Immunity, Vol. 10, 651–660, June, 1999, Copyright ©1999 by Cell Press

Interleukin 10–Mediated Immunosuppression by a Variant CD4 T Cell Epitope of *Plasmodium falciparum*

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Summary

The immunodominant CD4 T cell epitope region, Th2R, of the circumsporozoite protein of *Plasmodium falciparum* is highly polymorphic. Such variation might be utilized by the parasite to escape from or interfere with CD4 T cell effector functions. Here, we show that costimulation with naturally occurring altered peptide ligands (APL) can induce a rapid change from IFNγ production to the immunosuppresive mediator interleukin 10 (IL-10). This mechanism may contribute to the low levels of T cell responses observed to this pathogen in malaria-endemic areas.

Introduction

Despite repeated exposure to the parasite in malaria-endemic areas of the world, protective immunity to *Plasmodium falciparum* takes many years to develop (Greenwood et al., 1987; Gupta et al., 1994). Consistent with this observation, T cells specific for the preerythrocytic malaria antigens expected to participate in protective immunity are found infrequently in naturally exposed individuals (Esposito et al., 1992; Doolan et al., 1993, 1994; Aidoo et al., 1995; Plebanski et al., 1997). Malaria-specific T cell responses and, in some studies, even unrelated T cell responses are downregulated during the course of malaria infection (Ho et al., 1986, 1988; Wangoo et al., 1990; Hviid et al., 1991). Th1 type responses to preerythrocytic antigens by CD4 T cells can protect against malaria (Nardin et al., 1989; Moreno et al., 1991; Renia et al., 1993; Weiss et al., 1993; Takita-Sonoda et al., 1996; Wang et al., 1996; Doolan and Hoffman, 1997; Stoute et al., 1997), and it remains unclear whether and how the parasite can interfere with the induction of these responses.

The circumsporozoite (CS) protein of *Plasmodia* is one of the main targets in preerythrocytic immunity, capable of inducing protective antibodies, CD8 and CD4 T cells (Good et al., 1988; Marsh et al., 1988; Hoffman et al., 1989; Romero et al., 1989; Tsuji et al., 1990; Rodrigues et al., 1993; Stoute et al., 1997). Extensive polymorphism of the carboxyl terminus of *P. falciparum* CS was described over 10 years ago (de la Cruz et al., 1987; Lockyer et al., 1989), but much remains to be determined about the functional consequences of such variation. The highest level of variation coincides with an immunodominant proliferation-inducing CD4 T cell epitope denoted Th2R (326–347 aa) (Good et al., 1988). This region is frequently recognized by malaria-exposed individuals in West Africa, due in part to an ability to bind a multiplicity of HLA-DR and HLA-DQ types (Calvo-Calle et al., 1997). An HLA-A2-binding CD8 T cell epitope (327–335) has been described that is also contained within this polymorphic region (Tc2R) (Blum Tirouvanziam et al., 1995; Plebanski et al., 1995). The finding of exclusively nonsynonymous nucleotide changes in this epitope region suggested that its polymorphism is maintained by the parasite to escape protective T cell responses (Good et al., 1988). It has not been determined how CD4 or CD8 T cells exert such selective pressure on the malaria parasite. We have recently shown that variation in an adjacent region containing an HLA-B35-restricted CD8 T cell epitope can lead to impairment of cytotoxic T cell activity by altered peptide ligand (APL) antagonism (Gilbert et al., 1998). Antagonism has not been studied for the 326–347 region and, despite their likely impact on the maintenance of variability, APL-based immune evasion strategies by natural CD4 T cell epitope variants have not been investigated for any infectious pathogen.

