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Diverged Alleles of the *Anopheles gambiae* Leucine-Rich Repeat Gene *APL1A* Display Distinct Protective Profiles against *Plasmodium falciparum*

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Abstract

Functional studies have demonstrated a role for the *Anopheles gambiae* APL1A gene in resistance against the human malaria parasite, *Plasmodium falciparum*. Here, we exhaustively characterize the structure of the APL1 locus and show that three structurally different APL1A alleles segregate in the Ngousso colony. Genetic association combined with RNAi-mediated gene silencing revealed that APL1A alleles display distinct protective profiles against *P. falciparum*. One APL1A allele is sufficient to explain the protective phenotype of APL1A observed in silencing experiments. Epitope-tagged APL1A isoforms expressed in an *in vitro* hemocyte-like cell system showed that under assay conditions, the most protective APL1A isoform (APL1A2) localizes within large cytoplasmic vesicles, is not constitutively secreted, and forms only one protein complex, while a less protective isoform (APL1A1) is constitutively secreted in at least two protein complexes. The tested alleles are identical to natural variants in the wild *A. gambiae* population, suggesting that APL1A genetic variation could be a factor underlying natural heterogeneity of vector susceptibility to *P. falciparum*.

Introduction

Most of the global transmission of human malaria due to *Plasmodium falciparum* occurs in Sub-Saharan Africa and is vectored primarily by the mosquito *Anopheles gambiae* [1]. Studies on the *A. gambiae*- *P. falciparum* pair have revealed a large genetic influence upon mosquito resistance to the human malaria parasite [2–5], although the causative variants underlying the mapped quantitative trait loci (QTLs) have not yet been resolved. Separately, a number of functional studies show that the immune system of *A. gambiae* protects mosquitoes against infection with *P. falciparum*, and multiple immune factors have been identified [6–14].

In *A. gambiae* mosquitoes from West and East Africa, genetic loci strongly linked to *P. falciparum* resistance traits were repeatedly mapped to the same segment of chromosome 2L, and were resolved to a ~10 Mb genetic interval [2,4,5]. The *APL1* (*Anopheles Plasmodium*-responsive leucine-rich repeat 1) genes were identified as candidates within the resistance QTL cluster. The *APL1* locus is ~18 kilobases (kb) in length and comprises three paralogs, *APL1A*, *APL1B* and *APL1C* [15].

The three *APL1* genes encode a family of leucine-rich repeat (LRR) proteins that influence the survival of *Plasmodium* parasites, displaying distinct activities depending on the parasite species: APL1A protects against the human malaria parasite *P. falciparum* that is naturally transmitted by this vector, and APL1C against rodent malaria parasites [11,15]. The APL1C protein has been shown to form a heterodimer with another LRR protein, LRIM1, as part of a functional complex with the complement C3-like protein TEP1 [16–19]. Protein partners of APL1A and APL1B remain unknown.

The *APL1* genes show a high degree of similarity in sequence and intron-exon structure, and they likely originate from gene duplication and diversification events. Each of the encoded APL1 proteins is characterized by the presence of a N-terminal signal sequence and a LRR region followed by a cysteine-rich tract, and some variants have a C-terminal coiled-coil domain. The *APL1* locus comprises two additional genes also coding for LRR proteins, *AGAP007034* (LRIM1) and *AGAP0067037* (LRIM3), which are not members of the *APL1* family. Despite
the overall structural resemblance with the APL1 proteins, these
genes encode proteins belonging to the LRIM family [15,19,20].

In nature, APL1 genes display high genetic diversity, in a pattern
consistent with adaptive maintenance of polymorphism [21]. The
differential functional roles, if any, of APL1 alleles are not known.
However, the mapped P. falciparum resistance QTLs that include
the APL1 locus exert a strong influence on infection outcome [2,4].
The genetically mapped phenotypic effect is due to as yet
unknown causative allelic variant(s) found within the QTL locus
that includes APL1, which remains a strong candidate gene. One
previous report described fine genetic dissection of an A. gambiae
locus, but the locus was linked to protection against the rodent
parasite P. berghei, and does not correspond to the location of a P.
falciparum-protective QTL [22].

Here we measured the individual phenotypes of APL1 alleles
for protection against P. falciparum, using the Ngousso laboratory
colony, after first validating the existence of the same alleles in the
natural population. By genetic association studies using RNA
interference-mediated knockdown assays, we evaluated the
importance of the structurally different allelic variants of the
APL1 gene against the human malaria parasite P. falciparum and
observed distinct allele-specific protective profiles. Finally, in
assays using cultured hemocyte-like cells we detected different
subcellular localization and secretion patterns of the APL1 allele
isoforms.

Results

Structure of the APL1 Locus

We generated overlapping PCR fragments over ~18 kb
covering the APL1 locus from 20 individual Ngousso mosquitoes.
The sizes of the obtained amplicons corresponded to the physical
distances displayed in the A. gambiae PEST-strain reference
genome [23], with the exception of fragments from two intergenic
regions, the regions between APL1B – APL1A and APL1 –
AGAP007037. These size differences are due to the presence of
insertion-deletion variations (indels) and to the absence in Ngousso
of the TA-III-Ag miniature inverted transposon (MITF transpos-
able element) identified in PEST upstream of APL1 [15]. These
results confirm that the APL1 locus in Ngousso has the same
genomic organization as the PEST reference, and did not reveal
the presence of any additional copies of the APL1 genes (Figure 1).

