotic treatment for all rickettsioses is doxycycline.[102] This antibiotic has the advantage of covering other tick-borne bacterial infections. Rickettsiae are resistant to many antibiotics.[103] Other antibiotics, including chloramphenicol and fluoroquinolones may be effective, although there is evidence that they may have deleterious effects.[104,105] The antibiotic resistance of *Rickettsia* combined with the non-specific initial clinical presentation and lack of commercially available laboratory tests for confirmatory diagnosis during the acute presentation, lead to delayed diagnosis and inappropriate treatment; the consequence is excessive mortality.[104]

Rickettsial virulence

Many rickettsial genes have been predicted to participate in virulence based on bioinformatics analyzes;[72] several toxin-antitoxin systems are examples. One of them, encoded by the *vapB/C* genes was shown to be functional; *E. coli* transformed with rickettsial *vapC* significantly decrease their growth, while

VapB formed a complex with VapC to inhibit its RNase activity.[106] More importantly, microinjection of VapC to mammalian cells induced apoptotic death.

A large number of intracellular bacteria use type IV secretion systems to inject proteins into the host in order to produce a favorable niche. Interestingly, genomic analysis showed that multiple genes with the potential to encode a reduced type IV secretion system are conserved in *Rickettsia*.[107] Whether the system is actually functional or not remains to be tested.

The phospholipase D encoded by the gene *pld*, a likely mediator of phagosomal escape, is a virulence factor as suggested by the milder disease produced in guinea pigs infected with *R. prowazekii* with a mutated *pld*.[108] This study used homologous recombination for targeted knockout of a rickettsial gene. Previous studies using the difficult techniques of genetic manipulation of *Rickettsia*, including transposon-mediated mutagenesis, indicated that mutation of the open reading frames (ORFs) 243, 294, and 689 of *R. prowazekii* do not produce an observable phenotypic difference.[109] Thus, these genes may be non-essential genes (at least for growth in mouse cell line *in vitro*). Also, *R. rickettsii* mutants lacking expression of sca2, which participates in actin polymerization, do not cause apparent illness in guinea pigs.[110]

Loss of regulation due to genome decay has also been proposed as a mechanism of increased virulence;[111] however, this argument does not explain why *R. rickettsii* and *R. prowazekii* are almost equally pathogenic and the radical difference in virulence between the two typhus group rickettsiae, *R typhi* and *R. prowazekii*.

In the absence of genetic approaches that work well and consistently for *Rickettsia*, other methods have been introduced to identify virulence factors. One example is the comparison of the genomes of closely related *Rickettsia* with different pathogenicity. The *Dermacentor andersoni* endosymbiont *R. peacockii* was compared to virulent *R. rickettsii*; it was found that it had a plasmid, multiple transposons with intact transposase sequences, and many deletions, nonsense mutations, and split genes.[112] The authors proposed that some of the absent or mutated genes in *R. peacockii* might explain the lack of pathogenicity. Those genes include *DsbA* (a catalyzer of disulfide bond formation), *RickA*, *Sca0, Sca1*, a gene encoding Protease II, and a gene encoding a putative phosphoethanolamine transferase that could play a role in the formation of the prominent slime layer found in the pathogenic spotted fever-group rickettsiae. Interestingly, the hypothetical protein A1G_05165 of a virulent strain of *R. rickettsii* (strain Sheila Smith) is deleted in *R. peacockii* and it is also not present in other non-pathogenic rickettsiae. This hypothetical protein has ankyrin repeats; similar proteins in other members of this order (i.e., *Anaplasma*) appear to play a role in virulence through binding of host DNA and altered host gene regulation. A1G_05165 is also mutated in a non-pathogenic strain of *R. rickettsii* (strain Iowa). In addition, the genomic study that compared the pathogenic strains R and Sheila Smith with strain Iowa also found 23 deletions within predicted ORFs of *R. rickettsii* Sheila Smith and 24 deletions within predicted ORFs of *R. rickettsii* Iowa.[113] One of the genes deleted in *R. rickettsii* Iowa is the adhesin rOmpA (sca0). Also, *rompB* has four single nucleotide polymorphisms (SNPs) that may explain the defective processing of this important membrane protein in strain Iowa.[114] Finally, it should be emphasized that there is a good opportunity to understand virulence by comparing the genomes, transcriptomes, and proteomes of the two typhus group *Rickettsia* since they have very closely related genomes but very different virulence in humans, with *R. prowazekii* producing a much more severe infection (epidemic typhus) than *R. typhi* (murine or endemic typhus).

