Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming

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Variation in epitopes of infectious pathogens inhibits various effector functions of T lymphocytes through antagonism of the T-cell receptor. However, a more powerful strategy for immune evasion would be to prevent the induction of T-cell responses. We report here mutual 'interference' with the priming of human T-cell responses by a pair of naturally occurring variants of a malaria cytotoxic T-cell epitope. Interference with priming also occurs *in vivo* for a murine malaria T-cell epitope. Reshaping of the T-cell repertoire by such immune interference during naive T-cell induction may provide a general mechanism for observed patterns of immunodominance and persistence by many polymorphic pathogens.

The human immune system applies a constant selective pressure on infectious pathogens. Many persistent human pathogens have evolved families of closely related variant epitopes. Mutations can prevent immunodominant T-cell epitopes from binding to major histocompatability (MHC) molecules[1,2]. Also, the simultaneous presentation of closely related variant epitopes known as altered peptide ligands (APLs) can cause a transitory inhibition of memory T-cell effector functions such as cytolysis or lymphokine production[3,4]. Such immune evasion strategies may provide an immediate survival advantage for a particular strain in an individual. However, they do not adequately explain how APLs can initially emerge after a mutational event or how they can be stably maintained over time in an individual and in the population[5].

Mathematical modeling of the population dynamics of closely related variant epitopes in HIV (ref. 6), HTLV (ref. 7) and *Plasmodium falciparum* malaria[8,9] have demonstrated that a necessary component for the persistence of new variants is an ability to avoid specific *de novo* responses. However, it has been unclear how memory cytotoxic T lymphocytes (CTLs) may be selected from the naive T-cell repertoire during a natural infection with APL-bearing pathogens. A simple and reproducible method for the *in vitro* generation of primary cytotoxic $CD8^+$ T-cells from naive human individuals[10] makes exploration of this possible.

P. falciparum is the causative pathogen of severe malaria, a principal cause of death in the tropics[11]. The parasite has an early intracellular stage in the host liver, and protection by MHC class I-restricted CTLs may act during this life cycle stage[11–13]. The circumsporozoite protein (CS) of *P. falciparum* is present during the liver stage of the disease, and CS-specific CTLs can protect against malaria in murine models[11–13]. The *P. falciparum* CS is highly polymorphic at its carboxy terminus. Within this region there is an HLA-B35 binding motif shared between four natural variants found in The Gambia (cp26, cp27, cp28 and cp29)(ref. 14). Two of these variants (cp26 and cp29) bind HLA-B35 and elicit memory CTL responses from the peripheral blood mononuclear cells (PBMCs) of HLA-B35-positive, malaria-exposed donors[14,15]. Moreover, in some naturally exposed donors, cp26 and cp29 are able to antagonize CTL responses to each other[8]. Here we show how these APL variants may have gained a lasting advantage by perpetuating a population of functionally naive hosts, through a re-shaping of their immune response repertoire. This results from a newly described effect, which we refer to as immune 'interference' of variant peptides at the induction phase of specific T-cell responses.

CTL responses to cp26 and cp29 from naive individuals

To determine whether cp26 and cp29 can each prime CTL responses, we directly tested PBMCs from HLA-B35 individuals not exposed to malaria for the presence of cp26- or cp29-reactive T cells using an *in vitro* CTL priming model[10]. The naive $CD8^+$ T-cell compartment had both cp26- and cp29-specific as well as cross-reactive CTLs (Fig. 1). PBMC samples that gave responses in these primary assays failed to react in standard secondary CTL assays using the same peptide concentrations (not shown). Therefore, responses were not due to stimulation of secondary CTLs specific for some cross-reactive pathogen.

Negative results for cp26 or cp29 reactivity were also generated when cultures stimulated with the unrelated HLA-B35 binding peptide ls8 were re-stimulated with cp29 or ls8 (data not shown). Similar cp26- and/or cp29-reactive primary lines were generated from an additional three HLA-B35 naive donors. Reactivity to cp26 and cp29 was repeatedly found in all four HLA-B35 naive donors (donor A, $n = 2$ of 2; donor B, $n = 3$ of 3; donor C, $n = 2$ of 2; and donor D, $n = 1$ of 1). The peptide cp29 induced early primary lines with 300% the efficiency of cp26 (cp26, 7% + 0.9 s.e.m. positive wells; cp29, 21% + 3.7 s.e.m.; $P < 0.002$). An additional round of *in vitro* re-stimulation increased