Three APL1 Alleles Segregate in the Ngousso Colony

Each of the three APL1 genes was amplified with specific
primers flanking the coding region to avoid cross-amplification
of the other paralogs due to their high similarity (Table S1).
Sequencing of the diploid PCR fragments indicated a maximum
of two alleles in each PCR reaction. We chose two homozygotes
per allele and sequenced the cognate coding region. The
structurally most polymorphic of the paralogs is APL1A, with
three alleles: APL1A1, APL1A2 and APL1A3 (Figure 2, and
Supplementary Information S1), following the previous nomen-
clature of gene name with allele superscript [15,21]. All three
Ngousso APL1A alleles present a similar overall structure, but show
diagnostic indel polymorphisms (Figure S1). The diagnostic
differences allowed us to develop a genotyping assay for the
APL1A alleles. The APL1A allele is structurally nearly identical to
the APLA of the PEST reference.

The presence of allele specific differences in APL1A2
and APL1A3 leads to premature stop codons that result in proteins
without the coiled-coil domain (Figure 3, and Supplementary
Information S1). The alleles, APL1A1 and APL1A2, exist also in
the G3 colony and in field caught mosquitoes from Mali [15,21]. In
addition, we found four APL1A alleles in a sample set of wild
mosquitoes carrying the standard chromosomal form 2La*/2La+
from Burkina Faso (manuscript accepted [24]) and three of these
alleles were structurally identical to Ngousso alleles APL1A1,
APL1A2 and APL1A3, overall indicating that the APL1A alleles
observed in Ngousso are not colony artifacts or geographically
limited forms. Instead, there appears to be a limited repertoire of
allelic forms with major structural consequences for the encoded
APL1A proteins.

Alleles of APL1B and APL1C in Ngousso and Haplotype
Organization of the APL1 Locus

Three APL1B allelic variants exist in Ngousso. The APL1B1
and APL1B2 alleles display only single nucleotide polymorphism (SNP)
differences, and the encoded proteins correspond to the APL1B
protein in PEST. The APL1B3 allele, however, has a premature
stop codon due to a point mutation at the beginning of the second
exon (2L: 41268220), resulting in a 21 amino acid product
comprising essentially just the signal peptide. We confirmed the
existence of this truncated coding sequence variant in genome
sequences from multiple samples from the Ngousso colony.

For the APL1C gene, the major allelic differences are due to
variations in the copy number and composition of the encoded
consensus amino acid sequence Pro-Na-Aasn-Gly-Gly-Leu
(PANGGL) repeat in the N-terminal region of APL1C. The
PANGGL repeat or its variants is encoded by all APL1C alleles
and is also present in APL1B2 but absent in the other APL1A
alleles and all APL1B alleles. This amino acid repeat motif is not
found in any other Anopheles genes, and its function is unknown.

In order to detect linkage between alleles, we sequenced
the complete coding sequence of APL1B and APL1C from two
mosquitoes homozygous for each of the three APL1A alleles

Figure 1. Genomic organisation of the A. gambiae APL1 region. The genomic region of the 2L chromosome that comprises the APL1 locus
is indicated by a green bar (A. gambiae reference genome [23], genome assembly AgamP3). The bar delimits the region of the genome that has been
completely spanned with overlapping PCR amplicons. The chromosomal positions are indicated (in bp) on top of the bar. The yellow bars indicate
the regions for which overlapping PCR fragments have been sequenced. Blue arrows represent the genes in this chromosomal region and their direction
of transcription. The last two lines indicate gene identifiers and names.
doi:10.1371/journal.pone.0052684.g001
Alleles of the paralogs are linked to form three stable haplotypes in Ngousso (Figure 3). Thus, each APL1A allele is consistently part of a specific combination of APL1B and A alleles. For example, the APL1A3-bearing haplotype also encodes the highly truncated APL1B3 protein along with a complete APL1C protein. The haplotype structure of the locus in

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Figure 2. Alignment and comparison of the Ngousso APL1A alleles. A) Schematic view of the genomic regions of the different APL1A alleles identified in A. gambiae Ngousso. Each row represents the genomic region of one APL1A allele. The first row shows the APL1A gene from the A. gambiae reference genome. Each of the following three rows represents one of the APL1A alleles present in the Ngousso strain, APL1A1, APL1A2, APL1A3, respectively. The sequences belonging to the APL1A genes are represented as solid bars with white boxes corresponding to the 5' non-coding region and the region of the first intron. Dashed lines illustrate introduced gaps for a better alignment of the different alleles. DNA regions adjacent of the APL1A genes are shown as black horizontal lines. Predicted peptide domains are indicated as follows: signal peptide (black vertical bar), additional sequences and PANGGL repeat region (yellow), LRR repeat region (green), coiled-coil domain (orange). Yellow triangles indicate the positions and length of indels, red stars indicate the positions of the translation stop codons. At the bottom right, the black bar indicates the DNA region targeted by the dsRNA. B) Schematic representation of the labelled PstI-RFLP fragments of APL1A alleles obtained by the high-throughput RFLP genotyping assay in Ngousso. The first line represents the genomic DNA of this region with two arrows marking the positions of the forward and reverse fluorescent-labeled primers used in the assay. The red and the blue vertical bars indicate the respective position of the terminal PstI restriction sites in the different alleles. The fragment sizes of the labelled PstI fragment generated from the 5' end of the Ngousso APL1A alleles are indicated in red. The sizes of the labelled PstI fragment from the 3' end of the APL1A alleles are in blue.

