Spotlight

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Quantitative sequencing confirms VSG diversity as central to immune evasion by *Trypanosoma brucei*

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Antigenic variation is central to the virulence of African trypanosomes, where the VSG coat is used to evade the host immune system. Recent advances in technology have now allowed more secrets of this system to emerge, with the surprising insight that a broad repertoire of VSGs is rapidly expressed. This has major implications for how the parasite must evade the host immune response.

Changes to expression of the trypanosome variant surface glycoprotein (VSG), first characterised by Cross, have represented a paradigm for antigenic variation, a process of immune evasion that is exploited by many eukaryotic pathogens, bacteria and viruses [1]. At its heart, antigenic variation relies on sequential expression of surface protein variants that are central to the interaction between the host and parasite. Sequential switching between variants is essential for chronic infection due to pathogen elimination by the host immune response following recognition of expressed surface antigens, meaning that new variants must be continually expressed through the course of infection. To facilitate switching, a pathogen retains a repertoire of antigenically distinct surface protein genes, or can generate these by mutation or recombination on-the-fly, meaning that the host acquired immune system is continually challenged by novel epitopes. So successful is this strategy that African trypanosomes, *Trypanosoma brucei* and its relatives, are able, at the population level to withstand antibody-mediated onslaught for months or years. Despite recent advances in combating human trypanosomiasis disease, antigenic variation in *T. brucei, T. vivax* and relatives means that significant impact on agriculture remains across Africa, Asia and the Americas, with a huge global burden.

We have known for decades that trypanosome antigenic variation is fuelled by a large repertoire of VSG genes, more recently counted at around 2000 and shown to be largely subtelomeric and in rapid evolution [2]. The scale of the *VSG* repertoire and its distribution in the genome means the complexity of antigen switching strategies used by trypanosomes remains unsurpassed. VSG expression in the mammal is from one of ~15 subtelomeric VSG expression sites (ES), and switching can occur by coupled transcription activation and silencing between them [3]. However, most switching involves recombination, which feeds new *VSG* sequence information into the ES, perhaps promoted by DNA double strand breaks [4]. Significantly, many *VSG*s are present as partial or impaired gene copies, and only become useful if recombined to construct a complete, often novel *VSG* ORF.

While we have long known that antigen diversity is central to antigenic variation [5], the extent of VSG diversity has only recently begun to be evaluated in the course of infections. The first, comprehensive attempt to assess VSG diversity was conducted by Hall *et al.* [6], building upon work by Marcello and Barry [2], which captured expressed VSG mRNAs by specific RT-PCR and cloning. More recently, Mugnier and colleagues [7] have described 'VSGseq', which relies on the same VSG-specific RT-PCR but employs Illumina sequencing to define the VSG sequences being expressed. The avoidance of cloning removes a significant experimental bottleneck and should provide greater depth and quantitation of VSG diversity. Gratifyingly, VSGseq confirms many predictions made by Hall *et al.* for high levels of VSG diversity throughout a *T. brucei* infection: where Hall *et al.* characterised ~800 VSGs from 11 mice over ~30 days of infection with one *T. brucei* strain, Mugnier *et al.* recovered ~300 VSGs from four mice infections lasting 30–100 days with a distinct parasite strain. The importance of diversity is reinforced by the common observation of many VSG expressing variants in each parasite wave (Figure 1A); indeed, the average number of variants estimated by Hall *et al.* (~20/peak) compares remarkably well with that seen by Mugnier *et al.* (~28/peak). In both cases, it is likely that the measured levels of VSG diversity are an underestimate, and predictions by Hall *et al.* that the true number of VSG variants per peak may be close to 100 look close to the highest number detected by Mugnier *et al.* (83/peak), reflecting the greater depth of sampling made possible by Illumina sequencing. Within this diversity, both studies observed many VSGs that were common between the separate mice infections. The major implication is that a much larger proportion of the VSG repertoire is exposed during an infection than was appreciated and continuous generation of antigen diversity is the main output of trypanosome VSG switching, like antigenic variation in other pathogens.