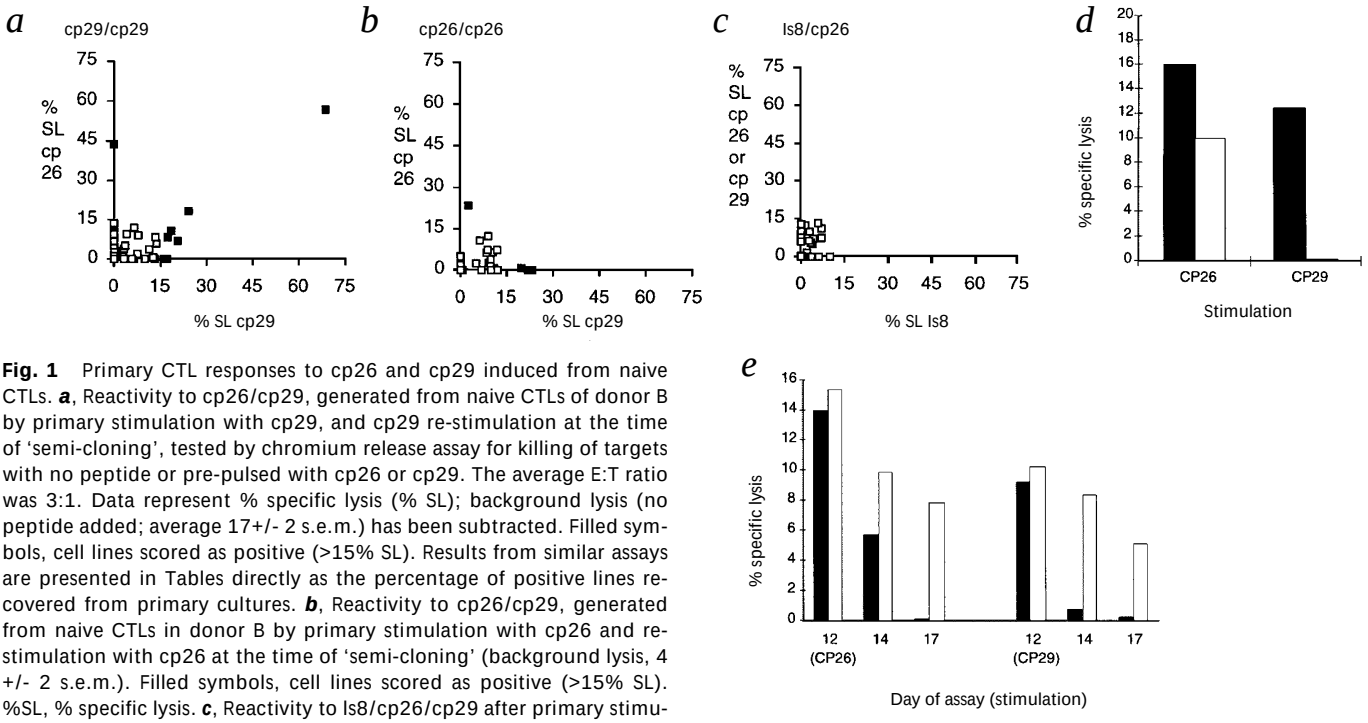

Fig. 1 Primary CTL responses to cp26 and cp29 induced from naive CTLs. ***a***, Reactivity to cp26/cp29, generated from naive CTLs of donor B by primary stimulation with cp29, and cp29 re-stimulation at the time of 'semi-cloning', tested by chromium release assay for killing of targets with no peptide or pre-pulsed with cp26 or cp29. The average E:T ratio was 3:1. Data represent % specific lysis (% SL); background lysis (no peptide added; average 17+/- 2 s.e.m.) has been subtracted. Filled symbols, cell lines scored as positive (>15% SL). Results from similar assays are presented in Tables directly as the percentage of positive lines recovered from primary cultures. ***b***, Reactivity to cp26/cp29, generated from naive CTLs in donor B by primary stimulation with cp26 and re-stimulation with cp26 at the time of 'semi-cloning' (background lysis, 4 +/- 2 s.e.m.). Filled symbols, cell lines scored as positive (>15% SL). %SL, % specific lysis. ***c***, Reactivity to ls8/cp26/cp29 after primary stimulation with ls8 (an unrelated HLA-B35 binding peptide) and 'semi-cloning' on cp26 (background lysis, 12 +/- 1 s.e.m.). %SL, % specific lysis. ***d*** and ***e***, Secondary CTL responses induced by cp26 and cp29 in malaria-exposed donors can be re-stimulated by their APLs. Memory cytotoxic T-cells from HLA-B35 Gambian donors naturally exposed to malaria were re-stimulated *in vitro* with cp26 or cp29 and tested in a chromium release assay for killing of targets pulsed with cp26 (■) or cp29 (□). E:T ratio, 10:1. Data represent % specific lysis (background lysis, 7.5 % +/- 4 s.e.m.). The low level of reactivity reflects the low precursor frequency number of these cells in Gambian donors[10]. *d*, Reactivity to cp26 induced from donor E by both cp26 and cp29, assayed on day 14 of culture. *e*, Specific reactivity to cp29 induced by both cp26 and cp29 from malaria-exposed donor F.

the percentage of positive lines induced by cp26 to a level similar to that of cp29 (cp26, 21% + 7 s.e.m. positive wells; cp29, 25% + 7 s.e.m.; *P*, not significant). We studied cp26/cp29 cross-reactivity further by cloning lines from two of these donors. A total of nine clones were recovered from donor A and twelve, from donor B. Cross-reactive clones were those having >50% of reactivity to the index. The peptide cp26 induced cp26-specific (A, 25%; B, 0%), cp29-specific (A, 25%; B, 0%) and cross-reactive (A, 50%; B, 100%) clones, and cp29 induced cp26-specific (A, 20%; B, 25%) and cross-reactive (A, 80%; B, 75%) clones, but no cp29-specific (A, 0%; B, 0%) clones.