Interferon gamma (IFNγ) has been shown to mediate protection in animal models of malaria when secreted by *Plasmodium*-specific CD8 (Doolan et al., 1996; Plebanski et al., 1998) or CD4 T cells (Renia et al., 1993; Weiss et al., 1993; Takita-Sonoda et al., 1996; Wang et al., 1996; Doolan and Hoffman, 1997). Both CD8 and CD4 T cells may also have lytic activity, but this effector function may not correlate with protection (Plebanski et al., 1998). Protection in humans by immunization with irradiated sporozoites (Nardin et al., 1989; Moreno et al., 1991) and by the recently described RTS,S vaccine may also involve IFNγ secretion by CS-specific CD4 T cells (Stoute et al., 1997). The present study addresses the impact of variation in the immunodominant CD4 T cell epitope of CS on IFNγ responses and on proliferation. Similarly to previous studies where proliferation was assessed in naturally exposed donors (Good et al., 1988; Zevering et al., 1994), we show that many adult Gambians (36/56) do not respond to any of the nine natural variants tested of the aa 326–347 CS epitope,

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utilizing an assay that specifically assesses IFNγ production. Moreover, IFNγ production was almost always variant specific.

Lack of IFNγ production to variant epitopes does not necessarily imply lack of recognition by T cells. Proliferation, as well as the secretion of Th2-type cytokines such as IL-4 and IL-10, can occur in the absence of IFNγ production (Mossman and Coffman, 1989; Evavold and Allen, 1991; Hollsberg et al., 1995; Jameson and Bevan, 1995). We report here the identification of variants that, in the absence of IFNγ production, can selectively induce IL-10 and IL-4 or IL-10 alone. IFNγ and proliferative responses to one variant of the aa 326–347 epitope of CS were inhibited by costimulation by other natural variants. Further characterization of this antagonistic effect demonstrated a direct and rapid change in the IFNγ to IL-10 production ratio. Recently, a new immunoregulatory CD4 T cell subset, Tr1, has been described that produces high levels of IL-10 and ocassionally TGFβ (Groux et al., 1997). This T cell subset suppresses primary proliferative T cell responses in vitro and downregulates autopathogenic T cell responses in vivo. The ability of altered peptide ligands to cause a rapid and direct change toward such a suppressive phenotype may therefore have important implications not only for infectious diseases but also for the maintainance of the homeostatic state. Indeed, autoreactive Th0 clones may become TGFβ producers upon APL stimulation (Windhagen et al., 1995). Here, IL-10 induction and IFNγ downregulation by APL occured with T cell clones, T cell lines, and peripheral blood mononuclear cells, suggesting a dominant suppressive effect in mixed T cell populations. Such immunoregulatory interactions by natural polymorphic CD4 T cell epitopes may prevent elimination of *P. falciparum* by IFNγ-mediated protective mechanisms and contribute to the overall low level of responses to malaria antigens in endemic areas. Such rapid IFNγ/IL-10 modulation could play a central role in susceptibility to a variety of autoimmune (O'Garra et al., 1997; Cobbold and Waldmann, 1998; Mason and Powrie, 1998) and infectious diseases (Trinchieri, 1997; Hill, 1998).

Results

IFNγ Responses to aa 326–347 Are Found in Less than Half of Naturally Exposed Adult Gambians and Show Limited Cross-Reactivity

With an average of five bites from malarious mosquitoes a year, a Gambian adult will have received in the order of 100 infectious bites (Greenwood et al., 1987; Gupta et al., 1994). Despite this continual antigenic exposure, many Gambian donors fail to respond to the CS protein in T cell proliferation assays (Good et al., 1988), even to the "universally binding" variant of the aa 326–345 epitope (Calvo-Calle et al., 1997). The reasons for this have been unclear, but generalized immunosuppression is unlikely since responses to nonmalarial antigens are found at the expected frequencies. We initially tested the simple hypothesis that polymorphism in the aa 326–347 immunodominant region of CS may generate variants with little cross-reactivity between them, even in polyclonal responses. Thus, T cell responses might not be detected unless all variants were tested. Moreover, responses of donors living in endemic areas would not be susceptible to heterologous boosting, limiting their persistence in vivo.