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Figure 3. Schematic representation of the three different APL1 haplotypes found in A. gambiae Ngousso. Each row shows one of the three identified APL1 haplotypes of Ngousso, numbered 1 to 3. The three APL1 proteins encoded by each haplotype are shown. Predicted peptide domains are indicated as follows: black vertical bar – signal sequence, yellow box – PANGGL repeat region, green – LRR repeat region, orange – coiled-coil domain.

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wild mosquitoes is unknown, but the founding effect of colonization should in general produce more extensive haplotype structure than exists in nature.

The APL1A² Allele is Highly Protective against P. falciparum Infection

We previously showed that APL1A displays a protective function against the human malaria parasite P. falciparum in A. gambiae Ngousso using gene silencing experiments [11]. Here, we examined the relative contribution of the three APL1A alleles to host protection. The high sequence similarity between the three APL1A alleles does not permit the design of double-stranded RNAs (dsRNAs) for allele-specific knockdown. Consequently, for gene silencing we injected a single dsRNA targeting all three APL1A alleles (Figure S3) followed by infection challenge and individual mosquito genotyping to test association of infection status with APL1A allele. We used gene silencing of APL1A to specifically measure the APL1A effect, and isolate it from the influence, if any, of the linked alleles on the APL1A-bearing haplotype. The haplotype is of unknown length, and could include the effects, possibly even contradictory in terms of phenotype, of tens or more linked alleles, including but not limited to APL1B and APL1C.

We compared the level of P. falciparum infection for each APL1A allele between the states of normal APL1A function (mosquitoes treated with control dsRNA dsGFP) and APL1A loss-of-function (treated with dsRNA for APL1A, dsAPL1A). In each of three independent replicate infections (Table S2A, Figure S2), APL1A was strongly protective, because its silencing rendered mosquitoes significantly more susceptible to P. falciparum infection by increasing infection prevalence (combined p-value for infection prevalence, 2.25e-05; Figure 4), but without affecting infection intensity (Figure S2).

When analyzing the results by APL1A allele, only the APL1A² allele displayed a consistent protective effect, because the global silencing of all APL1A alleles caused a highly significant increase of infection prevalence only among carriers of the APL1A² allele (p = 5.07e-07; Table 1). The protective phenotype was seen in APL1A² carriers regardless of the other APL1A allele present in the diploid mosquito, and thus demonstrates a genetically and functionally dominant protective effect of the APL1A² allele against P. falciparum infection. There was insufficient sample size to test whether the APL1A²/APL1A³ homozygous genotype is even more protective than the APL1A² allele alone.

No statistically significant allele specific effect on the infection intensities was detected in the mosquitoes of the three replicates injected with dsAPL1A (data not shown).

In contrast, mosquitoes carrying the APL1A² allele showed no significant difference in infection outcome after dsAPL1A treatment as compared to dsGFP-treated controls (p = 0.24722), indicating that function of the APL1A² allele does not underlie and is not required for the protection against P. falciparum mediated by the global silencing of APL1A. Finally, allele APL1A³ carriers displayed a significant difference of infection in only one replicate. The combined p-value for APL1A² influence on infection prevalence is significant (p = 0.00059), although three orders of magnitude less than for APL1A², but we regard it with caution because replicates 1 and 2, which were individually not significant, were still not significant when pooled to a single group before p-value calculation, indicating that the significant value in the APL1A² combined meta-analysis was generated solely by replicate 3.

Statistical power analysis indicates that sample sizes were sufficient to detect an effect of APL1A² and APL1A³, if one exists. For both APL1A² and APL1A³, there is power (>0.80 probability) to detect a difference in infection prevalence of the same magnitude as the effect of APL1A² (~30% infection difference) in either replicate 3 alone, or by pooling replicates 1 and 2. Thus, the experiments were properly powered and the result for APL1A² can be considered a robust negative. For APL1A², replicates 1 and 2 were robustly negative, but due to replicate 3 we regard APL1A² as at most weakly protective against P. falciparum. Taken together, these results show that, at least in the Ngousso colony, the APL1A² allele is necessary and sufficient to explain the protective effect of APL1A against P. falciparum, while APL1A³ plays no part in protection, and APL1A¹ has no, or at most weak, effect.

