Single-cell RNA sequencing of Trypanosoma brucei from tsetse salivary glands unveils metacyclogenesis and identifies potential transmission blocking antigens

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Edited by Anthony A. James, University of California, Irvine, CA, and approved December 20, 2019 (received for review August 29, 2019)

Tsetse-transmitted African trypanosomes must develop into mammalian-infectious metacyclic cells in the fly’s salivary glands (SGs) before transmission to a new host. The molecular mechanisms that underlie this developmental process, known as metacyclogenesis, are poorly understood. Blocking the few metacyclic parasites deposited in saliva from further development in the mammal could prevent disease. To obtain an in-depth perspective of metacyclogenesis, we performed single-cell RNA sequencing (scRNA-seq) from a pool of 2,045 parasites collected from infected tsetse SGs. Our data revealed three major cell clusters that represent the epimastigote, and pre- and mature metacyclic trypanosome developmental stages. Individual cell level data also confirm that the metacyclic pool is diverse, and that each parasite expresses only one of the unique metacyclic variant surface glycoprotein (mVSG) coat protein transcripts identified. Further clustering of cells revealed a dynamic transcriptomic and metabolic landscape reflective of a developmental program leading to infectious metacyclic forms preadapted to survive in the mammalian host environment. We describe the expression profile of proteins that regulate gene expression and that potentially play a role in metacyclogenesis. We also report on a family of nonvariant surface proteins (Fam10) and demonstrate sequential expression of antigenically unique VSG coat proteins, a process known as antigenic variation, enables trypanosomes to evade the vertebrate immune response and sustain an infection. Following ingestion by tsetse, the replicative bloodstream form of the parasites, known as slender cells, are lysed while insect-adapted and cell cycle-arrested stumpy cells differentiate to procyclic forms and acquire an invariant surface coat made up of procyclin proteins (6). To facilitate parasite midgut colonization, VSGs released into the midgut lumen by slender forms are taken up by tsetse’s cardia (also called proventriculus), where they transiently interfere with the production of a structurally robust peritrophic matrix (PM) midgut barrier (7). Following midgut colonization, procyclic parasites migrate to the cardia and foregut where they transform to long- and short-epimastigote forms (8). The short epimastigotes acquire yet another surface coat made up of brucei alanine-rich proteins (BARPs), colonize the SGs (9), and give rise to epimastigotes that undergo asymmetric division to give rise to metacyclic cells (10). The metacyclic

Significance

African trypanosomes, Trypanosoma brucei spp., are transmitted by the bite of infected tsetse flies. Mammalian vaccines are not available, and diagnosis and treatment remain difficult in the affected remote areas. The transcriptomic analysis of individual parasites from infected tsetse salivary glands provides insight into the developmental processes that give rise to infective metacyclic parasites transmitted to the host bite site. We describe proteins associated with the different parasite developmental stages in salivary glands and specifically highlight a family of nonvariant surface proteins associated with metacyclic parasites. Immunization with one member of this family reduced parasitemia early in the infection in mice, promising to be a potential candidate antigen for a transmission blocking vaccine approach.

Many parasites of medical and agricultural significance rely on insect vectors for transmission. Among these are Trypanosoma brucei spp., the causative agent of trypanosomiasis across sub-Saharan Africa, which are transmitted to their mammal hosts via the saliva of tsetse flies during blood feeding (1–4). Over the course of their life cycle, the parasites undergo multiple developmental stages, reflective of changes that allow them to adapt and survive in the different environments they encounter in their vertebrate host and invertebrate vector. For trypanosomes, these changes include nutrient-specific metabolic fluctuations, structural modifications related to the cellular localization of the kinetoplast and nucleus structures, and the expression of unique glycosylphosphatidylinositol (GPI)-anchored surface coat proteins.

It has not been possible to develop effective mammalian vaccines to prevent trypanosomiasis. This is largely because, in the mammal, the parasites are covered with the predominant surface coat proteins, variant surface glycoprotein (VSG). The continuous turnover of the VSG coat, in addition to the sequential expression of antigenically unique VSG coat proteins, a process known as antigenic variation, enables trypanosomes to evade the vertebrate immune response and sustain an infection (5). Following ingestion by tsetse, the replicative bloodstream form of the parasites, known as slender cells, are lysed while insect-adapted and cell cycle-arrested stumpy cells differentiate to procyclic forms and acquire an invariant surface coat made up of procyclin proteins (6). To facilitate parasite midgut colonization, VSGs released into the midgut lumen by slender forms are taken up by tsetse’s cardia (also called proventriculus), where they transiently interfere with the production of a structurally robust peritrophic matrix (PM) midgut barrier (7). Following midgut colonization, procyclic parasites migrate to the cardia and foregut where they transform to long- and short-epimastigote forms (8). The short epimastigotes acquire yet another surface coat made up of brucei alanine-rich proteins (BARPs), colonize the SGs (9), and give rise to epimastigotes that undergo asymmetric division to give rise to metacyclic cells (10). The metacyclic

Author contributions: A.V., B.L.W., and S.A. designed research; A.V., M.B.O., B.L.W., A.F.S., O.C.C., S.K., J.G.V., and S.A. performed research; A.V., M.B.O., A.F.S., O.C.C., S.K., J.G.V., and S.A. contributed new reagents/analytic tools; A.V., M.B.O., B.L.W., S.K., J.G.V., and S.A. analyzed data; and A.V., M.B.O., B.L.W., S.K., J.G.V., and S.A. wrote the paper.