Immune diversion

Both peptides could also elicit a proportion of CTLs that preferentially recognized the other variant in cytotoxicity assays (Fig. 1). We sought to assess whether such immune diversion of responses to another variant would also be found in naturally exposed donors. Responses from naturally exposed donors are often specific to the stimulating variant, but such studies did not test whether reactivity was induced to a variant not used to re-stimulate *in vitro*[8,14] . Memory cp26 and cp29 CTLs (n = 2 donors) could be diverted towards recognition of the variant used for re-stimulation (Fig. 1*d*). The ability to re-stimulate *in vitro* index peptide-specific CTL responses by an antagonist has been shown for memory CTLs to variant peptides derived from HIV (ref. 16) and HCV (ref. 17). In infected donors, this could be the result of preferentially stimulating memory index-specific CTLs or by immune diversion during re-stimulation. To determine whether responses could be diverted during the re-stimulation as well as the priming phase, we primed CTLs *in vitro* to cp26 or cp29 and then re-stimulated with either the same or the other epitope. Even in the presence of cp26 and cp29 cross-reactive CTLs primed *in vitro*, stimulation with the other variant indeed selected a narrow response, specific only to the variant that was not used in re-stimulation (Table 1).

Interference by APLs during priming

In The Gambia, more than 40% of infections are mixed, and 25% of infected individuals have both cp26- and cp29-bearing strains in peripheral blood samples during malaria infections[8]. Therefore, we determined what would occur in the priming of

Table 1 Re-stimulation with an altered peptide ligand generates fewer cross-reactive responses

Priming	Re-stimulation	Specificity of lines (% Positive wells)	
		CP26-specific	Cross-reactive
CP26	cp26	4	20
	cp29	4	6
		CP29-specific	Cross-reactive
CP29	cp26	10	4
	cp29	7	23

Primary CTL lines were generated and tested, with additional 'semi-cloning' and re-stimulation using cp29 for cp26-stimulated CTLs and cp26 for cp29-stimulated CTLs. For each condition, 36–40 wells were tested for specific cytotoxic activity against cp26 and cp29. The percentage of wells with positive reactivity (>15% SL average) for cp26, cp29 or for both for each condition was averaged from two similar experiments. There was no significant difference between the number of specific cells recovered by homologous re-stimulation, whereas the recovery of cross-reactive variants was significantly decreased for heterologous re-stimulation (P > 0.02, paired t-test).

cp26 and cp29 responses if both variants were presented simultaneously. Presentation of combinations of cp26 and cp29 epitopes abrogated CTL induction from naive precursors when used at equimolar concentrations (n = 4 experiments; Fig. 2 and Table 2). Complete inhibition was also seen in experiments (n = 2) using a dose of interfering peptide 10% that used previously (0.25 μg/ml to 2.5 μg/ml of index; percentage positive wells: cp26, 23 and 33; cp26/cp29 0 and 0; cp29, 23 and 33; cp29/cp26, 0 and 0). The peptide cp29 was consistently a strong inhibitor of cp26-induced responses, ranging from 76 to 100% (Table 2). In contrast, inhibition by an equimolar concentration of cp26 of the total cp29-induced response ranged from 25 to 100%. In each case, the remaining response, where still present (Table 2, EXP 1 and EXP 3) had considerably decreased levels of CTLs capable of recognizing both variants (Table 2 EXP 1, 100% decrease; EXP 3, 90% decrease). In similar experiments, the presence of an equimolar concentration of cp26 did not inhibit responses to the unrelated HLA-B35 binding peptide ls8 (percentage wells with ls8-specific CTL activity: ls8 alone, 21%; ls8/cp26, 21%), nor did the presence of cp29 (percentage wells with ls8-specific CTL activity: ls8 alone, 8%; ls8/cp29, 8%). Thus, co-stimulation with variant epitopes can prevent the induction of specific cytotoxic T cells, and could thus be used as an immune evasion strategy for these parasite strains in the human population. However, the extent to which it may be effective may depend on the cellular mechanisms that generate such mutual immune interference.

Table 2 Interference in the generation of CTL responses *in vitro* by co-culture with cp26 and cp29

Primary stimulus[a]	Re-stimulation	Total CTL lines % Positive wells			
		EXP1	EXP2	EXP3	EXP4
cp26	cp26	33	23	25	5
cp26 + cp29	cp26	0	3	6	0
	cp29	25	3	18	0
cp29	cp29	33	23	35	10

[a]Stimulation for the initial week of culture before 'semi-cloning'. Peptides were used singly or in combination. Interference was observed when both peptides were pulsed on to the same APCs simultaneously or sequentially. Wells were scored as positive regardless of whether their %SL (average, >15%) made them positive for recognition of cp26 only, cp29 only or for both. PBMCs were from two different HLA-B35, malaria-naive donors (EXPs 1,2 and 3: donor B; EXP 4, donor A). For each condition, 20–40 wells were tested for specific cytotoxic activity against both cp26 and cp29.