Table 1. Binding of Natural Variant Peptides of the 326–347 aa Region of the Circumsporozoite Protein of *Plasmodium falciparum*

Variant	Sequence	DR1302	DR1	DR15
V1	PSDQHI**E**KYL**K**TIQNSL**S**TEWS	++	++	++
V2	PSDQHI**E**KYL**K**TI**K**NSL**S**TEWS	++	++	+
V3	PSDQHI**E**KYL**Q**KIQNSL**S**TEWS	++	++	+++
V4	PSDQHI**E**KYL**Q**KI**K**NSL**S**TEWS	++	++	+
V5	PSDQHI**E**KYL**Q**KI**R**NSL**S**TEWS	++	+++	++
V6	PSD**K**HI**K**EYL**N**KIQNSL**S**TEWS	+++	++	++
V7	PSD**K**HI**E**QYL**N**TIQNSL**S**TEWS	++	++	++
V8	PSD**K**HI**E**QYL**K**KIQNSL**S**TEWS	−	−	+++
V9	PSD**K**HI**E**QYL**K**KI**K**NSI**S**TEWS	++	+++	++
pCLIP		++	++	ND
pTT		++	ND	++

Polymorphic positions are marked in bold. Peptides were scored as high (+++), medium (++), low (+), or undetectable (−) binding capacity (IC50 < 1, 1–25, or 25–100 or >100 mg/ml, respectively) to each HLA-DR allele tested. The universally binding epitopes from TT (pTT) and CLIP (pCLIP) were used as positive controls. ND, not determined.

The aa 326–347 epitope region of CS has at least nine natural variants (de la Cruz et al., 1987; Good et al., 1988; Lockyer et al., 1989). Variant V6 of this region has a broad spectrum of HLA-DR binding (Calvo-Calle et al., 1997). Table 1 shows that variation occurs at positions 329, 333, 336, 337, and 339. Positions 329, 332, 333, and 337 are dimorphic and positions 336 and 339 trimorphic (Table 1). Altering residues that contribute to HLA binding can be used as an immune evasion strategy for protective T cell responses (Phillips et al., 1991; de Campos Lima et al., 1994). However, the aliphatic or aromatic residues that are potential HLA-DR-binding anchor positions (Hammer et al., 1994) are conserved (aa 327, 328, 331, and 335) (Table 1). The binding potential of all variants of this region to three common African alleles (HLA-DRB1*1302, HLA-DRB1*0101, and HLA-DRB1*1501) was studied by an in vitro binding assay (Davenport et al., 1995b). Table 1 shows that all variants bound to HLA-DRB1*1501 and 8/9 to HLA-DRB1*1302 and HLA-DRB1*0101 (IC50<100 μg/ml). The nonbinding variant, V8, is absent in West Africa (de la Cruz et al., 1987; Good et al., 1988; Lockyer et al., 1989). No variation decreased binding to all three alleles.

Interferon γ released from T cells may mediate protection from malaria in both animals and humans (Nardin et al., 1989; Moreno et al., 1991; Renia et al., 1993; Weiss et al., 1993; Doolan et al., 1996; Takita-Sonoda et al., 1996; Wang et al., 1996; Doolan and Hoffman, 1997; Plebanski et al., 1998). Early IFNγ production induced from the peripheral blood mononuclear cells (PBMC) of malaria-exposed Gambian individuals by the nine aa 326–347 epitope variants displayed remarkable specificity in ELISPOT assays (Figure 1A). Thus, all aa changes could lead to lack of cross-recognition (3–8 variants) in individuals reacting to different variants. Cross-reactivity was investigated further by restimulating in vitro and testing for cross-reactivity against all variants after 14 days of expansion in culture (Figure 1B). PBMC from malaria-exposed Gambian (D20, D41,