Another system to study the physiology of *Rickettsia* in the absence of more efficient genetic systems is the use of *E. coli*-based assays. For example, to identify proteins transported out of the rickettsial cytoplasm, bioinformatic tools were used to uncover predicted secreted proteins (based on the presence of N-terminal signal peptides). The signal peptides of those proteins from *R. typhi* were then fused to the *E. coli* alkaline phosphatase *phoA* gene (lacking an intrinsic signal peptide sequence) to test if those signal peptides provided information to translocate PhoA into the periplasm of *E. coli*.[115] Eighty-four functional signal peptides were identified suggesting that those rickettsial proteins might be secreted using the rickettsial Sec system. Those proteins include sca1-3, sca5, Pld, and proteins that are believed to be part of a type IV secretion system.

Immunity and vaccines

An often overlooked but critical factor in the pathogenesis of rickettsial diseases is the transmission by arthropod vectors because their saliva is not a passive vehicle for transmission.[116-118] In fact, the tick saliva modifies the host environment in order to successfully complete the blood feeding, which occurs during extended periods (several days for nymph and adult ticks). Proteins in the tick saliva modulate host hemostasis, innate and adaptive immuntiy, complement activation,[119] angiogenesis, and extracellular matrix regulation.[120,121] Evidently, all of those factors could determine the final outcome of the infection. Furthermore, tick saliva can modulate the physiology of endothelial cells, the main target cells of *Rickettsia*. For example, salivary gland extracts from *D. andersoni* reduce the upregulation of ICAM-1 induced by TNF-α on a mouse endothelial cell line.[122] This change could contribute to reduce the migration of leukocytes into tick bite sites.

Endothelial cells are not passive actors in the anti-rickettsial immune response. Upon rickettsial infection, the transcription factor NFκB (a critical stimulating factor of the immune system) becomes activated in endothelial cells.[123-125]Other critical signaling mediators become activated as well. They include STAT1, STAT3,[126] and p38 MAPK.[127-129] As a consequence of the activation of these various signaling systems, endothelial cells respond by expressing a variety of chemokines,[130,131] cytokines such as IL-1 α, and IL-6,[132,133] adhesion molecules such as E-selectin, VCAM-1, ICAM-1,[134-136] and $\alpha V\beta 3$ integrin,[137] and secretion of prostanoids.[87,138]

NK cells are early producers of IFN-γ after infection with *Rickettsia*.[139,140] This cytokine is important because, together with TNF-α, it activates the bactericidal functions of the

endothelium.[141,142] Those functions are performed in part through expression of indoleamine-2,3-dioxygenase (IDO), which leads to tryptophan starvation.[143] Animal studies have demonstrated the importance of a T helper 1 (Th1) response in effective immunity against rickettsiae[144] with a particularly important role for $CD8^+$ T cells.[145,146] In fact, T cells are sufficient to mediate protection against a lethal rickettsial challenge, even in the context of a heterologous challenge where anti-typhus group T cells protect against a lethal challenge with SFG *Rickettsia* and vice versa.[147]

Despite the fact that rickettsiae are intracellular parasites and that cellular adaptive immunity is critical during a primary infection, there is clear evidence that the humoral immune response is very important in preventing the development of disease during secondary infections or after a lethal challenge following passive serum transfer. In fact, it was Ricketts himself who demonstrated this fact.[148] The anti-rickettsial humoral immune response is cross-reactive within rickettsiae of the same group but not across groups (e.g., between typhus and SFG groups).[149,150] The most abundant surface protein of *Rickettsia* is rOmpB (Sca5), which is an autotransporter. It is an immunodominant protein and antibodies against it are protective.[151]