Immune interference: antigen presentation requirements

The mechanism of interference during priming by cp26 and cp29 differed from antagonism during the effector phase of CTL responses[8] in that it was strictly dependent on the presentation of cp26 and cp29 together on the same antigen-presenting cell (APC) (Fig. 2*e*). The differences are not unexpected, as resting naive T cells and activated effector memory cells in general have different stimulation requirements. Thus, we refer to this inhibition of primary CTL responses as interference to distinguish it from effector-level antagonism. The same APC requirement is consistent with inhibition during the priming of mixed lymphocyte reaction proliferative responses from transgenic murine CD4$^+$ T cells by altered peptide ligands[18]. Thus, similar antigen-presenting requirements may exist for CD4$^+$ and CD8$^+$ T-cell interference. Moreover, the requirement for presentation on the same APC indicates that interference occurs through the T-cell receptor (TCR) to modulate early T-cell activation. Improper oligomerization is one of the mechanisms proposed for inhibition by altered peptide ligands consistent with a requirement for presentation by the same APC (refs. 19,20).

Immune interference: T-cell receptors

A unique feature of the variant pairing we studied here is that inhibition is mutual (Table 2): Mutual effects have not been observed for any other altered peptide ligands, to our knowledge. T cells reactive to cp26 and cp29 may share characteristics in TCRs that make them susceptible to a similar altered signaling effect[21]. We analyzed ten Vα and ten Vβ TCR sequences from cp26-specific and cp29-specific lines generated from separate naive T-cell cultures from donor B. Two identical related Vβ T-cell receptor sequences were used by cells of either cp26 or cp29 specificity (Vβ 4.1-YLCS-VERDGSDTQY-FGPG-Jβ2.9 and Vβ 4.1-YLCS VVDTASGNTIY FGPG-Jβ1.3). In contrast, Vα chains used by cp26-specific T cells (Vα 32.1-YFCA-GLSG-GYIPT-FGPG-Jα15.3 and 2.3-YLCV-VNRNYGQNFV-FGPG-Jα13.2) and cp29-specific T cells (Vα 24.1-YICV