Competing interest statement: The authors declare that a Provisional Patent Application describing the contents of this manuscript has been filed, Yale ref: O97835 || Saal ref: 047162-7268P1(10042).

This article is a PNAS Direct Submission.

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Data deposition: Sequencing read files have been deposited in the NCBI BioProject database (ID PRJNA562208).

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1914423117/-/DCSupplemental.

First published January 21, 2020.
cells acquire a different coat selected from ~20 to 30 VSGs, termed metacyclic VSG (mVSG) (11, 12). The acquisition of the mVSG coat is accompanied by morphological changes, including rounding up of the posterior end, elongation of the flagellum, and repositioning of the kinetoplast to the posterior end (10, 13). The metacyclic forms are quiescent, nondividing, and arrested in G1/G0 (14). Finally, an antigenically heterogeneous population of mammalian infective-metacyclic trypanosomes, with each individual cell expressing a single mVSG, are released into the SG lumen (15–17) and deposited at the bite site via the saliva of blood-feeding tsetse flies.

While extensive knowledge on the interactions between bloodstream-form parasites and their mammalian host exists, information on the in vivo tsetse-specific trypanosome stages is sparse. High-throughput RNA sequencing (RNA-seq) analysis from the midgut, cardia, and SG tissues of parasitized tsetse flies helped profile T. b. brucei transcripts from different developmental stages (18). However, as multiple developmental forms of the parasite reside within each organ, particularly in SGs where parasites undergo maturation to infective cells, these approaches could not provide sufficient resolution to identify development-specific processes. A better understanding of mechanisms that give rise to mammalian infective metacyclic parasites, known as metacyclogenesis, is fundamental and can help with the development of new methods to interfere with disease transmission success.

In this study, we applied single-cell RNA sequencing (scRNA-seq) to profile the transcriptomic landscape from a pool of 2,045 individual T. b. brucei isolated from SGs, which include multiple developmental forms (epimastigote and pre- and mature stages of metacyclic forms). We mined our data for stage-specific transcripts and identified metabolic profiles that reflect the process of preadaptation to the mammalian nutritional environment. We also present immunological and cellular microscopy data on one protein localized to the surface of mature metacyclic cells. We provide preliminary data that support the utility of this protein as a potential candidate transmission blocking antigen.

Results

scRNA-Seq Reveals Three Distinct Clusters. Multiple trypanosome developmental stages reside within infected tsetse SGs, ranging from proliferating epimastigotes to infective metacyclic forms adapted to survive in the mammalian host. We aimed to elucidate the molecular process of metacyclogenesis by characterizing the transcriptomic profiles of 2,045 individual parasites isolated from infected SGs that harbor a mix of different developmental stages. We obtained a total of 188,869,749 reads, 69% of which originated from identified cells (Dataset S1). We observed an average of 92,356 reads per cell and detected transcripts from 8,698 different genes across the profiled cells, with a median of 298 expressed genes detected per cell. Our gene expression analysis was based on unique molecular identifier (UMI) counts, with an UMI count corresponding to a unique transcript molecule. We observed a median of 410 UMI counts per cell.

After normalization and exclusion of genes expressed in less than 5% of the cells, our data segregated into three major clusters comprised of 647, 550, and 848 cells based on the calculation of the Davies–Bouldin index (Fig. L4 and SI Appendix, Fig. S1). While cluster 1 was distinct, clusters 2 and 3 represented subclusters of a broader continuous cluster. The number of genes detected expressed per cell in cluster 1 ranged between 91 and 1,126. Comparatively, we detected between 43 and 267 and 107 genes expressed from cells in clusters 2 and 3, respectively (Fig. 1B). Similarly, cluster 1 showed high variability in the number of UMI counts per cell, ranging from 184 to 8,402. Comparatively, UMI counts per cell in clusters 2 and 3 ranged from 183 to 844 and 186 to 1,210, respectively (Fig. 1B). For both measures, cluster 2 presented less variability concentrated around the median, while cluster 3 presented a broader distribution of data similar to cluster 1 but without the extreme variabilities. Altogether, these results indicate that parasites within clusters 1 and 3 are transcriptionally more active and diversified than those within cluster 2. Because gene expression is largely posttranscriptionally regulated in trypanosomes, we analyzed the translational activity associated with cells in different clusters by profiling the expression of 60S and 40S ribosomal RNA protein coding genes (SI Appendix, Fig. S2). Cluster 3 had the highest level of rRNA gene expression, indicative of greater translational activity associated with this cluster.