Role of Ngousso APL1A Haplotypes in P. falciparum Infections

We used the APL1A allele genotyping assay to test for an effect upon infection of the entire linked haplotype, rather than APL1A alone as tested in the dsRNA silencing assays. In three independent replicates (with n = 300 mosquitoes per replicate), mosquitoes were infected with P. falciparum in the absence of the dsRNA treatment used above (Table S2B, Figure S3). Despite the large sample sets, no significant association was observed between APL1A haplotypes as alleles or genotypes (data not shown). In each replicate, the observed genotype distribution was in Hardy-Weinberg equilibrium and each replicate had sufficient statistical power (>0.8 probability) to detect differences in infection
**Table 1. APL1A alleles display distinct protective function against *P. falciparum* infection.**

<table>
<thead>
<tr>
<th>APL1A alleles</th>
<th>EXP.</th>
<th>Inf. Prevalence (n)</th>
<th>APL1Akd</th>
<th>Inf. Prevalence (n)</th>
<th>p-value (1)</th>
<th>Combined p-value (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APL1A1</td>
<td>EXP.1</td>
<td>59% 22</td>
<td>EXP.1</td>
<td>73% 26</td>
<td>0.368</td>
<td>0.00059</td>
</tr>
<tr>
<td>APL1A2</td>
<td>EXP.1</td>
<td>41% 17</td>
<td>EXP.1</td>
<td>65% 23</td>
<td>0.194</td>
<td></td>
</tr>
<tr>
<td>APL1A3</td>
<td>EXP.1</td>
<td>28% 50</td>
<td>EXP.1</td>
<td>67% 46</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>APL1A2</td>
<td>EXP.2</td>
<td>37% 40</td>
<td>EXP.2</td>
<td>77% 35</td>
<td>0.0008</td>
<td>5.07E-07</td>
</tr>
<tr>
<td>APL1A3</td>
<td>EXP.2</td>
<td>43% 54</td>
<td>EXP.2</td>
<td>74% 68</td>
<td>0.0006</td>
<td></td>
</tr>
<tr>
<td>APL1A1</td>
<td>EXP.3</td>
<td>44% 113</td>
<td>EXP.3</td>
<td>62% 119</td>
<td>0.0050</td>
<td></td>
</tr>
<tr>
<td>APL1A2</td>
<td>EXP.3</td>
<td>33% 18</td>
<td>EXP.3</td>
<td>63% 19</td>
<td>0.104</td>
<td>0.24722</td>
</tr>
<tr>
<td>APL1A3</td>
<td>EXP.2</td>
<td>60% 23</td>
<td>EXP.3</td>
<td>71% 21</td>
<td>0.535</td>
<td></td>
</tr>
<tr>
<td>APL1A1</td>
<td>EXP.3</td>
<td>43% 53</td>
<td>EXP.3</td>
<td>52% 63</td>
<td>0.351</td>
<td></td>
</tr>
</tbody>
</table>

(1) Chi-square p-value comparing GFPkd versus APL1Akd for each experiment (EXP.).
(2) For each allele, the p-value of the 3 experiments were combined by meta-analysis.

This table presents the results from three independent APL1A gene-silencing assays (shown in Figure 4). From each mosquito both APL1A alleles were determined using the APL1A-RFLP test. For each gene-silencing treatment (GFPkd or APL1Akd) and for each experiment (EXP.), the oocyst infection prevalence (Inf. Prevalence) is given. The column labeled (n) indicates the sum of the corresponding APL1A allele in the mosquitoes of the experiment. Significance was calculated by Chi-square analysis, comparing the infection prevalence between GFPkd and APL1Akd mosquitoes for each experiment and for each allele (APL1A1, APL1A2, APL1A3). A meta-analysis using the Fisher method was also used to combine p-values of the three independent experiments.

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prevalence of $\geq 20\%$ between mosquitoes carrying different APL1A alleles, thus substantiating a robust negative result for haplotype effect. Sample sizes of haplotype combinations were too small (Figure S4) to test for genotype effects (data not shown).

**APL1A Alleles Encode Proteins with Different Subcellular Localization and Patterns of Secretion**

The large structural differences between predicted APL1A protein isoforms (Figure 3) likely have functional consequences, which could explain the different protective profiles against *P. falciparum* infection. It is not possible to raise APL1A isoform-specific antibodies due to high peptide sequence similarity between the three structural variants. Thus, to examine protein function, we transfected the *A. gambiae* hemocyte-like cell line 4a-3A [25] with constructs expressing either APL1A1, APL1A2 or APL1A3 bearing a C-terminal V5-tag. Immunostaining of transfected cells with anti-V5 monoclonal antibody (mAb) showed that the weakly protective APL1A1 isoform exhibited a diffuse distribution throughout the cytoplasm, while the protective APL1A2 and non-protective APL1A3 isoforms were essentially localized within large vesicle-like structures (Figure 5A).