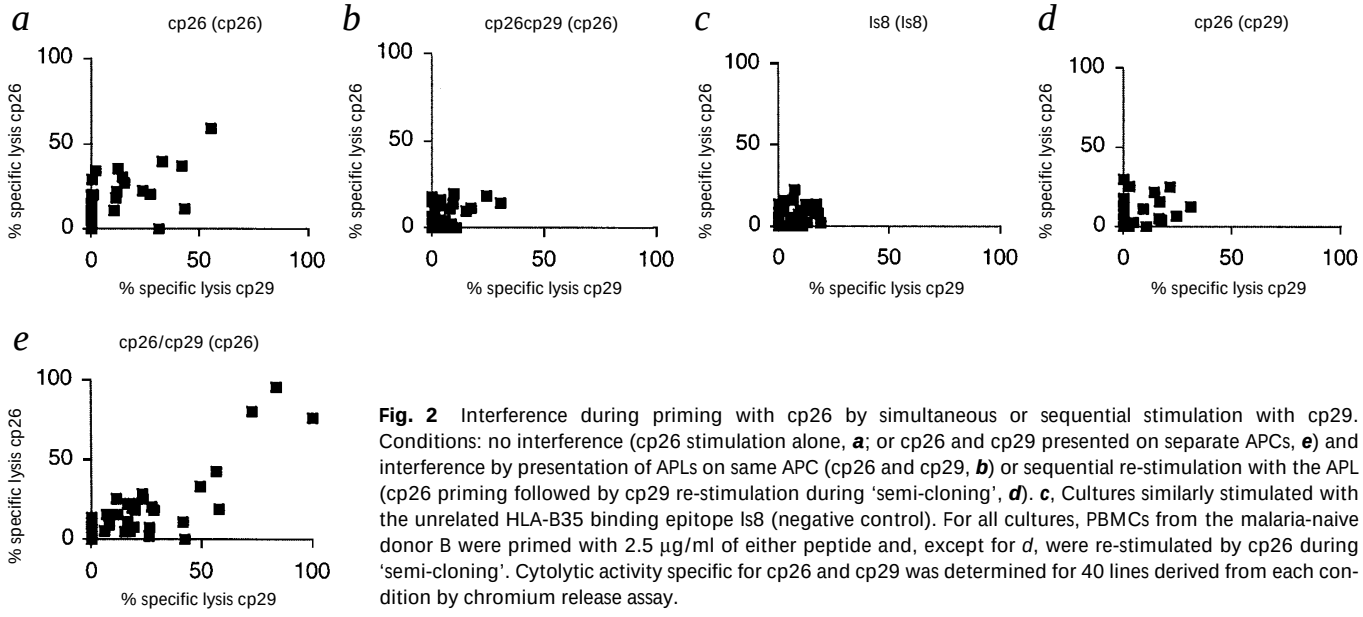

Fig. 2 Interference during priming with cp26 by simultaneous or sequential stimulation with cp29. Conditions: no interference (cp26 stimulation alone, ***a***; or cp26 and cp29 presented on separate APCs, ***e***) and interference by presentation of APLs on same APC (cp26 and cp29, ***b***) or sequential re-stimulation with the APL (cp26 priming followed by cp29 re-stimulation during 'semi-cloning', ***d***). ***c***, Cultures similarly stimulated with the unrelated HLA-B35 binding epitope ls8 (negative control). For all cultures, PBMCs from the malaria-naive donor B were primed with 2.5 μg/ml of either peptide and, except for *d*, were re-stimulated by cp26 during 'semi-cloning'. Cytolytic activity specific for cp26 and cp29 was determined for 40 lines derived from each condition by chromium release assay.

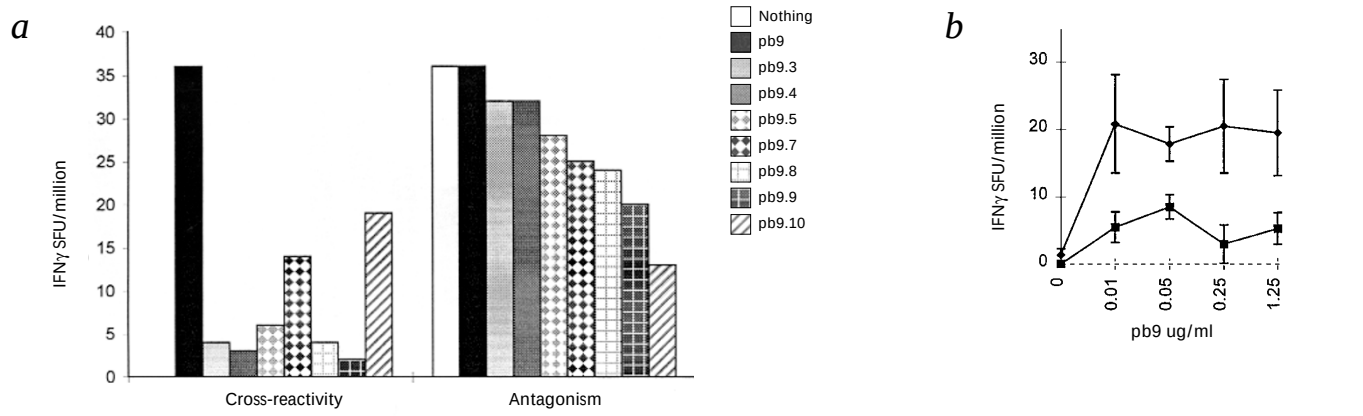

Fig. 3 *In vivo* immune interference with T-cell priming. ***a***, Variants of pb9 were assessed for their ability to re-stimulate or antagonize the pb9-induced IFN γ response from pooled splenocytes of mice (n = 3) immunized intravenously 2 weeks earlier with TyS3. Peptides were added to the ELISPOT assay directly (Cross-reactivity), or 1 h after the index pb9 epitope (Antagonism). Significant reactivity was observed for pb9 variants substituted at position 7 of pb9 (SYPSAEKI), pb9.7 (K→A) and pb9.10 (K→D) ($P < 0.05$, compared with background). Variant pb9.10 also inhibited strongly IFNγ pb9-induced production in the antagonism assay (64% inhibition; $P = 0.005$). The unrelated peptide NPKd failed to inhibit (not shown). SFU, spot-forming units. ***b***, Mice were immunized intradermally with TyS3 and unrelated peptide NPKd (control; diamonds) or the variant pb9.10mox (antagonism; squares). The number of IFNγ-producing cells in response to pb9 by co-immunization of TyS3 and pb9.10mox was significantly reduced (71%+/- 14 s.d. inhibition; $P < 0.000005$). One of three similar assays is shown. No inhibition was detected by co-immunization of TyS3 with the mox carrier alone (not shown).SFU, spot-forming units.

VKRDDMR-FGAG-Jα1.7 and Vα 24.1-YICV-ATHYGGSQGNLI-FGKG-Jα 9.11) were different. The use of identical Vβ TCR chains for recognition of cp26 and cp29 epitopes may provide a molecular basis for the T-cell susceptibility to very similar APL-mediated signaling events in donor B. In a TCR transgenic mouse (Vβ chain from a Hb^d(64-76)/I-Ek-specific T-cell clone recognizing the Ser69 variant), pairing with endogenous alpha chains leads to activity for an antagonist of the original clone. Responses in transgenics can be antagonized by the Ser69 variant. Thus, use of identical β chains leads, in this model as in ours, to the recognition of two related variant epitopes, and to T cells susceptible to similar antagonistic signaling events[22]. TCR sequencing from different donors, at different times after infection, will be necessary to determine the extent to which the use of similar Vβ TCRs can lead to mutual immune interference in the human population.

