# Association of Malaria Parasite Population Structure, HLA, and Immunological Antagonism

Sarah C. Gilbert,* Magdalena Plebanski,* Sunetra Gupta, Joanne Morris, Martin Cox, Michael Aidoo, Dominic Kwiatkowski, Brian M. Greenwood, Hilton C. Whittle, Adrian V. S. Hill†

Host-parasite coevolution has been likened to a molecular arms race, with particular parasite genes evolving to evade specific host defenses. Study of the variants of an antigenic epitope of *Plasmodium falciparum* that induces a cytotoxic T cell response supports this view. In African children with malaria, the variants present are influenced by the presence of a human leukocyte antigen (HLA) type that restricts the immune response to this epitope. The distribution of parasite variants may be further influenced by the ability of cohabiting parasite strains to facilitate each other's survival by down-regulating cellular immune responses, using altered peptide ligand antagonism.

There is increasing evidence that HLAs are subject to ongoing selection pressures by infectious pathogens, supporting the theory that natural selection by such parasites plays the central role in maintaining major histocompatibility complex (MHC) polymorphism (*1*, *2*). However, an understanding of the precise nature of this process has been impeded by a lack of field data from natural host-parasite populations on interactions between polymorphic antigens of the host and variable components of the parasite. The life cycle of the malaria parasite *Plasmodium falciparum* provides an opportunity to measure the outcome of a specific immunological interaction between genetic variants of a parasite and the MHC type of its host. This possibility arises from the occurrence of an HLA class I–restricted cytotoxic T lymphocyte (CTL) response against the parasite at the early liver-stage of infection (*3*, *4*). The response occurs directly after inoculation of malaria sporozoites by an infectious mosquito; analysis of parasites in the blood of infected individuals reveals which parasite variants (here termed "strains") have survived this potential in vivo selection step and have reached the subsequent blood-stage of the life cycle.

Studies of CTL responses to variant epitopes in human immunodeficiency virus (HIV) and hepatitis B virus have shown that some naturally occurring variants of these epitopes may specifically down-regulate the CTL response through altered peptide ligand (APL) antagonism, at least in in vitro assays (*5*, *6*). Thus, the simultaneous presence of the variant antagonist epitope appears to deliver an altered signal to the responding T lymphocyte that induces nonresponsiveness, or limited responsiveness, to its target agonist epitope.

**Antagonism of malaria CTL epitopes.** CTLs from individuals exposed to endemic malaria recognize epitopes in a variety of pre-erythrocytic antigens of *P. falciparum*, and indirect evidence indicates that these CTLs may play a role in protective immunity (*7*). CTLs restricted by the commonest HLA class I molecule in The Gambia, HLA-B35, often recognize a polymorphic epitope in a variable region of the circumsporozoite (CS) protein. Of the four allelic variants that are prevalent among strains of parasite in The Gambia, two (cp26 and cp29) are CTL epitopes that bind HLA-B35 (*4*); the other two (cp27 and cp28) are not epitopes and failed to bind to HLA-B35 in in vitro assembly (binding) assays (*8*). The cp26 and cp29 peptides are octamers that differ only at the second amino acid position, yet each interferes with activation of memory CTLs by the other (Fig. 1). In CTL responses to these malaria epitopes, cp29 was able to antagonize cp26-specific CTLs, and cp26 was able to antagonize cp29-specific CTLs, even when present on different antigen-presenting cells and at low antagonist:agonist ratios. A panel of cp26- and cp29-specific CTL lines generated from both malaria-naïve (Fig. 1A) and malaria-immune (Fig. 1, B through D) individuals were antagonized at the effector level. We also found that cp26 and cp29 could mutually interfere with the induction in vitro of primary CTL responses by the other peptide epitope in individuals unexposed to malaria. Hence, T cell responses to *P. falciparum* epitopes may be added to the list of immune responses shown to be subject to APL antagonism in vitro. However, the importance of the phenomenon in vivo and its possible impact on host-parasite evolution have been uncertain. Thus, we proceeded to examine the distribution of the allelic epitopes cp26 and cp29 among Gambian children with *P. falciparum* malaria.