Comparison of protein processing by immunoblotting of washed cells and culture medium revealed that APL1A1 was constitutively secreted into the culture medium, while APL1A2 and APL1A3 were retained in the cell cytoplasm (Figure 5B). The differential constitutive secretion is even more striking given that all proteins carry a classical N-terminal peptide signal sequence [21]. Under non-reducing conditions, APL1A1 formed two high molecular weight complexes, consistent with predicted sizes of homodimer and heterodimer, in addition to the monomeric form expected at 76 kDa (Figure 5B, left panel). In contrast, APL1A2 and APL1A3 only formed complexes with sizes consistent with homodimers. Their inability to form heterodimers with other partners may be consistent with the absence of a C-terminal coiled-coil domain, and, more importantly, with the absence of a cysteine residue corresponding to the Cys-562 of APL1C (AGAP007033), which is responsible for the disulfide-bridged complex formed between LRIM1 and APL1C (Figure 5C) [18,25]. These results suggest that the secretion of APL1A proteins may be dependent on domain interactions with binding partners. Interestingly, because these alleles are all found in nature, the observed polymorphism for the presence of a cysteine in APL1A equivalent to the APL1C C562 and consequent variation in protein partner interaction must be adaptive for APL1A function in the organism.

**Discussion**

Natural populations of *A. gambiae* display such high genetic diversity and low linkage disequilibrium that it will be difficult to carry out genetic association studies using the wild population [26,27]. This is particularly true for highly polymorphic genes, such as the *P. falciparum*-protective APL1A, coupled with the inherently noisy phenotype of *P. falciparum* infection [21]. Instead, it may be more efficient to perform initial variant discovery in a defined context that captures limited natural variation, followed by validation in the field.

In the current work, we show that the Ngousso colony has captured a repertoire of natural allelic variants of the *APL1* paralogs that result in structurally different proteins. The structurally most polymorphic paralog is APL1A, with three different alleles in Ngousso. The three APL1A alleles exist in nature, demonstrating that they are not laboratory colony mutations. Two alleles, APL1A1 and APL1A2, are also found in the *A. gambiae* G3 colony [15,21], which is much older than Ngousso and was sampled from a different geographic location in Africa [28]. Thus, the same APL1A alleles found in Ngousso have existed in nature over both space and time.

* A. gambiae colonies are expected to segregate longer haplotypes than found in nature due to small effective population size and the founder effect, and we confirmed the haplotype structure of the *APL1* locus in the Ngousso colony. Consequently, in order to specifically measure the relative contribution of the three APL1A alleles to protection against *P. falciparum*, we globally silenced all variants of the APL1A gene, determined individual phenotypes for *P. falciparum* infection, and genotyped mosquitoes for APL1A using a semi-automated assay. When APL1A is silenced, the APL1A allele accounts for essentially all of the dsAPL1A effect on *P.
Figure 5. Differential secretion pattern and sub-cellular localization of APL1A-V5 alleles in hemocyte-like cell culture. A) Immunofluorescence analysis of 4a-3A hemocyte-like cells transfected with plasmids encoding V5-tagged APL1A1, APL1A2 and APL1A3. Cells were stained with Hoechst 33342 to label nuclei (blue). Staining with a mouse anti-V5 mAb followed by anti-mouse Alexa 488-conjugated IgG (red) indicates that APL1A1 exhibits a diffuse pattern in the cytoplasm whereas APL1A2 and APL1A3 are essentially localized in vesicles. Pictures were taken under equivalent exposure conditions. B) Immunoblot analysis of cells (C) and culture medium (M) of the 4a-3A hemocyte-like cell line transfected with plasmids encoding V5-tagged APL1A1, APL1A2 and APL1A3 under reducing (R) and non-reducing (NR) conditions. Immunoblots were probed with a mouse anti-V5 mAb, protein quantities on the different blots are not comparable. APL1A1 is secreted in the culture medium as at least two protein complexes under non-reducing conditions, whereas APL1A2 and APL1A3 are retained in the cell cytoplasm and form only one protein complex under non-reducing conditions. Estimated sizes of monomeric APL1A forms including V5-tag are: 76 kDa (APL1A1), 60 kDa (APL1A2) and 51 kDa (APL1A3). C) Amino acid alignment of cysteine-rich regions of the three Ngousso APL1A proteins and *A. gambiae* PEST APL1C. Numbers correspond to the amino acid positions. Blue stars represent stop codons, cysteine residues are highlighted in red. The cysteine residue corresponding to the position 562 in the APL1C sequence (APL1CC562, black arrow) described by Povelones et al. [18], is involved in the disulfide-linked complex formed between LRIM1 and APL1C and is only conserved in APL1A1. The same cysteine is also referred to as APL1CC551 in the APL1C protein published by Baxter et al. [34].

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Plasmodium falciparum infection prevalence. The APL1A1 allele has weak or no effect, and APL1A2 has no influence on infection outcome. We did not detect an association between the haplotype bearing the APL1 locus and infection outcome, but the haplotypes have captured linked variation for an unknown number of genes, including ones that may be able to influence infection phenotype.

The considerable structural differences of APL1 proteins, particularly of APL1A, suggest that the isoforms may interact with distinct protein partners. This hypothesis is supported by the different migration profiles of APL1A isoforms under non-reducing conditions. The APL1C protein forms a complex with LRIM1 via conserved cysteine residues, and this complex can bind, through the coiled-coil domain, to certain TEP proteins [17,18]. Interestingly, APL1A2 and APL1A3, which lack a coiled-coil domain, form only one type of dimer under non-reducing conditions and are retained in the cytoplasm of hemocyte-like cells, whereas APL1A1, which has a coiled-coil domain and forms at least two different dimers, is secreted from the cell. The observed dimers of APL1A2 and APL1A3 show a size consistent with homodimers, though we cannot rule out that they could be heterodimers with unknown binding partners of similar molecular weight.