The peptides cp26 and cp29 vary only at position 2, a proline-to-serine difference. Structural studies indicate that the T-cell receptor does not interact directly with this residue[23]. Most of the TCR interaction with peptide–MHC complex consists of the TCR contacting the MHC molecule rather than the peptide, thus there may be a less-stringent recognition of antigenic determinant[24,25]. Therefore, the ability of these peptides to interfere may be due to conformational differences in the rest of the peptide and/or the HLA molecule itself. The use of identical TCR Vβ chains for cp26- and cp29-reactive cells emphasizes the similarities in their recognition and may provide the molecular basis for reciprocality during interference[22]. The peptides cp26 and cp29 can also be mutually antagonistic in cytotoxicity assays[8], and the APL requirements for interference during priming may thus overlap those required for effector antagonism.

Table 3 Combined statistical analysis of proliferation, cytotoxicity and TNF release after interference

Condition 1 (Control)		Condition 2 (interference)		Significance of inhibition in different effector assays (P value)		
Stimulation	Re-stimulation	Stimulation	Re-stimulation	Cytotoxicity	TNF	Proliferation
cp26	cp26	cp26	cp29	0.010	NS	NS
cp29	cp29	cp29	cp26	0.018	NS	ND
cp26	cp26	cp26cp29	cp26	0.012	0.001	NS
cp29	cp29	cp26cp29	cp29	0.006	NS	NS

Primary CTL lines from Table 2, EXP 2 were analyzed in detail. Each of the 40 wells derived from the 'semi-cloning' was assessed in parallel for cytotoxicity, proliferation and TNF production in response to cp26 or to cp29. Background reactivity comprised responses in the absence of the test peptide. The mean lysis and levels of cell proliferation and TNF release under the differing culture stimulating conditions were analyzed with general linear models. For analysis of specific lysis, background lysis was always controlled for in the statistical model. Initially, TNF level and proliferation were also controlled for; however, all variables behaved independently in response to interference. Thus, TNF level or proliferation did not significantly affect the observed relationship between culture stimulation conditions and subsequent lysis of cp26 or cp29 targets. These variables were then subsequently assessed separately. In each analysis, condition 1 (no interference) is compared with condition 2 (with interference) to assess the inhibition induced by the latter in the three readout effector assays. NS, not significant; ND, not determined.

Immune interference: induction of defective effector cells

Immune interference during priming could have stimulated either an apoptotic, 'anergizing' or partially activating signal in T cells recognizing both ligands[21–22,26–34]. Assessment of viability by trypan blue exclusion and FACscan analysis for $CD8^+$ $HLA\text{-}DR^+$ 'blast' T cells (dividing cells with increased size and granularity defined by forward and side scatter characteristics by FACScan) showed no decrease in viable responder cells recovered from conditions with immune interference on day 7 of culture (cp26cp29 cultures had 9.9% $CD8^+$ $HLA\text{-}DR^+$ blasts compared with 7.6% with cp26 alone or 8.7% with cp29 alone). Interference was seen in the presence of IL2. Thus, classical anergy induction was not the mechanism of interference. To determine whether a partial activating effect was delivered by immune interference, we analyzed different T-cell effector functions in parallel after conditions with

and without immune interference. We combined the statistical analyses of an experiment in which cytotoxicity, proliferation and tumor necrosis factor (TNF) release were assessed (Table 3). As expected, cytotoxicity was negatively affected by simultaneous or sequential presentation of cp26 and cp29. In contrast, proliferative responses to cp26 and cp29 were not affected by interference. TNF release showed a selective effect (decreased reactivity of cp26cp29 followed by cp26 re-stimulation, compared with cp26 followed by cp26). Thus, interference during priming led to the expansion of a population of defective effector cells capable of proliferating in response to antigen, but unable to mediate cytotoxicity or, in specific cases, to secrete TNF. The generation of defective effectors during priming may limit the extent to which protective T cells can be induced *in vivo*; this is important for the design of CTL-inducing vaccines to polymorphic pathogens. Moreover, natural polymorphism may have evolved to select for variants capable of such interference with T-cell priming.

In vivo effects of immune interference

It will be important to determine the distribution and local concentration of *P. falciparum* variant antigens *in vivo* to identify the extent to which these mechanisms may be used during infection. However, the effect of these mechanisms *in vivo* can be inferred from the distribution of variant parasite strains. In a study of more than 700 individuals with severe and mild malaria, cp26 and cp29 variant co-infections were found more frequently together than expected ($P > 0.000001$; ref. 8). Moreover, all infections with cp26 and cp29 were increased in HLA-B35 individuals compared with the rest of the population ($P > 0.02$; ref. 8). The unusual behavior of this pair of epitopes is explained by a mathematical model that specifies how interference during priming, and antagonism at the effector level together mediate increased co-habitation[8]. The model assumes that whereas an HLA-B35 donor infected with either cp26 or cp29 may prime epitope-specific immunity, a donor simultaneously co-infected with cp26 and cp29 will not. Effector-level antagonism in any individual infected with cp26 and cp29 together, even in the presence of pre-existent immunity to either epitope, will permit survival of co-infecting parasites. Thus, as defined[8], at the population level, interference and antagonism together enhance cp26 and cp29 transmission and co-transmission and are both strictly necessary in this model to reflect the observed parasite population structure.