Inactivated vaccines for *R. rickettsii* and *R. prowazekii* were produced early from a variety of sources including their vectors but they were very reactogenic and protection was incomplete. Later on, inactivated vaccines were produced from *Rickettsia* cultivated in eggs but antigenicity was variable and protection was poor.[152-155] In the 1950s a very effective vaccine for epidemic typhus was produced. It was an attenuated strain denominated Madrid E;[156] however, spontaneous reversion to a virulent phenotype precluded further development and testing.[157,158]. We now know that the attenuation is explained, at least in part, by a point mutation in the gene encoding a S-adenosulmethionine-dependent methyltransferase.[159] Given the nature of the mutation, it is not surprising that reversion was not an uncommon occurrence. Deletion of the entire gene would permit the production of a safer vaccine. Alternatively, strains with multiple genetic differences could prove to be safe vaccines. In this regard, it is interesting to note that the strain Iowa of *R. rickettsii*, which is attenuated and has multiple genetic differences when compared with virulent strains, can protect guinea pigs against a challenge with virulent *R. rickettsii*.[113]

Other recent efforts have focused on the production of a subunit vaccine. Fragments of rickettsial proteins that may trigger protective immunity were tested. They included rOmpA[160,161] and rOmpB[162-164] and results were encouraging; however, these approaches are limited and biased because of their focus on proteins that elicit a strong humoral response. A major effort for identification of immunogenic antigens is clearly needed, and the antigen discovery effort will need new tools to identify relevant conserved antigens recognized by T cells.

It will be possible to produce vaccines that cover more than one species of *Rickettsia* given the evidence of cross-protective immunity within the typhus or spotted fever groups[165-171] or even across groups.[147] The production of an effective anti-*Rickettsia* vaccine is a public health priority for several reasons. Firstly, some rickettsioses are highly lethal not only to humans but also to companion animals (i.e., dogs). Secondly, clinical diagnosis of rickettsioses is very difficult due to the non-specific initial clinical presentation. Thirdly, there are no commercially available diagnostic tests that can be used during the acute stage when antibiotic intervention is helpful.

The contemporary development of a vaccine has two initial essential aspects, namely identification of the relevant antigens and definition of immunological correlates of protection to guide the selection of vehicles, vectors, schedules, and adjuvants. In the case of infections caused by *Rickettsia*, due to the availability of excellent murine models, relevant correlates of protective immunity can be derived from the characterization of experimental infections because animals (as well as humans) that survive the infection become solidly immune to reinfection.

In regard to immunological correlates of protection, the magnitude of a response assessed by a single parameter (e.g., IFN-γ for intracellular pathogens, as frequently reported), is not enough. Now we know that there is functional heterogeneity of the T cell effector responses (including cytokine secretion, cytolytic activity, and development of various memory phenotypes) and that there are particular subsets of T cells, which express unique combinations of effector functions, that are more protective.[172-174] We probably should approach the definition of correlates of protective immunity in a way that parallels the complexity of physiological immunity, which is a multifaceted and integrated response that includes many different cells, receptors, ligands, and signaling modules that function in a combinatorial mode. For infections in which cellular immunity plays a predominant role, there is evidence from experimental models that multifunctional T cells are the best correlate of protection described thus far. More importantly, this has been demonstrated in humans as well.[175-177]

The technologies for understanding the integrated functioning of the immune system are now available and accessible. Those are the tools of Systems Biology, the "omics" methods and bioinformatics tools that permit the analysis of complex interactions in biological systems through the investigation of massively parallel data acquired from each experimental condition.[178] The application of Systems Biology to vaccinology is already identifying transcriptional signatures of protective immune responses that include sub-signatures of appropriate innate and adaptive responses.[179] Moreover, early predictive signatures of appropriate adaptive immune responses immediately after vaccination have been defined and verified using the Yellow fever (17D) vaccine as a model.[180] It is expected that such knowledge will provide paradigms for the development of novel vaccines for which limited data from humans is currently available. That is certainly the case for infections caused by *Rickettsia* because it is unlikely that we will be able to collect sufficient human samples from clinical cases with diverse outcomes in order to define broad signatures of

protective immunity. A promising solution to this problem is to use our current understanding of well-known effective immune responses as guiding principles. The study of the response to two of the most successful human vaccines in history, the yellow fever vaccine[177,181] and the smallpox vaccine,[176] is likely to yield relevant paradigms that we could use as guiding posts in rickettsiology.