The mechanism underlying APL1A secretion remains elusive and it is not clear whether the secretion pattern is related to protection against Plasmodium infection. The behavior of APL1A alleles in culture does not necessarily translate to what happens in vivo. For example, absence of secretion of APL1A2 and APL1A3 may indicate that these alleles are not correctly folded in 4a-3A cells or that epitope tagging impairs their secretion. In addition, the APL1A3 allele lacks the Cys-Cys pair common to the entire LRIM family of LRR proteins (highlighted in Figure 5G) and this motif has been experimentally shown to be important for LRIM1 folding and secretion [18]. This suggests that APL1A3 may not be correctly folded and secreted even in vivo. Moreover, if APL1A secretion is dependent on interaction with binding partners, the putative APL1A2 and APL1A3 binding partners may not be expressed in 4a-3A cells. An alternative hypothesis could be that APL1A1, APL1A2 and APL1A3 are secreted through distinct mechanisms, and that the variants participate in resistance against Plasmodium in different ways. In vivo, APL1A1 might be constitutively secreted, while APL1A2 and APL1A3 secretion might be stimulated by immune elicitors or other factors. The potentially constitutive presence of APL1A1 in the hemolymph, if associated with a long protein half-life, could then explain why gene silencing experiments did not show a consistent protective effect for the APL1A1 allele. Finally, another hypothesis is that APL1A2 functions as a dominant negative inhibitor of a factor required for P. falciparum development. Further work will be necessary to distinguish between these possibilities.

Our findings highlight the need to consider allelic variation of putative immune factors, including the spectrum of alleles segregating in a colony, in studies aimed at deciphering their function. We also demonstrate the utility of performing a discovery step in a recent colony that segregates a defined set of allelic variants that can be confirmed to exist in nature. The long haplotypes in colonies limit resolution, so in general it may not be meaningful to directly test genotype-phenotype association in colonies, because the allele genotypes are just a proxy for the longer haplotype. Rather, it is best to distinguish the effects of the candidate gene effects, and the influence of the carrying haplotype, either by allele-specific silencing when possible [22], or if the alleles offer insufficiently distinct dsRNA targets as for APL1A1, by global gene silencing with allele-specific genotyping as we did here. The information gained should then permit the design of statistically well-powered tests of small numbers of candidate variants in the natural population, where resolving power is greater due to the low linkage disequilibrium. Based on the current findings, it would be interesting to directly compare phenotypic outcomes of APL1A1, APL1A2 and APL1A3 carriers in the wild population after challenge with P. falciparum.

Materials and Methods

Mosquito Rearing

The A. gambiae s.s. colony Ngousso was established with mosquitoes captured in Yaounde, Cameroon in January 2006 [29]. The mosquitoes are of the M molecular and Forest chromosomal form, fixed for the standard 2La chromosomal inversion. The colony was reared at the CEPIA mosquito production facility at the Institut Pasteur under standard rearing conditions at 26°C and 80% relative humidity, under a 12 h light/dark cycle as described elsewhere [29].

P. falciparum Gametocyte Culture and Ngousso Infection

Parasite culture and experimental feedings with the P. falciparum isolate NF54 were done as previously described [11]. Briefly, P. falciparum NF54 was cultured using the automated tipper-table system of Ponnudurai [30] implemented in the CEPIA mosquito facility of Institut Pasteur. Fourteen days after initiating the parasite subculture and prior to each infection experiment, gametocyte maturity was assessed by testing exflagellation of male microgametes. Gametocytaemia and proportions of mature male and female gametocytes were determined on Giemsa stained slides (Table S2).

For an infectious blood meal, 10 ml of the gametocyte culture was then centrifuged at 2000 rpm, and the cell pellet was resuspended in an equal volume of normal type AB human serum. The infected erythrocytes were added to fresh erythrocytes in AB human serum and transferred into a membrane feeder warmed to 37°C. Female mosquitoes (4–5 days old) were allowed to feed for 15 min, unfed mosquitoes were removed and only fully engorged females were maintained on 10% sucrose solution for further analysis.

Each infection experiment replicate was done with a new generation of Ngousso females and a new gametocyte culture of P. falciparum NF54.

Analysis of Infection Phenotypes

As infection phenotypes we analyzed prevalence and intensity. Infection prevalence is the fraction of mosquitoes carrying at least one oocyst, while parasite intensity is the number of oocysts per mosquito determined only in the subset of mosquitoes with ≥1 oocyst.