***P. falciparum* population structure.** Table 1 shows the frequencies of the allelic variants of the CS gene in parasite DNA from Gambian children with clinical malaria. Parasite DNA was amplified from pe-

**Table 1.** Frequency of infection with cp26 to cp29 strains in 795 Gambians with malaria. The expected frequency of single and mixed infections with each of the strains, assuming random mixing, are shown for different values of the parasite rate (PR) in the population. The actual PR in the locality from which the malaria cases were sampled (from August to November) is in the range of 10 to 50%. Analysis of the distribution showed cp26 and cp29 present together significantly more often than expected, whatever the PR (*, $\chi^2$ = 10–50; **, $\chi^2$ = 50–100; ***, $\chi^2 > 100$, $P < 10^{-20}$) (*23*). Methods are described in (*24*).

| Phenotype | Observed | Expected at PR of | | |
|---|---|---|---|---|
| | | 100% | 50% | 10% |
| 26 only | 36 | 58 | 138** | 195*** |
| 27 only | 405 | 257** | 329* | 378 |
| 28 only | 15 | 10 | 30 | 48* |
| 29 only | 16 | 33 | 89** | 134*** |
| 26 and 29 | 124 | 22** | 22** | 6*** |
| 27 and 29 | 4 | 99** | 53* | 11 |
| 26 and 27 | 71 | 171* | 82 | 16* |
| 26 and 28 | 2 | 7 | 7 | 2 |
| 28 and 29 | 1 | 4 | 5 | 1 |
| 27 and 28 | 34 | 29 | 18 | 4* |
| 26, 27, 29 | 58 | 66 | 13* | 0** |
| 27, 28, 29 | 2 | 11 | 3 | 0 |
| 26, 27, 28 | 9 | 19 | 4 | 0 |
| 26, 28, 29 | 6 | 3 | 1 | 0 |
| 26, 27, 28, 29 | 12 | 7 | 1 | 0* |

S. C. Gilbert and M. Cox are at Wellcome Trust Centre for Human Genetics, Nuffield Department of Medicine, University of Oxford, Windmill Road, Oxford OX3 7BN, UK. M. Plebanski and M. Aidoo are at Molecular Immunology Group, Institute of Molecular Medicine, Nuffield Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK. S. Gupta is at Wellcome Trust Centre for the Epidemiology of Infectious Diseases, Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK. J. Morris is at Department of Epidemiology and Population Sciences, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK. D. Kwiatkowski, B. M. Greenwood, and H. C. Whittle are at Medical Research Council Laboratories, Fajara, Post Office Box 273, The Gambia. A. V. S. Hill is at Wellcome Trust Centre for Human Genetics, Nuffield Department of Medicine, University of Oxford, Windmill Road, Oxford OX3 7BN, UK, and Molecular Immunology Group, Institute of Molecular Medicine, Nuffield Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK.

*These authors contributed equally to this research article.

†To whom correspondence should be addressed. E-mail: adrian.hill@imm.ox.ac.uk

ripheral blood, so these parasites have survived clearance at the earlier liver-stage of infection. Infection by more than one variant was found in 41% of the children, similar to previous studies (9). The distribution of parasite strains differed markedly from that expected under random mating without selection. In particular, cp26 and cp29 were found together more than twice as frequently, as expected from their prevalence in the host population ($P < 1 \times 10^{-8}$). Pairing of cp26 with cp29 (with or without cp27 or cp28 as well) was present in children with HLA-B35 [observed, 55; expected, 12; at 50% parasite rate (PR) in the population] and without HLA-B35 (observed, 144; expected, 26; at 50% PR). There was no difference in the distribution of strains between severe ($n$ = 482) and mild ($n$ = 313) malaria cases.

We then assessed whether the presence of HLA-B35 affected the distribution of the four allelic types in the host population (Table 2). We found cp26 and cp29 more frequently in children with HLA-B35 ($P$ = 0.012). This association was independent of numerous potentially confounding factors, and similar analysis of the possible influence of 12 other class I and class II alleles on the distribution of these parasite variants showed no significant association with these host alleles.

Several lines of evidence suggest that the observed cohabitation results from a functional effect of the cp26, cp27, cp28, and cp29 variation itself, rather than of other parasite genes. Genetic recombination occurs during the sexual stage of the *P. falciparum* life cycle, and field studies (*10*) do not show evidence of clonality. Furthermore, analysis of a polymorphic region of the CS gene only 180 base pairs 5′ to the epitopes studied (*11*) revealed in this parasite population only very weak linkage disequilibrium between these alleles. The strongest allelic association (Table 1) is between cp26 and cp29, suggesting that this is the primary interaction. Finally, the differing frequencies of the variants that are epitopes for HLA-B35 in hosts with and without this HLA type support a mechanism involving the antigen-presenting function of this molecule.

Our immunological findings on APL antagonism suggest that the cohabitation of cp26 and cp29 could result from these strains facilitating each other's survival through mutual antagonism of CTL to the other variant at the liver stage of infection. But can this mechanism account for the cohabitation observed in individuals both with and without HLA-B35?