We next analyzed the expression of barp and mVSGs, which encode the surface coat proteins of epimastigote and metacyclic forms, respectively (19). We observed that a total of 655 cells, almost exclusively within cluster 1, expressed barp (Fig. 1C). The remaining cells in clusters 2 and 3 expressed one of the four mVSGs previously identified from parasitized SGs (18): ILTat1.22, 1.61, 1.63, and 1.64 (Fig. 1C). This finding supports the previous observations that each metacyclic parasite presents a unique mVSG protein coat and that the metacyclic population transmitted to the mammal in saliva represents a heterogeneous mix of cells, each with a different surface coat antigen (20, 21). The predominant mVSGs expressed in clusters 1 and 3 were ILTat 1.22 and 1.61 detected in 447 and 314 cells, respectively. The next most abundant mVSGs were ILTat 1.64 and 1.63 detected in 162 and 142 cells, respectively. When we compared the expression of coat proteins at the cluster level, instead of at the individual cell level, again barp was predominant in cluster 1, while mVSGs showed highest expression in cluster 2, with less expression in cluster 3 and none in cluster 1 (Fig. 1D). Interestingly, we also noted that 325 cells (16% of the total cells we analyzed) expressed neither barp nor one of the four known mVSGs. Because these cells are distributed within clusters 2 and 3, we speculate that they may encode other mVSGs that remain to be classified in the parasite strain we used here. We also conclude that barp and mVSGs, which are the most differentially expressed (DE) genes between cells in cluster 1 and clusters 2 and 3 (Dataset S1), serve as relevant biomarkers during development.

Altogether, our results indicate that based on transcriptional activity profiles and the identity of the surface coat proteins expressed, cluster 1 includes proliferative epimastigote stages (called cluster Epim), while clusters 2 and 3 represent different stages of metacyclics (called clusters Meta1 and Meta2, respectively) (22). Our scRNA-seq data indicate differences within the two metacyclic populations, with the rRNA profile indicating less translational activity in cells within Meta1 (SI Appendix, Fig. S2). In addition, cells within the Meta1 cluster had fewer UMI counts and fewer genes detected relative to Meta2 (SI Appendix, Fig. S3), suggesting that Meta1 is transcriptionally more quiescent than Meta2, which exhibits a more diverse transcriptome and higher transcriptional activity. This profile could reflect a process of transition within the metacyclic population, toward parasites that are ready to survive in the different nutritional and immune environment of the mammalian host.

Predicted Metabolome of Parasites in Different Clusters Reflects a Process of Adaptation to Different Host Environments. To better understand the biological differences between the epimastigote and metacyclic parasites, we identified DE genes between cluster Epim and the combined clusters Meta1 and Meta2 (Dataset S1). We then applied a gene ontology (GO) enrichment analysis and summarized the redundant terms using the REVIGO webtool (23) for functional differences (Dataset S2 and Fig. 2A). GO terms enriched in Epim were preferentially associated with energy metabolism, such as mitochondria-associated anion transport and tricarboxylic acid (TCA) cycle reflective of oxidative
phosphorylation, which is the major path for energy production in insect stage trypanosomes (24). By contrast, metacyclic form clusters were enriched in GO terms associated with cytosolic and transmembrane transport, amine biosynthesis, and spermidine biosynthesis, as well as regulation of cytokinesis (Fig. 2A). Spermidine biosynthesis is particularly relevant as spermidine is a precursor of trypanothione (25), a derivative of glutathione that acts as an antioxidant necessary for trypanosomes to infect their vertebrate hosts (26). However, these results would need further functional validation due to a single gene associated with the GO term “spermidine biosynthesis.” Altogether, these results support the expected metabolic profile for epimastigote forms that utilize mitochondria and an amino acid-based metabolism. In contrast, the putative metabolome of metacyclic forms reflects a process of adaptation to the mammalian glucose-rich nutritional environment where the bloodstream-form parasites depend upon glycolysis for ATP production (22, 27).

We observed that while epimastigote cells clustered tightly, metacyclic trypanosomes were distributed over two clusters. To further investigate the differences between the two metacyclic clusters, we identified DE genes between Meta1 and Meta2. Similar GO enrichment analyses revealed that Meta1 is enriched in functions associated with DNA biosynthesis and microtubule-based movement, while Meta2 is enriched in functions associated with gene expression and translation, including for CDP-choline pathway, nitrogen-based metabolism, and general biosynthesis pathways (Dataset S2 and Fig. 2B). We hypothesized that these metabolic differences, along with our earlier observations showing greater translational activity in Meta2, indicate that Meta1 parasites begin the reorganization of cellular structures, while Meta2 parasites are transitioning to a metabolically more active state as precursors of bloodstream forms in the mammalian host.

Subcluster-Specific Expression Profiles. To dissect the process of differentiation into mature metacyclics, we further split the original three clusters into seven subclusters: three within Epim, two in Meta1, and two Meta2 (Fig. 3A). We manually formed an eighth subcluster (called Inter for intermediary subcluster) that contains the cells lying at the junctions of the original three clusters. These subclusters reflected a finer scale of varying gene expression capacity, based on the number of genes expressed and phosphorylation, which is the major path for energy production in insect stage trypanosomes (24). By contrast, metacyclic form clusters were enriched in GO terms associated with cytosolic and transmembrane transport, amine biosynthesis, and spermidine biosynthesis, as well as regulation of cytokinesis (Fig. 2A). Spermidine biosynthesis is particularly relevant as spermidine is a precursor of trypanothione (25), a derivative of glutathione that acts as an antioxidant necessary for trypanosomes to infect their vertebrate hosts (26). However, these results would need further functional validation due to a single gene associated with the GO term “spermidine biosynthesis.” Altogether, these results support the expected metabolic profile for epimastigote forms that utilize mitochondria and an amino acid-based metabolism. In contrast, the putative metabolome of metacyclic forms reflects a process of adaptation to the mammalian glucose-rich nutritional environment where the bloodstream-form parasites depend upon glycolysis for ATP production (22, 27).