From the perspective of antigen identification for vaccine development, until recently it was almost exclusively biased towards the humoral immune response. This bias was partly due to the effectiveness of antibodies in protection against almost all of the currently approved vaccines for human use, the relative technical simplicity of working with serum and antibodies, and the methodical challenges of working with T-cells. Presently, the barriers to identify potent vaccine antigens recognized by T-cells need to be addressed because most of the vaccines that remain to be produced require a strong T-cell component to afford significant protection. In particular, there is an urgent need to develop appropriate techniques to identify antigens recognized by T-lymphocytes because antigen discovery is the most important aspect of any vaccine development project; without appropriate antigens, a vaccine is unlikely to succeed.

Given the evidence that $CD4^+$T cells and $CD8^+$T cells target different antigens,[182] it is clear that antibody-based screening methods are not suitable to identify antigens recognized by $CD4^+$T cells or, particularly, $CD8^+$T cells. Several approaches to more directly identify antigens recognized by T-cells have been used; many of them rely on Reverse Vaccinology, a branch of Systems Biology that analyses entire microbial genomes to predict immunogenic proteins based on predefined rules derived from the analysis of large empirical datasets.[183] On the other hand, the predicting power of those immunoinformatic strategies has not been thoroughly tested by direct experimentation. Moreover, at least for bacterial proteins, known protective antigens actually have less predicted epitopes than randomly selected bacterial protein sets used as a control.[184]

Empirical methods for identification of antigens recognized by T-lymphocytes rely on T-cells from animals or individuals that are immune to the pathogen. Those memory T-cells had been selected during the physiological immune response to persist and recognize a limited number of antigens (i.e., immunodominant antigens). Thus, methods that use memory T-cells for antigen identification are more likely to miss potentially protective subdominant antigens. One strategy for T-cell antigen identification that is not biased towards immunodominant antigens is genomic immunization or Expression Library Immunization (ELI).[185] In this technique, pools of eukaryotic expression vectors with cloned pathogen genes are used to directly immunize animals. The animals are then challenged with lethal doses of the microbial pathogen. The gene pools that trigger protection are subsequently deconvoluted by testing each component of the pool one at a time. This method allows the priming of naïve T-cells by the expressed cloned microbial genes regardless of whether they are subdominant or dominant during a natural infection as long as the appropriate T-cell receptors are present. Although ELI has been successfully used,[186] it has its own problems as it relies on a DNA immunization strategy; thus, antigen expression is not guaranteed in all cases. Accordingly, it is not possible to know which pathogen genes were not screened validly; a negative response can be due to lack of an immunological response or to failed expression of the microbial gene.

As an alternative, we produced a new *in vivo* screening platform; the idea is to easily produce antigen presenting cells (APCs) expressing individual open reading frames (ORFs) from any sequenced *Rickettsia* and use them for immunization of naive mice. Immunization with pooled APCs containing 4 to 5 rickettsial ORFs is followed by challenge with live virulent pathogen and measurement of an indicator of protection such as decreased bacterial load. Once protective pools are identified, each member of the pool is tested individually to identify ORF(s) responsible for a protective immune response. With this platform, one can easily test for cross-protective responses by immunizing with the ORFs of one species of *Rickettsia* and challenging with another. Importantly, the proposed methodology is not biased by immunodominance because T cells from immune animals are not used to select antigens. This aspect is potentially important for vaccine development because subdominant or cryptic antigens have been shown to elicit protective immune responses in other systems.[187-189] The ability of our platform to discover relevant antigens for vaccine development independently of their ranking in the natural hierarchy of immunodominance dramatically expands the universe of possible antigens; thus, this platform offers a possible solution to the identification of protective antigens that are conserved among different strains of a microbe or even different species within a genus.

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