**A model of antagonism.** To address this question quantitatively, we developed a mathematical model (*12*) to explore the dynamics of two parasite strains within two different host classes. In individuals with HLA-B35, each strain (that is, cp26 or cp29) elicits short-lived pre-erythrocytic strain-specific infection-blocking immunity. Individuals without HLA-B35 develop short-lived pre-erythrocytic non–strain specific immunity (acting equally against cp26 and cp29) upon infection with either strain. We assume that there is very little cross-reactivity between immune responses to cp26 and cp29, as indicated by the data on secondary CTL responses (Fig. 1) (*4*).

We incorporated APL antagonism into this system at both the effector and induction level. HLA-B35 individuals who are already immune to a given strain (for example, cp26) would, in the absence of effector-level antagonism, become infected with the other strain (cp29) when subjected to simultaneous inoculation. The degree of effector-level antagonism may thus be represented by a parameter, $\alpha$, which determines the probability that an individual immune to a given strain will become infected with both strains upon simultaneous inoculation, as a result of the existing CTL being antagonized. Induction level antagonism is incorporated by assuming that no immunity develops after simultaneous inoculation with cp26 and cp29.

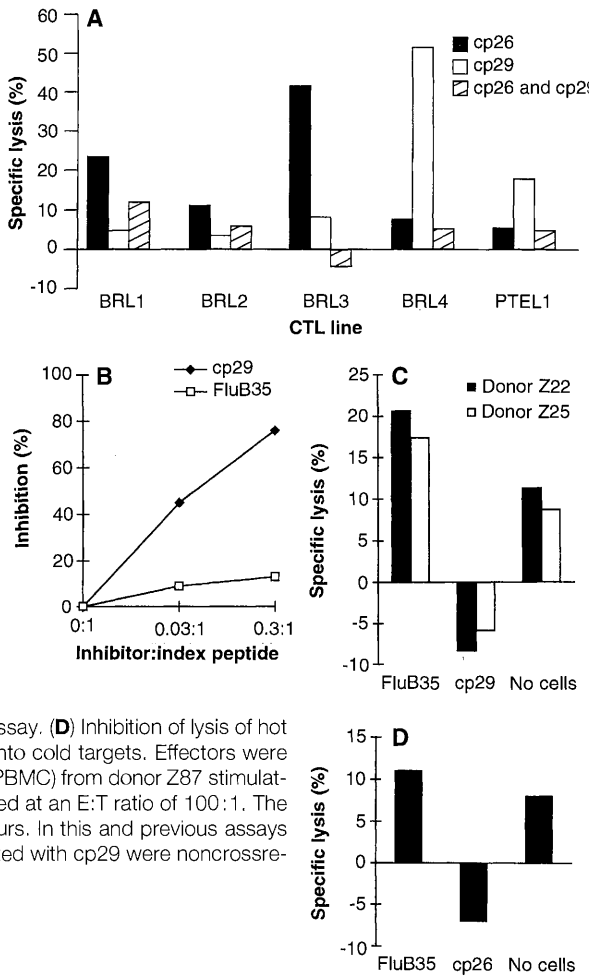


**Fig. 1.** Reciprocal CTL antagonism in PBMC cultures by two naturally occurring *P. falciparum* CS protein variants. See (*8*, *21*). (**A**) Inhibition of lysis of cp26- or cp29-pulsed cells by cp29 or cp26 at an antagonist: index peptide ratio of 1:1. Effector CTL lines were generated from malaria-naïve donors BR and PTE as described (*26*) and used at an E:T ratio of 20:1. Specific lysis is shown at 4 hours for BRL1 and BRL2, and at 18 hours for BRL3, BRL4, and PTEL1. (**B**) Inhibition of lysis of cp26 pulsed cells by cp29 at antagonist: index peptide ratio of 0.3:1-0.03:1 (*27*). (**C**) Inhibition of lysis of labeled (hot) cp26-pulsed cells by cp29 variant pulsed onto unlabeled (cold) targets. As a control, FluB35 peptide pulsed cold targets were used. The assay shown was harvested at 4 hours. Effectors were PBMC from donor Z22 or Z25 stimulated with cp26 14 days before and used at an E:T ratio of 50:1. Similar levels of antagonism were again observed using cells from this donor in a subsequent study 12 months after this assay. (**D**) Inhibition of lysis of hot cp29-pulsed cells by cp26 pulsed onto cold targets. Effectors were peripheral blood mononuclear cells (PBMC) from donor Z87 stimulated with cp29 14 days before and used at an E:T ratio of 100:1. The assay shown was harvested at 8 hours. In this and previous assays (*4*), PBMC from donor Z87 restimulated with cp29 were noncrossreactive with cp26.