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UMI counts, than those described for the original three clusters (SI Appendix, Fig. S3). We next investigated the spatial expression profile of putative proteins known to regulate developmental processes in African trypanosomes within the eight subclusters. We focused on DE genes expressed in at least one of the three original clusters based on pairwise comparisons (Dataset S1 and Fig. 3B).

Prior studies had identified two putative lipid phosphate phosphatases (LPPs) expressed in SG stages (28), which our data confirmed to be epimastigote specific. We also investigated flagellar calcium-binding proteins, calflagins, which have been associated with prometacyclic and metacyclic forms, but not with epimastigote parasites (10). Our data confirmed the preferential expression of calflagin in the Meta1A–Meta1B cluster, which further supports that these clusters represent the prometacyclic form. We next evaluated expression profile of calpains, which enable the development and interaction of the related parasite Trypanosoma cruzi with the kissing bug vector's midgut cells (29, 30). We noted high expression of calpain-related (skcp1-4 and skcp1-5) genes in epimastigote forms, while calpain genes (calp, calp1.1, and calp1.3) were abundantly expressed in Inter, Meta1A, and Meta1B clusters, with the highest expression noted in the subcluster Meta1A. Hence, changes in the expression of calpains could be one factor that regulates tsetse–parasite interactions in SGs during metacyogenesis.

African trypanosomes produce polycistronic immature mRNAs, which are processed to mature monocistronic mRNAs by transsplicing and polyadenylation events. Essential components of this process are structural motifs enriched in 3′-untranslated regions of mRNAs that serve as ligands for different RNA binding proteins (RBPs), which inhibit the translation of their target mRNAs (31, 32). Proteins that regulate these processes, including members of RBPs, zinc-finger (ZF), pumilio (PUF), and U-rich RNA binding protein (UBP) families, presented a varying profile with the highest number expressed in Meta1, including the zinc-finger domain-containing proteins (ZC3H20, ZFP1, ZC3H1, ZFC3H11, and ZFCH38) and UBP1 (Fig. 3B). Moreover, both the number of genes expressed and UMI counts in Meta1A were lower than in Meta2B (SI Appendix, Fig. S3), indicating that Meta1A is the least transcriptionally active group of cells as we proposed earlier. We noted that RBP6 was predominant in EpimC, which represents cells transitioning to metacyclic forms. In fact, RBP6 overexpression in cultured procyclic forms resulted in differentiation to long and short epimastigotes as well as mammalian infective metacyclic parasites, supporting the role of RBP6 in parasite development regulation (33). We also observed the highest expression of rpb10 in the Meta2 population, which are preparing to transition to proliferative bloodstream forms in the mammal. Similar overexpression of RBP10 in procyclic forms increased the abundance of bloodstream form-specific transcripts (34) and initiated the transition of cells to the bloodstream stages (35, 36).

Fig. 2. GO enrichment analysis of epimastigote and metacyclic forms. (A) Biological process-associated GO terms significantly enriched in epimastigote forms (Right bars) in comparison to metacyclic forms (Left bars). (b) Biological process-associated GO terms significantly enriched in cells in Meta1 cluster (Right bars) in comparison to parasites in Meta2 (Left bars). In both comparisons, the GO terms have been filtered using the REVIGO webtool to avoid redundancies. Bars represent the level of significance of the term enrichment. Bars of the same color represent terms that are closely associated.
High levels of *rpb10* expression were also noted in an in vitro metacyclic cell line generated by RBP6 overexpression in comparison to procyclic cells (22). Finally, the mVSG genes were expressed abundantly within the Meta1 subclusters, signifying the transition of the epimastigote state cells to the premetacyclic forms at this time.

Taken together, our results suggest that trypanosomes undergo three sequential developmental steps in tsetse’s SGs, each with varying transcriptomic and metabolomic profiles. Specifically, epimastigote forms (Epim cluster) transition to premetacyclic forms (Meta1 cluster), which give rise to mature metacyclic forms (Meta2 cluster). We hypothesize that mature metacyclic forms populating cluster Meta2 represent the free mammalian infective population transmitted to the bite site in fly saliva.

Unique Surface Proteins Associated with Epimastigote and Metacyclic Forms. We searched within the different clusters for abundant and differentially expressed genes that encode surface associated proteins. The first family we noted, here named salivary gland epimastigote 1 (SGE1), includes five closely related members exclusively expressed in clusters EpimA – EpimC (Fig. 3A and SI Appendix, Fig. S4). Previous in silico analysis identified the SGE1 family as putative cell surface proteins of African trypanosomes (Fam50, lineage iv) (37). Expression profiling in different tsetse tissues indicated its preferential expression by SG parasites (38). Further, SGE1 proteins were not detected within the surface phylome of bloodstream forms when analyzed by biochemical methods (39).

We identified a second gene family (family 10, Fam10), comprised of six closely related genes, expressed at high levels exclusively in Meta1 to Meta2 subclusters (37) (Fig. 3A and SI Appendix, Fig. S5) (18). Least-square means (LSM) expression values for the Fam10 genes were highest in cluster Meta2 (with the exception of *sgm1.6*, Fig. 3C). The peak expression of Fam10 genes was within the subcluster Meta1B (Fig. 3B). This expression pattern could be indicative of their preferential function during transition from pre- to mature metacyclic forms.