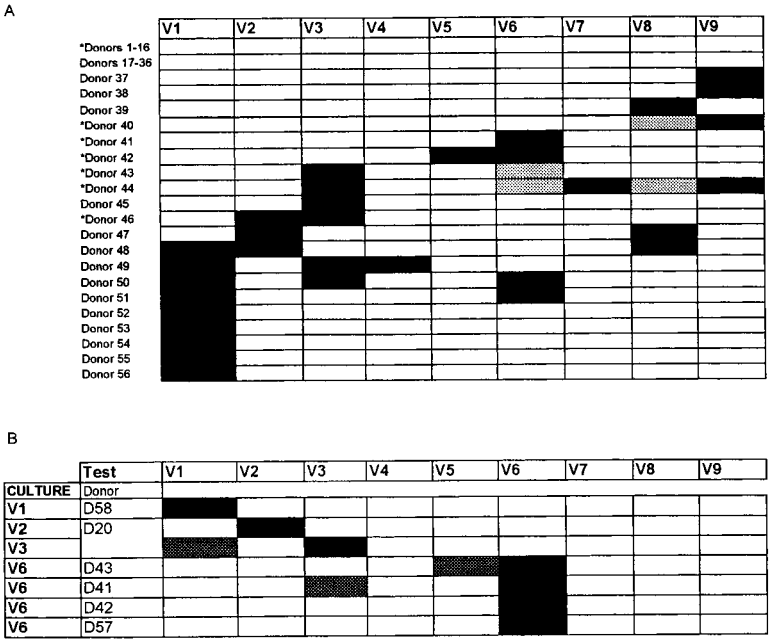

Figure 1. Limited Cross-Reactivy of IFNγ Responses to Naturally Occuring aa 326–347 Variants

(A) Peripheral blood mononuclear cells from adult Gambians were stimulated with aa 326–347 region variants in a 16 hr early IFNγ release assay. All individuals were tested for reactivity to PPD, with the expected responses seen (data not shown). Positivity (filled squares) reflects a precursor frequency of >10 cells/million of IFNγ-producing cells in response to the test epitope and 95% confidence that the response is above background ($p > 0.05$). The 21 individuals where IL-4 reactivity was tested in parallel are asterisked, and responses where both IFNγ and IL-4 were detected are shaded in gray. No reactivity was detected to IL-4 alone. All assays of IL-4 production were positive when stimulated with PHA (data not shown). A further 20 naive individuals tested for reactivity to the same panel of variants failed to react to any variant but reacted at the expected frequencies to the control antigens PPD and TT and to the mitogen PHA (data not shown). (B) PBMC were cultured with the stated variant and tested 14 days later for cross-reactivity against the other variants by IFNγ ELISPOT. As well as Gambian donors 20, 41, 42, and 43, two naturally malaria-exposed European donors who reacted specifically to variants V1 (donor 58) or V6 (donor 57) in ex vivo ELISPOT assays were also tested to evaluate the specificity of V1 or V6 stimulated short-term T cell lines. Donors 41, 42, and 43 gave significant responses to V6 by ex vivo IFNγ ELISPOT whereas donor 20 responses to V2 and V3 were undetectable prior to in vitro expansion. Significant responses over background (SFU > 10/million and $p > 0.05$) for variants other than the stimulating variant are indicated by hatched squares.

D42, D43) or malaria-exposed European (D57, D58) donors were cultured separately with variants and then each line tested for reactivity against all the other variants by IFNγ ELISPOT. Only statistically significant responses from positive lines are shown. Figure 1B shows that all responses detectable after culture were either monospecific or recognized only two of the nine variants.

Out of 56 Gambian individuals tested, only 20 responded to any aa 326–347 region variant by IFNγ production, despite exposure of all individuals in the tested age group (18–65) to *P. falciparum* (Greenwood et al., 1987). Moreover, many infections in The Gambia are coinfections of at least two different variant CS strains (Gilbert et al., 1998). We speculated that nonresponders for IFNγ production, which is a characteristic Th1 type lymphokine, might have generated instead a Th2 type response, characterized by IL-4 production. However, in the 21 Gambian adults assessed in parallel for stimulation of early IFNγ and IL-4 production, the few (3/21) individuals who responded by IL-4 secretion also secreted IFNγ to the same stimulating variant (Figure 1A). None of the 20 malaria naive individuals tested reacted to any of the variants in identical early lymphokine release assays.