**Table 2.** Frequency of cp26 and cp29 in individuals with HLA-B35 compared to the rest of the population. There is an increased occurrence of cp26 and cp29, both together and separately, in individuals with HLA-B35. This association was found in both individuals with mixed [$P$ = 0.02, OR = 1.54 (1.05–2.27)] or single infections [$P$ = 0.006, OR = 2.3 (1.20–4.36)]. HLA type was determined by PCR as described (*4*). Analysis is described in (*25*).

| Parasite type | HLA-B35 ($n$ = 304) | | Non–HLA-B35 ($n$ = 903) | |
|---|---|---|---|---|
| | $n$ | Percent | $n$ | Percent |
| cp26 | 92 | 30.3 | 223 | 24.7 |
| cp27 | 140 | 46.1 | 450 | 49.8 |
| cp28 | 10 | 3.3 | 71 | 7.9 |
| cp29 | 62 | 20.4 | 159 | 17.6 |
| cp26 or cp29* | 154 | 50.7 | 382 | 42.3 |

*For all infections, $P$ = 0.012 and odds ratio (OR) = 1.4 [95% confidence intervals, 1.07–1.83].

Figure 2 shows how the distribution of parasites within the two host classes changes in the model with increasing effector-level antagonism, when there is total antagonism at the induction level. We assume in this example that cp29 is more likely to induce protective immunity than cp26, consistent with the observations that cp29 binds better than cp26 to HLA-B35 and induces primary CTL more readily (*8*, *13*). Two nonintuitive results are apparent. Firstly, a small difference in duration of immunity to cp26 and cp29 in a small segment of the host population (the 30% with HLA-B35) can translate into a large difference in the relative population prevalence of the two strains. This is because the two strains are in direct competition with each other within the non–HLA-B35 host population as a result of strain-transcending immunity (acting equally against cp26 and cp29) (*14*). Second, APL antagonism can act to structure the parasite population such that there is a preponderance of mixed infections. A high degree of antagonism is required at the effector level as well as at the level of induction of CTL responses; the latter alone is insufficient to generate the observed levels of mixed infections (*15*). Importantly, the altered parasite population structure is reflected in both the HLA-B35 and the non–HLA-B35 populations in proportions commensurate with the levels of immunity induced by the relevant epitopes. Levels of infection with cp26 and cp29 in non–HLA-B35 hosts, for whom these are not the immunodominant epitopes, can either be higher or lower than in HLA-B35 hosts who do respond in an epitope-specific manner (Fig. 2).

**Evolutionary, immunological, and parasitological implications.** We have shown previously that HLA type affects a child's risk of developing severe malaria in this West African population (*1*). Here, we report that HLA class I antigens may affect the strain of parasite found in children with malaria. Both observations are compatible with an effect of HLA class I–restricted CTLs acting at the liver stage of *P. falciparum* infections. However, the present observations have particular implications for the coevolution of host and parasite diversity. The different distribution of allelic types of the CS gene in children with and without HLA-B35 provides evidence of an ongoing selective effect of HLA antigens on the strain of parasite causing blood-stage malaria infections. This, in turn, will influence the strain of parasite that is transmitted to further individuals and, ultimately, the population prevalence of the strains. Over time, such small effects are sufficient to markedly affect parasite frequencies. The cohabitation of the alleles cp26 and cp29 can be explained by mutual down-regulation of each other's CTL responses at the liver stage of infection. Although this will occur only in HLA-B35–positive individuals, the mathematical model indicates that, over time, the cohabitation should spread to individuals of all HLA genotypes, consistent with the observed lack of a detectable effect of HLA genotype on the extent of cohabitation. Thus, host HLA type may affect the parasite allele distribution, but the converse also applies. The observed antagonistic interactions of parasite alleles implies that geographical variation in parasite allele frequencies may affect the local magnitude of HLA associations with infectious diseases and thus the selection pressure exerted by that parasite on HLA frequencies.