To understand the temporal processes during differentiation from metacyclic to bloodstream-form parasites in the mammal, we measured the transcript levels for stage-specific genes (Dataset S3 and Fig. 4A). Expression of *mVSG1.22*, which is one of the most abundant genes expressed in our metacyclic parasite line in the SG organ, persisted in the mammalian host (mouse blood) for the first 36 h, but was undetectable thereafter as cells differentiated to bloodstream forms (Fig. 4A). Transcripts for *sgm1.3* and *sgm1.7*, which are two members of the Fam10 family most abundantly expressed in metacyclic cells (Fig. 3C), continued to be detected in the bloodstream-form cells in the mammalian host, albeit at significantly lower levels compared to the metacyclic stage (Fig. 4A). In accordance, a biochemical analysis of parasite surface proteome identified both SGM1.3 and SGM1.7 in the...
bloodstream-form trypanosomes (39). Similar profiling of epimastigote-specific SGE1 (here analyzed one member, \textit{Tb}927.7.360) showed high expression in the SG organ followed by a sharp decline in the mammalian host by 36 h and beyond (Fig. 4A). Collectively, it appears that metacyclic cells persist in the mammalian host for about 36 h before transforming into the bloodstream-form cells, and that SGM1.7 and SGM1.3 proteins, most abundant in metacyclic forms, continue to be expressed in the bloodstream forms, albeit at significantly lower levels.

We next evaluated the cellular localization of SGM1.7 and SGE1 using immunofluorescence and immunogold microscopy analyses. In support of our transcript data, SGM1.7 is localized specifically to the surface of metacyclic cells (Fig. 4B and C) while SGE1 is detected only on the surface of epimastigote forms (Fig. 4D and E). Interestingly, while we detected \textit{sgm1.7} expression in the bloodstream forms, we could not establish its presence on the surface of cells by immunofluorescence. It may be due to the thick VSG coat of the bloodstream form that prevents detection of nonvariable surface antigens. It is also possible that the abundance of the SGM1.7 protein may vary between the metacyclic and bloodstream-form parasites, independent of the \textit{sgm1.7} transcript levels associated with each developmental state.

\textbf{SGM1.7 Explored as a Transmission Blocking Vaccine Antigen.} Because SGM1.7 is exposed on the surface of infective metacyclic parasites, we explored whether SGM1.7 could be a potential target for transmission blocking studies. We used SGE1 as a control since this protein is exclusively expressed in the epimastigote forms that are not infectious or free in saliva to be introduced into the mammalian host.
We first tested whether coinoculation of metacyclic parasites together with anti-SGM1.7 immunoglobulin G (IgG) could interfere with trypansome development and block or reduce the onset of parasitemia in the mouse system. Using needle injection, we coinjected intradermally 500 SG isolated parasites with anti-SGM1.7 or naive IgG, respectively. We followed the parasitemia in mice blood daily over the first week postchallenge and noted that presence of anti-SGM1.7 IgG reduced the parasitemia early in the infection process relative to control mice that received the same infectious parasites together with naive mouse IgG (Fig. 5A). This outcome indicates that an antibody-mediated response could interfere with parasite survival or differentiation at the bite site.

We next vaccinated mice with rSGM1.7 or rSGE1 in combination with adjuvant and confirmed the presence of high antibody titers (<1:312,500) in vaccinated animals (Dataset S5). We next challenged all vaccinated animals and age-matched naive mouse controls similarly with 500 parasites obtained from SGs. In two independent experiments, the parasitemia levels in rSGM1.7-vaccinated animals were lower than naive controls early in the infection (Fig. 5B). On the other hand, rSGE1-vaccinated mice did not show any difference in parasitemia levels relative to naive controls over the same time span analyzed (Fig. 5C). We also infected mice vaccinated with rSGM1.7 through a single infected tsetse bite and observed significantly lower parasitemia levels relative to naive controls at 3d postinfection, the earliest time point measured (Fig. 5D). Although we did not observe sterile immunity via rSGM1.7 antigen, the significant decrease in the parasitemia measured early in vaccinated animals, suggests that some, but not all, metacyclic parasites were cleared from progressing to bloodstream infections at the bite site.

Conclusions

The ability to capture the transcriptomes of individual parasites in infected tsetse SGs provides unmatched depth of insight into the developmental processes that give rise to mammalian infective metacyclic cells. Although posttranscriptional mechanisms that affect mRNA stability, translation, and protein stability regulate trypansome gene expression, we observed temporal expression of several genes, which correlates well with the experimentally confirmed roles of their products during development, i.e., calpains, RBP6, and RBP10. In addition, the predicted metabolomes of parasites in different clusters reflect a changing landscape from the known biochemical profile of the epimastigotes, which utilize mitochondria and an amino acid-based metabolism, to metacyclic forms, which undergo development to survive in the glucose-rich environment of the mammalian host. Our data also confirm that each metacyclic cell expresses a single gene encoding one of the known mVSG surface coat proteins. In addition, within the pool of metacyclic cells that express mVSCs, we observed varying physiological states which likely reflect the process of gaining mammalian infectivity and adaptation to the mammalian host environment. Finally, our cell level data indicate that the metacyclic state represents a spectrum of cells with low but varying gene expression activity. Interestingly, the mature metacyclic parasites (Meta2 cluster) express higher numbers of genes, UMI counts, and rRNA transcripts relative to the premetacyclic stage (Meta1 cluster). This heightened transcriptional state may promote the process of cellular development that takes place as the parasites entering into the mammalian bite site differentiate to bloodstream-form cells.