Altered Peptide Ligand Antagonism in aa 326–347

Naturally exposed individuals responded frequently to the "universally" binding variant V6 (Figure 1; Table 1) (Calvo-Calle et al., 1997). PBMC from four naturally exposed individuals who responded to variant V6 were tested in an early IFNγ secretion assay to assess the effect of the presence of each one of the other variants on the V6 response. These variants were added after binding with a suboptimal dose of V6 had occurred in a standard antagonism assay (Evavold and Allen, 1991). Figure 2 shows that responses to V6 could be partially inhibited by coculture with some of the naturally occurring aa 326–347 variants. Surprisingly, the same variants could be inhibitory in different individuals (V2, V5, V7, and V9 in 2/4, 3/4, 4/4, and 2/4 individuals showed >50% inhibition, $p < 0.05$). This putative antagonistic APL effect was investigated in detail for donor 57. The response of donor 57 was specific to V6 (Figure 1B). Over the 12 month period when IFNγ antagonism assays on freshly isolated PBMC were performed in donor 57, V5 and V7 were usually inhibitory (V5 in 11/12 and V7 11/13 experiments), despite variation in the absolute level of inhibition (mean inhibition for V5 39% ± 7 SE, $p < 0.0005$; V7 39% ± 9 SE, $p < 0.0009$). The universally binding HLA-DR peptide from TT (pTT) did not inhibit V6 responses when used in antagonism assays ($n = 3$), and PPD responses were not inhibited by V5 or V6 ($n = 2$) (data not shown). Figures 2B and 2C show a representative assay in which proliferative responses in this donor induced by V6 were strongly inhibited by V5 and V7 ($n = 6$ assays). Significant inhibition by V5 but not V7 could be found even if it was presented on a different antigen-presenting cell (APC) from V6 (Figure 2B). This *trans*-inhibition by V5 was surprising and suggested a soluble suppressive mediator.

Selective Induction of IL-10 Production by aa 326–347 Region Variants

Both TGFβ and IL-10 have been shown to have suppressive effects on T cell proliferation (Moore et al., 1993; Groux et al., 1997) and on cytokine production in

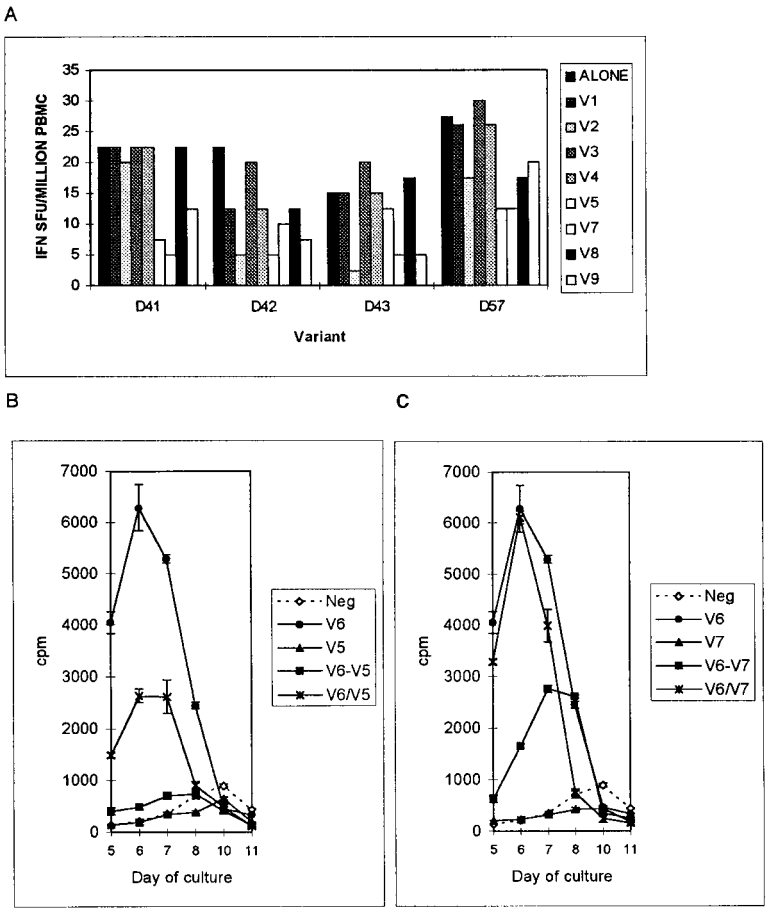

Figure 2. Altered Peptide Ligand Antagonism of V4 IFNγ Responses and Proliferation