To determine infection phenotype, midguts of blood-fed females were dissected 7–8 days post-infection, stained in 1×PBS buffer with 0.4% mercury dibromofluorescein (Sigma) and the number of oocysts per midgut was determined using a light microscope. Carcasses of the dissected mosquitoes were immediately transferred individually into a fresh tube and stored at −20°C until DNA extraction. Amongst the infection experiments for the association study we analyzed only those trials where infection prevalence was between 35% and 35% (Table S2A). Differences in infection prevalence were analyzed using the Chi-Square test. For intensity-related infection, non-parametric statistical tests were used, including the Mann-Whitney Rank Sum
Test and the Kruskal-Wallis ANOVA on ranks, excluding mosquitoes with zero oocysts. Three independent replicate infections were performed and data were pooled prior to statistical analysis.

DNA Preparation
Genomic DNA was extracted from individual female mosquitoes by homogenizing in 100 µl DNAzol (Invitrogen, CA, USA) using a disposable pestle, essentially following the manufacturer’s protocol.

PCR Reactions
The APL1 paralogs correspond to the VectorBase identifiers APL1A (AGAP067036), APL1B (AGAP067033) and APL1C (AGAP067033). PCR primers were designed based on the sequence of the A. gambiae PEST reference genome or on available Ngousso sequences. Primers were designed to bind in the exon regions of the APL1 genes. Genomic DNA of a single mosquito was used for PCR and sequencing reactions. For evaluation of size differences of PCR fragments, amplification reactions were performed in a final volume of 20 µl using Taq DNA polymerase (Invitrogen). PCR cycles were as follows: a denaturation step at 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 sec, 62°C for 45 sec, 72°C for 3 minutes and a final extension of 72°C for 10 minutes. Primers and their positions are given in Table S1.

DNA amplification for sequencing was done using AccuPrime SuperMixII (Invitrogen). Primers carried a 5’t extension for sequencing with the universal forward (M13, 5’TGT AAA TCG GAC GGG AGC TGC TTT CGA GTG GTA TGA TTG GCG AG 3’) or reverse (M13reverse, 5’TGT AAA TCG GAC GGG AGC TGC TTT CGA GTG GTA TGA TTG GCG AG 3’) primers [31]. Final reaction volume was 30 µl and cycling conditions for amplification used denaturation at 94°C for 3 minutes, followed by 40 cycles at 94°C for 30 sec, 62°C for 45 sec and 72°C for 3 minutes and a final extension step of 72°C for 10 minutes.

Sequencing
PCR products were sequenced using ABI Big Dye Terminator v.3.1 Cycle Sequencing kit (LifeTechnologie) and an ABI Prism 3730 DNA Analyzer (Applied Biosystems). The sequences were assembled using CodonCode Aligner (CodonCode Corporation). Heterozygous peaks were identified manually. Sequences were analyzed using eBioX/eBiotools [32]. Coordinates of features including lengths of the indels are based on the PEST reference sequence. APL1 sequences were deposited into Genbank under accession numbers JX292981 to JX292986.

Development of a RFLP Assay to Determine the APL1A Alleles in Ngousso
We developed a high-throughput Restriction Fragment Length Polymorphism test (RFLP) to facilitate the identification of the APL1A alleles present in Ngousso mosquitoes. This assay takes advantage of the allele specific indel patterns in the APL1A genes. These indel patterns are stable in the Ngousso colony, distinguish the APL1A allelic variants and provide information on the haplotype of the APL1 region.

The APL1A-RFLP test is based on the PCR fragment spanning the complete APL1A coding-region. The full-length amplicon is generated with two fluorescent primers labeled with different fluorophores and then cleaved with PstI. Resulting fluorescent-labeled restriction fragments, which span the indel bearing regions at the 5’ and 3’ end of the APL1A alleles (Figure 2) are distinguishable by size and color. Fragment separation and sizing were done on an ABI Prism 3730 DNA Analyzer. In combination, the two sized, end-labeled fragments provide information about the presence or absence of the PANGGL repeat region and determined the patterns of the small indels in the 3’region of the APL1A gene based on alterations of the restriction fragment length. The APL1A-RFLP distinguishes the three allelic APL1A variants present in the Ngousso laboratory colony and all combinatorial genotypes of the APL1A alleles were found in the colony.

Double-stranded RNA Synthesis and Injection
Double-stranded RNAs were synthesized from PCR amplicons using the T7 Megascript Kit (Ambion) as described previously [11]. 500 ng of dsAPL1A and dsGFP in a maximum volume of 200 µl were injected into the thorax of cold-anesthetized 1 day-old A. gambiae females using a nano-injector (Nanoject II; Drummond). Mosquitoes were challenged with P. falciparum parasites 4 days after dsRNA injection.

Gene Knockdown Verification
The efficiency of transcript knockdown was monitored 4 days after dsRNA injection. cDNA synthesis was performed by using M-MLV reverse transcriptase and random hexamers (Invitrogen). In each case, 500 ng of total RNA was used in triplicate assays. The triplicates were pooled and the mixture was used as template for PCR analysis. Gene knockdown verification was performed as described in Mitri et al. 2009 [11].