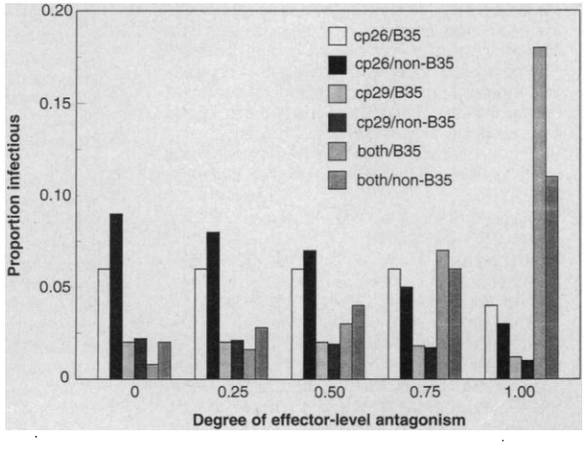


**Fig. 2.** Output of the model. The proportions of hosts infectious for cp26, cp29, and both, in hosts with HLA-B35 and without HLA-B35 is dependent on the degree of effector-level antagonism. As this increases, there is a nonlinear increase in cohabitation. There is also a reversal from lower to higher levels of cp26 and cp29 variants in HLA-B35 compared to non-HLA-B35 hosts. In the absence of antagonism, there is more infection in the non-HLA-B35 hosts because their average duration of immunity to any strain is effectively only 0.5 years ($\gamma$ = 0.01) compared to 1.25 years for cp26 ($\gamma_{cp26}$ = 0.025) and 5 years for cp29 ($\gamma_{cp29}$ = 0.1) in the HLA-B35 hosts. However, as antagonism increases, the advantage this provides to parasites within the HLA-B35 population outweighs the effects of higher levels of immunity, leading to levels of infection that are higher than in the non-HLA-B35 population, which responds more weakly to other epitopes in parasites bearing these variants. Other parameter values used are as follows: $\beta$ = 50, $\sigma$ = 10, $\delta$ = 0.975, $\mu$ = 0.02 (*12*).

*Plasmodium falciparum* strains may be structured at a primary level by immune responses against a polymorphic and antigenically varying blood-stage antigen, PfEMP1 (*16*). The observations on HLA-B35 and CS variants we present may thus reflect a particular substructure of the parasite population that results from interaction between a less effective liver stage–specific immune response and its target epitope. This raises the possibility that several such substructures of association between particular host and parasite variants might be observed as the result of each parasite locus evolving away from the specific host molecule regulating immune responses to it.

The discovery of APL antagonism has been of considerable immunological interest, in part because it might allow interventions to specifically down-regulate pathological immune responses. Although it has been observed for both HLA class I– and class II–restricted T cell responses, and in CTL responses to hepatitis B and HIV-1 infections (*5*, *6*), the in vivo significance of antagonism and the potential evolutionary advantage of this type of putative immune escape mechanism have been unclear. As discussed elsewhere (*17*), it might appear more efficacious for pathogens to simply evade an immune response by mutations that prevent binding of epitopes to MHC molecules rather than by those that produce APL antagonism. A novel feature of the antagonist peptide epitopes displayed here is that the antagonism is mutual. The antagonist is effective at very low antagonist:agonist ratios and, unusually, inhibits the polyclonal CTL response (*18*) to the agonist. These unusual features suggest that this pair of antagonists might reflect natural selection in the malaria parasite of variants that are mutually inhibitory. The data on the CS allele distributions argue that antagonism is employed as an escape mechanism in natural pathogen populations. They also suggest that malaria parasites may manifest strains with mutually antagonistic epitopes because the use of an antagonist is a more efficient method of escaping an immune response than avoidance of MHC binding: the mutually antagonistic variants cp26 and cp29 were found more frequently in HLA-B35 individuals than were the nonbinders cp27 and cp28. We speculate that, by maintaining binding, cp26 and cp29 may prevent the emergence of an immunodominant response to another HLA-B35–restricted epitope in *P. falciparum* such as the ls8 epitope in liver-stage antigen-1 (*4*).

Some malaria vaccine programs are

aimed at inducing CTL responses against epitopes expressed by the liver-stage parasite (*19*). Our results imply that inclusion of all allelic peptide variants in such vaccines may not offer a solution to the well-recognized problem of antigenic polymorphism in malaria: inclusion of a variant epitope might even lead to antagonism of naturally acquired immunity to other variants, resulting in increased susceptibility.

This study has identified a specific association between variable components of a parasite and its mammalian host. Combined genetic, immunological, and mathematical analyses of further such examples from natural populations should provide a molecular understanding of the mechanisms driving host-parasite coevolution.