African trypanosomiasis continues to be a debilitating disease of great medical and socioeconomic importance. Because trypanosomias have evolved efficient mechanisms to manipulate and escape host immune responses, development of effective mammalian vaccines has not been possible (40). However, the ability to restrict infections at the bite site where relatively few parasites are inoculated can provide a powerful new method for disease prevention. In fact, experiments where mice were challenged twice with Trypanosoma brucei bloodstream trypanosomes followed up by experimental cure, resulted in sterile immunity upon subsequent challenge (41). However, immunity to challenge was short-lived and did not provide long-term protection. It was thought that this loss of immunity could have resulted from the small number of parasites the animals typically received via fly challenge (42).

Building on the molecular knowledge of metacyclic infective forms, we identified a prominent nonvariant coat protein (SGM1.7) present on the surface of metacyclic cells. The putative role of Fam10 surface proteins in metacyclogenesis remains to be elucidated. Future experiments with genetically modified parasites can shed light on processes leading to survival in the SG, or during infection establishment in the mammalian host bite site. Our results with anti-SGM1.7 antibodies implicate a B cell antibody-mediated response that can interfere with parasite survival or differentiation during the initial stages of infection in the mammal. Although we did not observe sterile immunity in our experimental system, future experiments with SGM1.7 antigen can identify critical epitopes that are effective for parasite interference, thus improving the protein’s efficacy as a subunit vaccine antigen. In addition, including different members of the Fam10 family proteins, which are also highly expressed in metacyclic cells, together with SGM1.7 as vaccine antigens, could enhance immune protection. Given that Fam10 proteins are conserved in the Trypanosoma brucei spp. parasites, including the human infective T. b. rhodesiense and T. b. gambiense, the availability of

Fig. 5. Prophylactic efficacy of SGM1.7. (A) Antimetacyclic-specific rSGM1.7 IgG and naive mice IgG coinoculated with 500 SG parasites intraperitoneally in two groups of mice, respectively. Parasitemia was followed via eye bleeds. (B and C) Two independent groups of mice vaccinated with metacyclic-specific rSGM1.7 or epimastigote-specific rSGE1 were challenged with 500 SG parasites injected i.d. in the ear. As a control, nonvaccinated age-matched groups of mice were similarly infected. Parasitemia was followed via eye bleeds. (D) Infections in mice vaccinated with metacyclic-specific rSGM1.7 or age-matched naive controls were initiated through a single infected fly bite intraperitoneally. For statistical analysis, a mixed-effect linear model was fitted, where the logarithm in base 10 of the parasitemia counts was taken as the dependent variable. For day-to-day pairwise comparison of parasitemia between vaccinated and nonvaccinated mice, significance was evaluated with Tukey’s contrasts. Statistically different data points are shown by asterisks: * > 0.05, *** > 0.0001. Complete statistical results are available in Dataset S4.
induced, purified, and analyzed for purity using standard methods (SI Appendix, Materials and Methods).

Immunofluorescence Analysis. For immunostaining, parasites obtained from infected SGs were fixed in 4% paraformaldehyde (PFA) and 0.1% gluteraldehyde and then processed as described in SI Appendix, Material and Methods. Cells were not permeabilized in order to detect surface exposure of the antigens. Slides were incubated with either rabbit anti-rSGE1 or mouse anti-rSGM1.7 or their respective preimmune sera. Alexa Fluor 488-labeled goat anti-rabbit IgG (Invitrogen) and Alexa Fluor 594-labeled goat antimouse IgG were used as secondary antibodies. Slides were visualized at 400× and 1,000× using a Zeiss Axios Imager M2 microscope and images were captured using an AxioCam Mrm (Zeiss) and the AxioVision software (v4.8.2.0, Zeiss). Images were processed using the Fiji version of the ImageJ software (46). Only contrasts and luminosity were adjusted on images.

Electron Microscopy. Parasites were collected by incubating infected SGs in phosphate saline glucose (PSG) at room temperature for 45 min and processed as described in SI Appendix, Material and Methods. For SGM1.7, sections were incubated with either preimmune or anti-rSGM1.7 mouse sera, then with a rabbit anti-mouse IgG secondary antibody used as a bridge to improve binding with 10 nm colloidal gold-labeled protein A (University Medical Center Utrecht [UtrechUMC]). For SGE1, sections were incubated with either preimmune or anti-rSGE1 rabbit sera, and then with 10 nm colloidal gold-labeled protein A (UtrechUMC). In both cases, sections were processed for viewing with an FEI Tecnai Biotwin transmission electron microscope at 80 kV. Images were taken using a Morada CCD camera and ITEM software (Olympus).

Coinoculation of Parasites with Immune Sera. Five hundred SG purified parasites were mixed with 20 μg rSGM1.7 IgG in a volume of 20 μl or with 20 μg naive mice IgG and introduced intradermally (i.d.) in the ear via needle injection, respectively. Parasitemia was followed by retroorbital sampling of blood on days 4 to 7 in two independent experiments (Exps) (Dataset SS, Exps 2 and 3).