Insect Cell Culture and Transfection
A. gambiae derived 4a-3A hemocyte-like cells were cultured in monolayer at 27°C in Insect Xpress medium (Lonza) supplemented with 5% foetal bovine serum (GIBCO BRL) and 50 µg/ml gentamycin (Sigma). The three APL1A alleles were amplified from selected mosquitoes with known APL1 haplotypes by PCR using the following primers flanking the coding regions of each gene: APL1A5’EcoRI (5’TGGG AAT TCC CTC TTT CGA GTC TAA TG 3’), APL1A3’V5XbaI (5’TGG GTG GTA TAG TTG GCG AG 3’), APL1A3’EcoRI (5’TGGGAAT TCC CTT GTA CGA GTA GAA TCC CTG TTT CGA GTG CTA TAA TG 3’), APL1A3’V5XbaI (5’TGG GTG GTA TAG TTG GCG AG 3’), APL1A5’EcoRI (5’TGGG AAT TCC CTC TTT CGA GTC TAA TG 3’), APL1A3’V5XbaI (5’TGG GTA GAA TCC CTG TTT CGA GTA CGA GTA GAA TCC CTG TAA TG 3’), APL1A3’V5XbaI (5’TGG GTG GTA TAG TTG GCG AG 3’). The amplicons were cloned into a dual His and V5-tag insect expression vector (pAc5.1 V5/His, Invitrogen) or a dual Strep and V5-tag insect expression vector that was modified to replace the His-tag by a Strep-tag. 4a-3A cells were co-transfected with the expression vector and the selection vector pCoBlast Puromycin in a ratio 9:1.

Mosquitoes were challenged with P. falciparum parasites 4 days after dsRNA injection.

Western Blotting
To analyze the secretion pattern for tagged proteins, the culture medium was collected and spun for 10 min at 800 rpm to remove floating cells and large debris. Adherent cells were washed in 1×PBS and collected by scraping in 10 mM Tris pH 8. Proteins from culture medium and cells were extracted in XT sample buffer (Bio-Rad), heated at 95°C for 5 min and separated on 4–12% Criterion SDS-PAGE gels (Bio-Rad). Reduced samples were prepared by adding XT reducing agent (Bio-Rad) before heating. After protein transfer to PVDF membrane, immunoblots were
blocked for 1 hour in 5% nonfat dry milk, probed with a mouse mAb anti-V5 antibody at 1:5000, followed by 1 hour with horseradish peroxidase-conjugated anti-mouse IgG secondary antibodies (Promega) at 1:10,000. The detection step was performed using the Pierce chemiluminescence system (Pierce) following the manufacturer’s instructions. Immunofluorescence Assays

Transfected cells were allowed to grow in an 8-well Lab-Tek chamber slide system (Thermo Scientific) for one hour. The culture medium was then removed, the cells were washed in 1xPBS, fixed in 1xPBS with 4% paraformaldehyde for 30 min at room temperature, and permeabilized in 0.2% Triton X-100 and 5% Bovine Serum Albumin in PBS 1X for 45 min at room temperature. Cells were incubated with a mouse mAb anti-V5 antibody at 1:500 in 1xPBS containing 10% foetal bovine serum for 1 hour at 4°C, followed by 1 hour with Alexa Fluor 594-conjugated anti-mouse IgG secondary antibody at 1:2000 and Alexa Fluor 488-conjugated phalloidin (Molecular Probes) at 1:500 in 1xPBS 1X. Nuclei were stained with Hoechst 33342. After mounting in SlowFade Gold antifade reagent (Molecular Probes), samples were observed at 100x magnification using a Leica DM 5000 B fluorescent microscope.

Supporting Information

Figure S1 Alignment of genomic regions coding for APL1A, APL1B and APL1C alleles in Ngousso mosquitoes. Nucleotide sequences have been aligned using ClustalW. Prettyplot (EMBOSS package) has been used for boxing and colouring with plurality = 4 to calculate the consensus. Red colour indicates nucleotides identical to the consensus. Start codons of the APL1 alleles are highlighted in blue and the stop codons in green. The deletions-insertions described in Supplementary Information are indicated by arrows.

Figure S2 Observed infection intensities in silencing experiments. The experiment number (Exp 1, Exp 2 and Exp 3) and the RNAi knockdown target are shown on the horizontal axis. The number of infected mosquitoes (n) from each knockdown experiment is indicated. GFPkd was used as a control dsRNA. The vertical axis shows the number of midgut oocysts 7–8 days following a P. falciparum infectious blood meal. The median number of oocysts is indicated by the solid horizontal bar.

Figure S3 Observed infection intensities without knockdown. The experiment number (Inf1, Inf2 and Inf3) is shown on the horizontal axis. The number of infected mosquitoes (n) from each experiment is indicated. The vertical axis shows the number of midgut oocysts 7–8 days following a P. falciparum infectious blood meal. The median number of oocysts is indicated by the solid horizontal bar.
Table S2 Parameters of the *P. falciparum* gametocyte (NF34) cultures and results of the different infection experiments. (PDF)

Supplementary Information S1 (PDF)

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References

1. TDR/WHO (2012) Malaria

Author Contributions

Conceived and designed the experiments: IH CL CM TG KE KDV. Performed the experiments: IH CL CM TG KE MMR. Analyzed the data: IH CL CM TG KE MMR. Contributed reagents/materials/analysis tools: EBF EB ET IT AZ SP CB. Wrote the paper: KE CL CM TG IH MMR KDV.