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8. Peripheral blood mononuclear cells (PBMCs) from malaria-exposed individuals determined to be responders to cp26 or cp29 12 months before (*7*) were stimulated in vitro with peptides cp26 (KPKDELDY) (*20*) and cp29 (KSKDELDY). Binding of the peptides cp26 and cp29 was assessed in HLA assembly assays using the T2 cell line transfected with HLA-B35, as described [J. Elvin, V. Cerundolo, T. Elliott, A. Townsend, *Eur. J. Immunol.* **21**, 2025 (1991)]. The cp27 and cp28 peptides failed to show binding at 100 μM; cp26 and cp29 showed 50% maximal assembly at 30 μM and 2 μM, respectively. The induction of primary CTL responses with these peptides has also been explored in detail (*13*). In brief, the generation of primary CTL lines from malaria-naïve donors by peptide cp26 was inhibited 91% in the presence of an equimolar dose of cp29 ($n = 4$), and the generation of lines by peptide cp26 was inhibited 64% by an equimolar dose of cp29 ($n = 4$). The cp29 peptide induced short-term CTL lines from malaria-naïve donors threefold more efficiently than did cp26 at the same peptide concentration. Methods are described in (*21*).
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12. We consider two host genotypes, A and B, and two parasite strains corresponding to specific epitopes (that is, cp26 and cp29) recognized by hosts of genotype A (that is, HLA-B35). We may describe a system with full antagonism at the induction level and a variable level of antagonism at the effector level (as measured by the parameter α) within hosts of genotype A (as referred to by the superscripts) by the following set of equations

$$\frac{dz_i^A}{dt} = \gamma_i\lambda_i(1 - z_i^A) - \mu z_i^A$$

$$\frac{dy_{ij}^A}{dt} = [\lambda_{ij}(1 - w^A) + \alpha\lambda_{ij}(w^A - z_i^A)](1 - y_{ij}^A) - (\sigma + \mu)y_{ij}^A$$

$$\frac{dy_i^A}{dt} = [\lambda_i(1 - z_i^A) + (1 - \alpha)\lambda_{ij}(w^A - z_i^A)](1 - y_i^A) - (\sigma + \mu)y_i^A$$

$$\frac{dw_i^A}{dt} = \Sigma\gamma_i\lambda_i(1 - w^A) - \mu w^A$$

Here, $z_i$ is the proportion immune to strain $i$, $w_i$ is the proportion immune to either strain, $\gamma_i$ represents the fraction of individuals who become immune upon exposure to strain $i$, $\sigma$ is the rate of loss of infectiousness, $\mu$ is the death rate, $y_i$ is the proportion infectious for strain $i$, and $y_{ij}$ is the proportion infectious for both strains as a result of simultaneous inoculation. A fraction of individuals ($y_i y_j$) may also be coinfectious as a result of sequential inoculation by the two strains. The per capita force of infection with strain $i$, $\lambda_i^A$ (in the absence of the other host genotype) is thus given by $\beta[y_i - \delta y_i y_j + 0.5(1 - \delta)y_{ij}]$, where $\delta$ is the probability that both strains will be transmitted. The per capita force of coinoculation of strains $i$ and $j$, $\lambda_{ij}^A$ is given by $\beta\delta(y_{ij} + y_i y_j)$. Non–HLA-B35 hosts do not recognize the epitopes defining the strains, and therefore respond to infection by either parasite strain in a cross-reactive manner. The proportion immune to either parasite strain in hosts of genotype B (that is, non–HLA-B35) may thus be represented by a single variable $z^B$, whose dynamics may be characterized by the equation

$$\frac{dz^B}{dt} = \gamma(\Sigma\lambda_i + \lambda_{ij})(1 - z^B) - \mu z^B$$

where $\gamma$ represents the fraction of individuals who become immune upon exposure to any strain or combination of strains. The proportions infectious are given by