Further questions are raised and addressed by these two studies. First, how much of the *VSG* gene repertoire is used? Though the number of VSGs captured is large, in each infection examined this was significantly smaller

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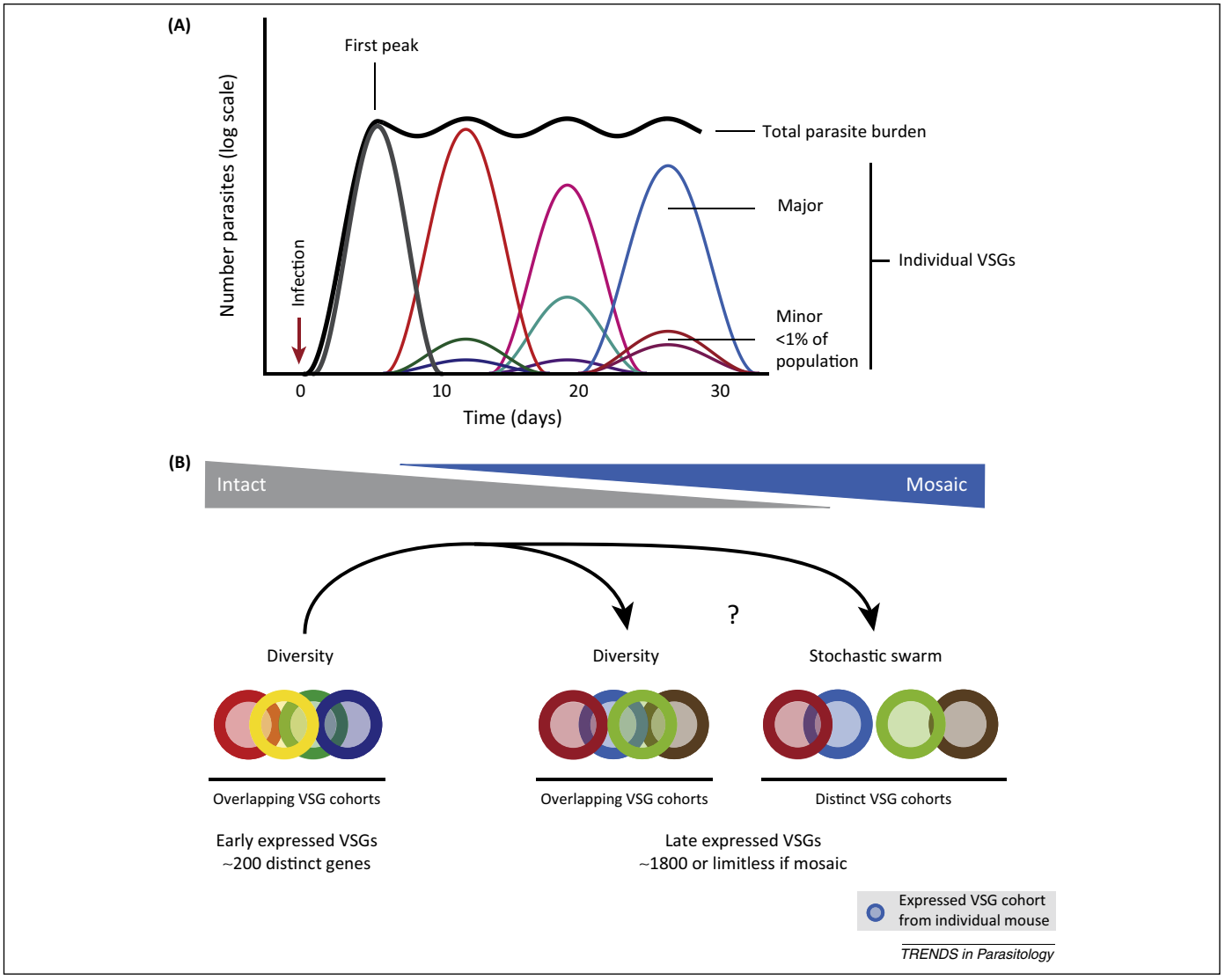