Parasite Challenge of Vaccinated Mice. For each experiment, two groups of mice with a minimum of five individuals were vaccinated with purified rSGE1 and rSGM1.7 with Magic Mouse adjuvant (Creative Diagnostics, product no. CDN-A001), receiving two boosts in 2-wk intervals. ELISA was performed on all vaccinated animals 2 wk postfinal boost (Dataset SS, Exps 4, 6, 8, and 10) and 3 wk after the final boost using a BioTek Synergy HT plate reader. Subsequently, mice were infected with 500 SG isolated parasites (containing epimastigote, premetacyclic, and mature metacyclic forms) via needle injection in the ear. Parasitemia was followed in two separate experiments (Dataset SS, Exps 5 and 7 for rSGE1 and Exps 9 and 11 for rSGM1.7). Age-matched naive mice were similarly infected as controls. Mice vaccinated with rSGM1.7 and naive age-matched controls were also infected via single tsetse bite and parasitemia similarly monitored (Dataset SS, Exps 12 and 13).

Data Availability. Sequencing read files have been deposited in the NCBI BioProject database (ID PRJNA562204).

ACKNOWLEDGMENTS. We acknowledge the Center for Cellular and Molecular Imaging for their kind assistance with the electron microscopy. We thank members of the Yale Center for Genome Analysis for consultations on bioinformatics analysis. We are grateful to Yuling Lei, Yineng Wu, and Bu Lei Tu for their excellent technical help with the experimental methods. This work received support from the Bill and Melinda Gates Foundation (OPP1139763), the Ambrose Monell Foundation, and NIH AI051584.

1. P. P. Simarro et al., Risk for human African trypanosomiasis, Central Africa, 2000-2009. Emerg. Infect. Dis. 17, 2322–2324 (2011).
2. P. P. Simarro et al., The Atlas of human African trypanosomiasis: A contribution to global mapping of neglected tropical diseases. Int. J. Health Geogr. 9, 57 (2010).
3. P. P. Simarro, A. Diarra, J. A. Ruiz Postigo, J. R. Franco, J. G. Jannin, The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000-2009: The way forward. PLoS Negl. Trop. Dis. 5, e1007 (2011).
4. P. P. Simarro, J. Jannin, P. Cattand, Eliminating human African trypanosomiasis: Where do we stand and what comes next? PLoS Med. 5, e55 (2008).
5. L. J. Morrison, L. Marcello, R. McCulloch, Antigenic variation in the African trypanosome: Molecular mechanisms and phenotypic complexity. Cell. Microbiol. 11, 1724–1734 (2009).
6. I. Roditi, M. J. Lehane, Interactions between trypanosomes and tsetse flies. Curr. Opin. Microbiol. 11, 345–351 (2008).
7. E. Aksoy et al., Mammalian African trypanosome VSG coat enhances tsetse vector competence. Proc. Natl. Acad. Sci. U.S.A. 113, 6961–6966 (2016).
8. J. Van Den Abbeele, Y. Claes, D. Van Bockstaele, D. Le Ray, M. Coosemans, Trypanosoma brucei spp. development in the tsetse fly: Characterization of the post-mesocyclic stages in the foregut and proboscis. Parasitology 118, 469–478 (1999).
9. S. Uswiler, E. Studer, C. K. Renggli, I. Roditi, A family of stage-specific alanine-rich proteins on the surface of epimastigote forms of Trypanosoma brucei: Mol. Microbiol. 63, 218–228 (2007).
10. B. Rotureau, I. Subota, J. Buissory, P. Bastin, A new asymmetric division contributes to the continuous production of infective trypanosomes in the tsetse fly. Development 139, 1842–1850 (2012).
11. C. M. Turner, J. D. Barry, I. Maudlin, K. Vickerman, An estimate of the size of the metacyclic variable antigen repertoire of Trypanosoma brucei rhodesiense. Parasitology 97, 269–276 (1988).
12. M. R. Mugnier, C. E. Stebbins, F. N. Papavasiliou, Masters of disguise: Antigenic variation and the VSG coat in Trypanosoma brucei. PLoS Pathog. 12, e1005784 (2016).

13. L. Tetley, C. M. R. Turner, J. D. Barry, J. S. Crowe, K. Vickerman, Onset of expression of Vigneron et al. PNAS 12. M. R. Mugnier, C. E. Stebbins, F. N. Papavasiliou, Masters of disguise: Antigenic variation and the VSG coat in Trypanosoma brucei. PLoS Pathog. 12, e1005784 (2016).

14. S. Z. Shapiro, J. Naessens, B. Liesegang, S. K. Moloo, J. Magondu, Analysis by flow cytometry of DNA synthesis during the life cycle of African trypanosomes. Acta Trop. 41, 313–323 (1984).

15. L. Tetley, V. K. Peterman, D. G. Baskerville, J. S. Crowe, K. Vickerman, Antigenic variation and the VSG coat in Trypanosoma brucei. J. Cell Sci. 87, 363–372 (1987).

16. S. Z. Shapiro, J. Naessens, B. Liesegang, S. K. Moloo, J. Magondu, Analysis by flow cytometry of DNA synthesis during the life cycle of African trypanosomes. Acta Trop. 41, 313–323 (1984).

17. K. Vickerman, L. Tetley, K. A. Hendry, C. M. Turner, Biology of African trypanosomes in the tsetse fly. Med. Bull. Sci. Med. Bull. 41, 105–114 (1985).