$$\frac{dy_i^B}{dt} = \lambda_i(1 - z^B) - (\sigma + \mu)y_i^B$$

$$\frac{dy_{ij}^B}{dt} = \lambda_{ij}(1 - z^B) - (\sigma + \mu)y_{ij}^B$$

The two host populations are coupled by the force of infection of strain $i$, $\lambda_i = p\lambda_i^A + (1 - p)\lambda_i^B$, where $\lambda_i^B$ is calculated in the same manner as $\lambda_i^A$ shown above. The latter assumes that mixing between vectors and hosts occurs in a homogeneous fashion. Spatial heterogeneities may create differences in the force of infection terms between A and B such that the proportionate contribution from the same genotype is larger; in this case, we may expect to see a stronger effect of antagonism on the parasite distribution within A (that is, the host genotype in whom antagonism occurs) than within B. In the limit of no mixing, the force of infection terms will be entirely separate, and the effects of antagonism will only be seen in the hosts with HLA-B35.
13. M. Plebanski *et al.*, in preparation.
14. In reality, they will also be competing by virtue of other common immune responses (such as blood-stage immunity); within this theoretical framework, this competition is mediated by the non–strain specific component of immunity within the non–HLA-B35 population. When two strains are in competition, even slight differences in reproductive success can result in the elimination of one or the other and can precipitate large differences in frequency.
15. Strong APL antagonism is observed for polyclonal CTL populations at both induction and effector levels (Fig. 1) (*13*).
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20. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
21. Peptides were synthesized, and $^{51}$Cr release assays were performed as described (*4, 7*), using HLA-matched B lymphoblastoid cell lines as targets. Targets were prepulsed with 10 mM peptide. Based on previous studies in this population on malaria-specific CTL (*1, 7*), a threshold level for a positive response is taken as 10% specific lysis, a level further validated by limiting dilution analysis [M. Plebanski, M. Aidoo, H. C. Whittle, A. V. S. Hill, *J. Immunol.* **158**, 2849 (1997)]. Antagonism was investigated in two ways. For donor Z22 (cp26 responder), labeled targets were prepulsed for 1 hour with cp26, washed, and incubated (1 hour) alone or with varying concentrations of cp29 (putative antagonist) or FluB35 {an unrelated HLA-B35–binding peptide ASCMGLIY [T. Dong *et al.*, *Eur. J. Immunol.* **26**, 335 (1996)] derived from influenza matrix protein}. Inhibition was calculated as 100 × percent of specific lysis (% SL) [cp26 alone – cp26 + (cp29 or FluB35)]/cp26 alone. Donors Z22 and Z25 (both cp26 responders) were tested in a subsequent separate experiment in an antagonism assay where the antagonist is present on a different target cell from the index peptide. The assay was set up with d14 effectors from the cp26-stimulated PBMC cultures. Briefly, radiolabeled, or "hot," targets were prepulsed with or without cp26 and unlabeled, or "cold," targets were prepulsed with either cp29 or FluB35. Targets were then used together at a cold:hot ratio of 1:1. Assays were performed in duplicate, and differences between wells were always <10% of the averaged counts. Data are presented as % SL by subtracting the corresponding background lysis controls, for example, percent of lysis (% lysis) [cp26 pulsed targets (hot) with FluB35 pulsed (cold)] – % lysis [no peptide targets (hot) with FluB35 (cold)]. A similar assay was set up for the cp29 responder Z87, but in this case the hot targets were pre-pulsed with either cp29 or in the absence of peptide and cold targets with either cp26 or FluB35. A fourth HLA-B35 donor studied showed an early cross-reactive response without antagonism (*22*). Subtracted background was in all cases % lysis [no peptide targets (hot) and cp26-pulsed targets (cold)]. For lines derived from the malaria-naïve donors (BR and PTE), antagonism was investigated by using as targets autologous cells prepulsed for 1 hour with 10 μM cp26 or cp29, washed, and then incubated for a further 1 hour in the absence or presence of 10 μM of the corresponding antagonist. These lines were not tested on recombinant vaccinia-infected targets, but other malaria-specific CTL lines from this population have been shown to lyse target cells infected with recombinant vaccinia virus [M. Aidoo, A. Lalvani, H. C. Whittle, A. V. S. Hill, K. J. H. Robson, *Int. Immunol.* **9**, 731 (1997)].
22. M. Plebanski, data not shown.
23. Expected values were calculated for three different parasite rates assuming a binomial distribution with the proportion of the total population uninfected being 1-PR. Pairing of cp27 with cp26 or cp29 was found significantly less often than expected. Linkage disequilibrium with variants of another polymorphic region of the CS protein, Th2R (*11*) was significant for only 3 of the 12 combinations assessed: cp38-cp28 ($D = 0.041$), cp39-cp26 ($D = 0.09$), and cp39-cp29 ($D = 0.064$). Correcting for allele frequency variation by calculating the ratio of $D$ to its maximum possible value ($D'$) [M. Nei, *Molecular Evolutionary Genetics* (Columbia Univ. Press, New York, 1987)] yielded a mean $D'$ of 0.28 for the 12 combinations, representing relatively weak linkage disequilibrium for loci only 26 amino acids apart.
24. DNA was prepared from blood samples collected as part of a case-control study of malaria (*1*). To identify the four variants, part of the *P. falciparum* CS protein gene was amplified by polymerase chain reaction (PCR), transferred to nitrocellulose membrane and probed with oligonucleotides specific for the variants as described (*4*). The probes (5′ to 3′) and wash temperatures were: cp26, TAAACCTAAAGACGAATT, 47°C; cp27, AAAGACCAATTAGATTAT, 43°C; cp28, ACCAATTAAATTATGAAA, 41°C; and cp29, AAATCTAAAGACGAATTAG, 47°C. To confirm the coexistence of cp26 and cp29, allele-specific PCR was carried out on a subset of individuals in whom both cp26 and cp29 had been detected on dot-blots. Samples containing only cp26 or cp29 as determined on dot-blots were used as controls. Primers were: cp26-specific, CATAATCTAATTCATCTTTAGG; cp29-specific, CATAATCTAAT-