To determine whether an epitope with antagonistic activity *in vitro* would be capable of interfering with the generation of T-cell responses *in vivo*, we studied the effect of variant epitopes in an animal model. Sterile protection against *P. berghei* infection can be achieved in mice by priming with DNA or Ty particles and 'boosting' with modified vaccinia Ankara strain (MVA) to a single CS-derived CTL epitope, pb9 (refs. 35,36). Protection specific to pb9 is IFNγ-dependent in Balb/c mice, as detected by rapid ELISPOT assays[35,36,37]. Initially, we searched for a pb9 variant capable of antagonizing IFNγ production by ELISPOT. We identified a peptide with a single amino-acid substitution of the pb9 epitope (pb9.10) that is a partial agonist with antagonist activity on IFNγ secretion by pb9-primed splenocytes (Fig. 3*a*). Similar patterns of reactivity were found in another two experiments in which Ty particles with pb9 were administered intradermally (not shown). The peptide pb9.7 was similarly an agonist, and pb9.10, an agonist/antagonist, of responses from pb9-primed mice with different Ty particles containing pb9 administered subcutaneously, intravenously, intradermally and intranasally (mean inhibition by pb9.10 in antagonism assays was 72% +/- 11 s.e.m. for the four groups, three mice per group; not shown). Co-immunization with pb9.10 and pb9 as immunogens substantially interfered with *in vivo* pb9-specific priming (Fig. 3*b*). Thus, immune interference during priming can also be induced *in vivo*.

Here, we have identified two immune mechanisms that can be exploited by *P. falciparum* strains to persist in the population. Both act at the induction rather than the effector phase of CTL responses. We first described a polymorphic malaria epitope that stimulates naive and memory CTLs whose effector functions focus on another epitope: immune diversion. We then characterized immune interference, the priming of CTLs with impaired cytolytic and lymphokine secreting ability *in vivo* and *in vitro* as a result of co-stimulation with closely related variants.

Methods

Antigens. The HLA-B35 binding epitopes cp26 (KPKDELDY) and cp29 (KSKDELDY) from the circumsporozoite antigen, ls8 (KPNDKSLY) from the liver-stage antigen 1 (LSA1) of *P. falciparum*[15], the murine H-2K^d binding epitopes NPKd from influenza virus[38] (TYQRTRALV) and pb9 epitope from *P. berghei* circumsporozoite antigen[35,36] (SYIPSAEKI) and its variants substituted at a single amino acid (pb9.3, SYIASAEKI; pb9.4, SYIPAAEKI; pb9.5 SYIPSVEKI, pb9.6 SYIPSAAKI, pb9.7 SYIPSAEAI, pb9.8 SYYPSAEKI, pb9.9 SYIPSAVKI, pb9.10 SYIPSAEDI) were made with an automated Zinsser Analytical synthesizer or purchased from Severn Biotech (Kidderminster, UK). For use *in vivo*, an immunogenic form of the pb9.10 variant was made by conjugation to an oxidized mannan carrier (pb9.10mox), keyhole limpet hemocyanin (KLH; Calbiochem, La Jolla, California), as described[39]. The immunogenic Ty particles containing the pb9 epitope (TyS3, construct CABD from ref. 40) and modified vaccinia virus (MVAS) with pb9 as part of a string of epitopes were generated and purified as described[35,36,40].

Cells and culture conditions. Blood donors were HLA-B35 volunteers without exposure to *Plasmodium* antigens (that is, UK donors who have never traveled to malarial areas), or *P. falciparum*-exposed Gambians. Heparinized blood was separated on Ficoll and the PBMC fractions were collected. Unless otherwise stated, PBMCs were pre-pulsed for 1 h at 37 °C with 50 μg/ml (unless otherwise stated) of the HLA class I binding peptide(s) cp26, cp29 or ls8 alone or in simultaneous or sequential combination. Cells were then washed or diluted 1:20 by the addition of culture medium (αMEM or RPMI1640) supplemented with 10% heat-inactivated normal human serum (for primary cultures) or fetal calf serum (for secondary cultures) and 4 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin sulphate (all from Life Technologies). After being pulsed with peptide(s), cells were cultured at 2×10^6 cells/ml in 2-ml wells either alone (for secondary responses) or with the addition of 5 μg/ml KLH and 25 ng/ml interleukin 7 (IL7, recombinant human; R&D Systems, Minneapolis, Minnesota) (for primary responses). Interleukin 2 (IL2, recombinant human, Lymphocult HT; Biotest, Shirley, Solihull, UK) was added at 10 U/ml on day 4. For secondary responses, fresh medium with 10 U/ml IL2 was added on day 7. For primary responses, day-7 cells recovered from bulk cultures were 'semi-cloned' at 5,000 cells/well in 96 U-bottomed well plates with 100,000 autologous irradiated (2,000 rads), peptide pre-pulsed (10 μg/ml) PBMCs per well and 10 U/ml IL2 (ref. 10). A week later, wells were re-stimulated using autologous PBMC. CTL lines were generated from selected cp26 and cp29-reactive wells as described[10]. Cloning was done by limiting dilution. The anti-CD8-PE and anti-HLA-DR-FITC antibodies for FACScan analysis were from Serotec (Raleigh, North Carolina).