18. A. F. Savage et al., Transcriptome profiling of Trypanosoma brucei development in the tsetse fly vector Glossina morsitans. PLoS One 11, e0156877 (2016).

19. K. Vickerman, C. M. Turner, J. D. Barry, J. S. Crowe, K. Vickerman, Antigenic variation and the VSG coat in Trypanosoma brucei. J. Cell Sci. 74, 1–19 (1985).

20. K. Vickerman, Developmental cycles and biology of pathogenic trypanosomes. Br. Med. Bull. 41, 105–114 (1985).

21. K. Vickerman, L. Tetley, K. A. Hendry, C. M. Turner, Biology of African trypanosomes in the tsetse fly. Biol. Cell 64, 109–119 (1988).

22. A. F. Savage et al., Transcriptome profiling of Trypanosoma brucei development in the tsetse fly vector Glossina morsitans. PLoS One 11, e0156877 (2016).

23. F. Supek, M. Božič, T. Skunca, N. G. Kolev, E. Ullu, C. Tschudi, The emerging role of RNA-binding proteins in the life cycle of Trypanosoma brucei. PLoS Negl. Trop. Dis. 11, e1006560 (2017).

24. E. Mugo, C. Clayton, Expression of the RNA-binding protein RBPI0 promotes a bloodstream-form differentiation state in Trypanosoma brucei. Mol. Microbiol. 83, 1048–1063 (2012).

25. A. Fairlamb, P. Blackburn, P. Ulrich, B. T. Chait, A. Cerami, Trypanothione: A novel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. Mol. Biochem. Parasitol. 154, 203–213 (2007).

26. S. Krieger, K. R. Matthews, The cell biology of Trypanosoma brucei differentiation. Curr. Opin. Microbiol. 10, 539–546 (2007).

27. D. J. Creek, V. M. Nantulya, J. J. Doyle, L. Jenki, Studies on Trypanosoma (nannomonas) congolense III. Biol. Cell 39, 191–198 (1988).

28. T. L. Alves e Silva, A. F. Savage, S. Aksoy, Transcript abundance of putative lipid phosphate phosphatases during development of Trypanosoma brucei in the tsetse fly. Am. J. Trop. Med. Hyg. 94, 890–893 (2016).

29. L. S. Sangenito et al., Arrested growth of Trypanosoma cruzi by the calpain inhibitor MDL28170 and detection of calpain homologues in epimastigote forms. Parasitology 136, 433–441 (2009).

30. V. Ennes-Vidal, R. F. Menna-Barreto, A. L. Santos, M. H. Branquinha, C. M. d’Avila-Levy, MDL28170, a calpain inhibitor, affects Trypanosoma cruzi metacyclogenesis, ultra-structure and attachment to Rhodnius prolixus midgut. PLoS One 6, e18371 (2011).

31. N. G. Kolev, E. Ullu, C. Tschudi, The emerging role of RNA-binding proteins in the life cycle of Trypanosoma brucei. Cell. Microbiol. 16, 482–489 (2014).

32. M. A. Romaní, G. Cervini, A. Cassola, Regulation of RNA binding proteins in trypanosomatid protozoan parasites. World J. Biol. Chem. 7, 146–157 (2016).

33. M. Wurst et al., Expression of the RNA recognition motif protein RBPI0 promotes a bloodstream-form transcript pattern in Trypanosoma brucei. Mol. Microbiol. 83, 1048–1063 (2012).

34. M. A. Romaní, G. Cervini, A. Cassola, Regulation of RNA binding proteins in trypanosomatid protozoan parasites. World J. Biol. Chem. 7, 146–157 (2016).

35. E. Mugo, C. Clayton, Conversion of procytocform Trypanosoma brucei to the bloodstream form by transient expression of RBPI0. Mol. Biochem. Parasitol. 216, 49–51 (2017).

36. A. F. Savage et al., Transcript expression analysis of putative Trypanosoma brucei GPI-anchored surface proteins during development in the tsetse and mammalian hosts. PLoS Negl. Trop. Dis. 6, e1708 (2012).

37. C. Gadelha et al., Architecture of a host-parasite interface: Complex targeting mechanisms revealed through proteomics. Mol. Cell. Proteomics 14, 1911–1926 (2015).

38. B. Stijlemans, M. Radwanska, C. De Trez, S. Magez, African trypanosomes undermine humoral responses and vaccine development: Link with inflammatory responses? Front. Immunol. 8, 582 (2017).

39. V. M. Nantulya, J. J. Doyle, L. Jenki, Studies on Trypanosoma (nannomonas) congolense III. Antigenic variation in three cyclically transmitted stocks. Parasitology 80, 123–131 (1980).

40. V. M. Nantulya, Immunological approaches to the control of animal trypanosomiasis. Parasitol. Today 2, 168–173 (1986).

41. G. Caljon et al., Tsetse fly saliva accelerates the onset of Trypanosoma brucei infection in a mouse model associated with a reduced host inflammatory response. Infect. Immun. 74, 6324–6330 (2006).

42. R Core Team, R: A Language and Environment for Statistical Computing (R version 3.5.1, 2018).

43. M. A. Rojas, TriTrypDB: A functional genomic resource for the Trypanosomatidae. Nucleic Acids Res. 38, D457–D462 (2010).

44. J. Schindelin et al., Fiji: An open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).