Figure 1. Patterns and hierarchy of variant surface glycoprotein (VSG) expression. **(A)** A small number of VSG variants ('major') can dominant parasite peaks within the infecting population, but the total number of variants expressed at any one time is considerably greater: Hall *et al.* and Mugnier *et al.* indicate that this can surpass 80 VSGs for an individual wave. The first peak is frequently dominated by the VSG expressed at the point of infection (red arrow) and is shown in grey. The overall parasitaemia does not vary greatly, and there may be many VSGs that are present in low abundance ('minor'). **(B)** Each circle represents a VSG repertoire for an individual animal, and which is common to a differential extent between individuals. Throughout infection many VSGs are expressed. Intact *VSG* genes that directly encoding a functional VSG coat, are expressed early in infections (bright colors) and represent ~10% or more of the repertoire of 2000 *VSG* genes. Later in infection the formation of mosaic VSGs likely predominates (duller colors), where multiple *VSG* genes and pseudogenes recombine by segmental gene conversion. The number of VSG coats that are generated in the later phase of the infection may be significantly greater than the ~2000 *VSG* genes available, and it is possible that host-specific cohorts are selected for, a 'stochastic swarm', as opposed to a more ordered progression where the VSG cohorts are similar between individuals.

than oft-cited 2000 *VSG*s in the genome. Does this indicate than that the usable VSG repertoire is smaller than the gene number? This appears unlikely, as both studies stress that sampling and sequencing strategies likely underestimate the true level of VSG diversity. Indeed, it is possible that diversity is distinct in larger hosts, such as cattle. More importantly, antigenic variation and the VSG repertoire does not merely contribute towards extending the length of infection in a single host but also towards host immunity at a population level, and facilitating superinfection. One significant challenge to understanding VSG expression potential is cross reactivity. Many *VSG*s are part of families [2], which likely also decreases the effective number of antigenically distinct VSGs. More work is needed in this regard, but Hall *et al.* have demonstrated that VSGs closely related at the sequence level and generated late in infections can retain antigenic distinctness [6].

A second question is, how is the *VSG* gene repertoire used? Both studies agree that, for the most part, expressed VSGs are distinct between early and late infections, consistent with the suggestion that the VSG repertoire is activated in a semi-predictable hierarchy (Figure 1B) [8]. A key feature of this hierarchy is that generation of novel, 'mosaic' *VSG* genes predominates late in infections, after the more readily activated functional *VSG*s have been utilised. Addressing this was problematic for Mugnier *et al.*, due to lack of an annotated *VSG* gene repertoire in the *T. brucei* strain used, but Hall *et al.* make it clear that VSG mosaics predominate in long-term infections. The route by which mosaic *VSG* genes are formed, segmental gene conversion, allows the parasite to activate

VSG pseudogenes. Though we have limited knowledge of the recombination mechanisms or where mosaic *VSG* assembly occurs (in the active ES?), segmental gene conversion is the sole strategy used to generate surface antigen diversity during immune evasion for many pathogens, such as *Anaplasma* and *Borrelia* [5]. The prolific use of segmental gene conversion by *T. brucei* reflects its long-predicted importance.

The greater quantitation provided by next generation VSGseq revealed that many VSG variants arise but never reach significant levels in the population (<1.0%). Moreover, there is also heterogeneity within VSG persistence, with some variants surviving for two weeks and others decaying over a more canonical eight day period of immune recognition, but all are clearly eliminated efficiently by the immune system.

The data that have emerged from the complementary studies by Hall *et al.* and Mugnier *et al.* are intriguing, and appear consistent with modelling predictions and experimental data suggesting that dynamics of *T. brucei* infections are heavily influenced by differentiation of replicative 'long slender' parasites to non-replicative 'short-stumpy forms' [9] Whether this means some VSG variants do not elicit an antibody response, and how the host immune response intersects with parasite switching and differentiation are important questions for the future. For instance, reports that stumpy stages can constitute a significant proportion of parasite populations [10] and which probably do not switch VSG, may have a distinct contribution to sampling by the immune system. It also remains to be understood if the expressed VSG diversity levels and patterns in large and/or trypanotolerant hosts follow a similar pattern to these small animal models.

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