Tests of T-cell activity. PBMCs from malaria-exposed individuals stimulated *in vitro* with peptides cp26 and cp29 were tested for specific cytotoxic activity against both peptides in standard chromium release assays[15]. Targets were ^{51}Cr- labeled autologous or HLA-matched Epstein-

Barr virus-transformed B-cell lines alone or pre-pulsed with peptide(s). Preliminary titration CTL assays (in the absence of immune interference or antagonism) indicated that 10 μg/ml was the optimal concentration (not shown). Targets were incubated in 96 round-bottomed wells at 37 °C with 5×10^3 targets per well with either effector cells at an E:T ratio of 20:1, media or 5% Triton X in media. The supernatant was assayed at 4 h and the percentage specific lysis (% SL) was calculated as $100 \times$ [(experimental lysis–spontaneous lysis)–(background lysis–spontaneous lysis)]/(maximum lysis–spontaneous lysis).

Cytotoxic T-cell activity from malaria-naive donors was assessed in chromium release assays as described[10]. Unless otherwise stated, the data shown corresponds to CTL assays on day 21 of culture (two weeks after 'semi-cloning'). Each of the 24–40 wells from each condition after 'semi-cloning' was divided into three groups aliquots and assessed for killing of ^{51}Cr-labeled autologous or HLA- matched B-cell lines alone, or pre-pulsed for 1 h at 37 °C with 10 μg/ml of cp26 or cp29. When ls8 was used to stimulate the primary culture, wells were also tested for killing of B-cell lines pre-pulsed with 10 μg/ml of ls8. The average E:T ratio was 5:1. Unless otherwise stated, assays were collected after 4 h. The percentage specific lysis was calculated for each well as described above.

On average, we have scored as positive wells with >15% SL for the peptide tested[10]. The number of such lines recovered out of those initiated indicates the magnitude of the primary response[10]. Similar thresholds are used to identify responses by limiting dilution analysis[41]. SL greater than 15% has been validated to identify *in vitro*-primed CTLs that continue to show significant levels of specific lysis for more than 3 months[10].

In another experiment, 24 h after the chromium release assay, the supernatants were also assessed for tumor necrosis factor activity by the WEHI cell bioassay[42] and the cells were assessed for proliferation by ^{3}H-thymidine incorporation[39]. For each starting condition, 40 wells were tested.

To assess IFNγ production to the *P. berghei* pb9 epitope, splenocytes from Balb/c mice were used in IFNγ ELISPOT assays as described[35,36]. Unless otherwise stated, peptides were used at 1 μg/ml . The antagonists were added 1h after the index pb9 peptide as described[3,4,21]. Data are presented as precursor frequency in IFNγ spot-forming units per million murine spleen cells tested.

Sequencing of T-cell receptor α and β chains. The sequences of the V, CDR3 and J regions of the T-cell receptors in lines specific for cp26, cp29 or cross-reactive was determined by RNA extraction, cDNA synthesis, dGTP tailing and PCR as described[43]. The primers used were: polyC anchor, 5'–GCATTCAGCTGCGGCCGCCCCCCCCCCCCCCC–3'; Ca primer, 5'–TGACCGCAGTCGACAGACTTGTCACTGGATT–3'; Cb primer, 5'–ATACTGGAGTCGACGGAGATCTCTGCTTCTGATG–3'. PCR products were purified from agarose gels (Qiaquick columns; Qiagen, Valencia, California), digested with *Not*I and *Sal*I and ligated to pBC-SK+ (Stratagene, La Jolla, California) between the *Not*I and *Sal*I sites. After transformation of *E. coli* DH5a, plasmids were prepared using Qiawell columns (Qiagen, Valencia, California). Sequencing reactions used a Taq FS dye terminator kit (Perkin-Elmer ABI, Warrington, UK) with the M13 forward primer, and were analyzed on an ABI 373 automated sequencer.

***In vivo* interference with priming in mice**. Balb/c mice were immunized intravenously with TyS3 (refs. 36,40). Then, 2 weeks later, pooled spleen cells from three mice were assayed. Peptides were added at 1 μg/ml to the standard ELISPOT assay directly (cross-reactivity), or 1 h after the index pb9 epitope (antagonism)(refs. 3,4,21).

Balb/c female mice 6–8 weeks old were immunized intradermally with 30 μg of TyS3 particles in PBS in the presence or absence of 30 μg of the antagonist pb9.10mox in PBS (epitope molar ratio, 1:30). Three mice were immunized per group. After 2 weeks, pb9 and pb9.10 specific activity was assessed in splenocytes from individual mice by the IFNγ ELISPOT assay[35,36]. Differences in reactivity between differently immunized groups were assessed by the Student's *t*-test; *P* values are presented.

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