TCGTCTTTAGA; and conserved primer CAATCAAGGTAATGGACAAGG. PCR was carried out in 16 mM $(NH_4)_2SO_4$, 67 mM tris HCl (pH 8.8), 0.01% Tween 20, 0.5 mM $MgCl_2$, and 0.5 mM spermidine, with deoxynucleotide triphosphates (0.2 mM) and a 0.5 mM concentration of each primer, for an initial denaturation step of 99°C for 5 min, followed by 35 cycles each of 1 min 30 s at 94°C, 2 min at 55°C, and 2 min at 72°C. The results from all 20 samples tested by allele-specific PCR agreed with the dot-blot results. PCR products were also cloned into pGEM-T (Promega) and sequenced to directly confirm the dot-blot results. This showed that all cp29 sequences had the codon GGT immediately before the B35 epitope [N. Yoshida *et al.*, *Exp. Parasitol.* **71**, 386 (1990)], whereas cp26 sequences had AAT. A combination of allelle-specific PCR sequencing was used to confirm the presence of both cp26 and cp29 sequences in 10 randomly selected samples that were positive for both cp26 and cp29 on dot-blots. There was complete concordance between the dot-blot, allele-specific PCR, and sequencing results. Linkage disequilibrium with the polymorphic TH2R region (amino acids 334 through 342) close to the cp26-cp29 variants (amino acids 368 through 390) was assessed by typing 478 samples for three TH2R variants [cp36 (YLKTIQNSL), cp38 (YLQKIKNSL), and cp39 (YLNKIQNSL)], using sequence-specific oligonucleotides.

25. The numbers of infections by either cp26 or cp29 strains were compared to the number of cp27 or cp28 infections in the children with and without HLA-B35 using $\chi^2$ analysis. This was the primary comparison because of the functional difference between these strains: cp26 and cp29 are HLA-B35 epitopes, cp27 and cp28 are not. Confounders were allowed for by log-linear regression analysis using SPSS 6.0. The significant increase in the number of cp26 or cp29 infections, or both, in the HLA-B35 control group was found to be independent of age, malaria condition, ethnic group, hospital site, and area of residence. Further analysis of the influence of two class I alleles (A2 and B53) and 10 class II haplotypes on strain distribution showed no significant association.
26. M. Plebanski, C. E. M. Allsopp, M. Aidoo, H. Reyburn, A. V. S. Hill, *Eur. J. Immunol.* **25**, 1783 (1995).
27. Effectors were PBMC from donor Z22 stimulated with cp26 14 days before and used at an E:T ratio of 20:1. Specific lysis of cp26 alone was 15% at 4 hours. In accordance with our previous observations (*4*), cp26- or cp29-stimulated cells from the previously detected cp26 responders Z22, Z25, and Z58 did not show reactivity to cp29 in standard 4-hour $^{51}Cr$ release assays after 7 days of culture. Limiting dilution analysis performed on donors Z22 and Z58 confirmed the existence of low levels of CTL to cp26 in these donors in the absence of cp29 CTL precursors. However, consistent with data on antagonistic HIV variants (*6*), a low level of killing of cp29 pulsed target could be detected for cp26-stimulated CTLs if the assay was harvested after 18 hours instead of 4 hours (*22*).
28. We thank the Gambian children and their parents and guardians, and the villagers of Brefet for their participation. This study was approved by the Gambian government–U.K. Medical Research Council (MRC) joint ethical committee. We thank C. Allsopp, A. Gallimore, and A. Jepson for assistance, and A. McMichael, A. McLean, R. Anderson, and C. Newbold for advice. A.V.S.H. is a Wellcome Trust Principal Research Fellow. Funded by the Wellcome Trust and the MRC.

11 June 1997; accepted 23 December 1997