(A) Donors 41, 42, 43, and 57 respond to V6 by IFNγ secretion. The impact of the presence of the other variants on the V6 response was assessed in an antagonism assay. Index peptide was prepulsed first at 25 μg/ml onto PBMC and the other variants an hour later at 50 μg/ml. The assay was then run as a standard ELISPOT. For donor 43, the antagonism assay was repeated another two times with similar results and for donor 57, another 12 times. Inhibition in donor 57 was observed from 5 μg/ml of V5 or V7 (n = 2 assays) (data not shown). Proliferation to V6 was induced from PBMC of donor 57 by mixing 1:1 irradiated PBMC that had been sequentially prepulsed with V6 followed by V5 (V6-V5) (B) or V7 (V6-V7) (C) or prepulsed separately (V6/V5 or V6/V7), washed, and then mixed 1:1. Tritiated thymidine incorporation was monitored from days 5–10. Results are shown as the mean cpm ± SE of triplicate samples. The parallel IFNγ ELISPOT assay with the same PBMC showed SFU/million background of 1, V6 response of 13, V6/V5 of 8 (36% inhibition), and V6/V7 of 7 (45% inhibition). A representative experiment of six is shown. The average inhibition for all experiments at the time of peak proliferation for V6 by V5 was 62% ± 7 SE (sequential pulse) and 41% ± 19 SE (separate pulse). For V7 it was 59% ± 13 SE (sequential pulse) and 18% ± 9 SE (separate pulse).

malaria (Ho et al., 1988). Figure 3 shows that inhibition of early V6-induced IFNγ responses by V5 could be completely reversed by neutralizing anti-IL-10 but not anti-TGFβ monoclonal antibodies (n = 3 assays). Assessment of intracellular IL-10 content of a V6-specific line (antagonizable by V5) from the same donor showed doubling of IL-10 production in $CD3^+$ cells by V6/V5 costimulation (Figures 3B and 3C). V5 and V7 also stimulated early IL-10 release from the PBMC of donor 57 both alone and when utilized with V6 in antagonism assays, as detected by ELISPOT (Figure 3D). V5 and V7 alone failed to stimulate significant IFNγ or IL-4 production at any concentration tested (5–100 μg/ml, n = 6 assays) (data not shown).

The effect of the V5 variant on lymphokine production by V6-specific T cells was further studied utilizing clones from donor 57. Figure 4 shows that stimulation of V6-specific clones with V6 induced IFNγ (C2, C5, C6) or IFNγ and IL-10 (C3, C4) secretion. Stimulation of these clones with V6 in the presence of V5 led to downregulation of IFNγ production and a concomitant increase in IL-10. Thus, IL-10 production in CD4 T cell clones may be increased by costimulation with an altered peptide ligand. Oligoclonal responses (when clones were mixed) reflected the dominant change in IFNγ/IL-10 ratio toward IL-10 by the presence of V5 on V6 responses (Figures 4C and 4D). No IL-4 could be detected under any experimental condition in parallel ELISPOT assays.

Consistent with the observation that C3 and C4 use a different TCR (data not shown), their reactivity pattern to V5, V6, and V7 was also different. Thus, clone C4 failed to produce IL-10 to V5 and V7 but still showed significant IL-10 induction by V6-V5 or V6-V7 costimulation. By contrast, clone C3 responded to V6, V7, and V5 alone by IL-10 secretion. As expected, both clones responded by IFNγ secretion to V6 but not V5 or V7 alone (data not shown). Fresh PBMC responses were more similar to C3 reactivity, with IL-10 produced to V5 and V7 and partial downregulation by V5 of V6-induced IFNγ responses (Figure 4F).

Discussion

CS-specific CD4 T cells capable of IFNγ production can protect against malaria in animals and are induced by protective vaccination in humans (Nardin et al., 1989; Tsuji et al., 1990; Renia et al., 1993; Rodrigues et al., 1993; Weiss et al., 1993; Takita-Sonoda et al., 1996; Wang et al., 1996; Doolan and Hoffman, 1997; Stoute et al., 1997). In the present study, we have observed that selected APL variants of the immunodominant CD4 T cell epitope of CS downregulate IFNγ and proliferative responses by preferential induction of IL-10.

Clones capable of high IL-10 secretion with low or absent secretion of other lymphokines, such as IL-2, IL-4, IL-5, or IFNγ, have been denoted Tr1 (Groux et al., 1997). Here, we show that the ratio of IFNγ to IL-10 can be changed during both rapid polyclonal responses and

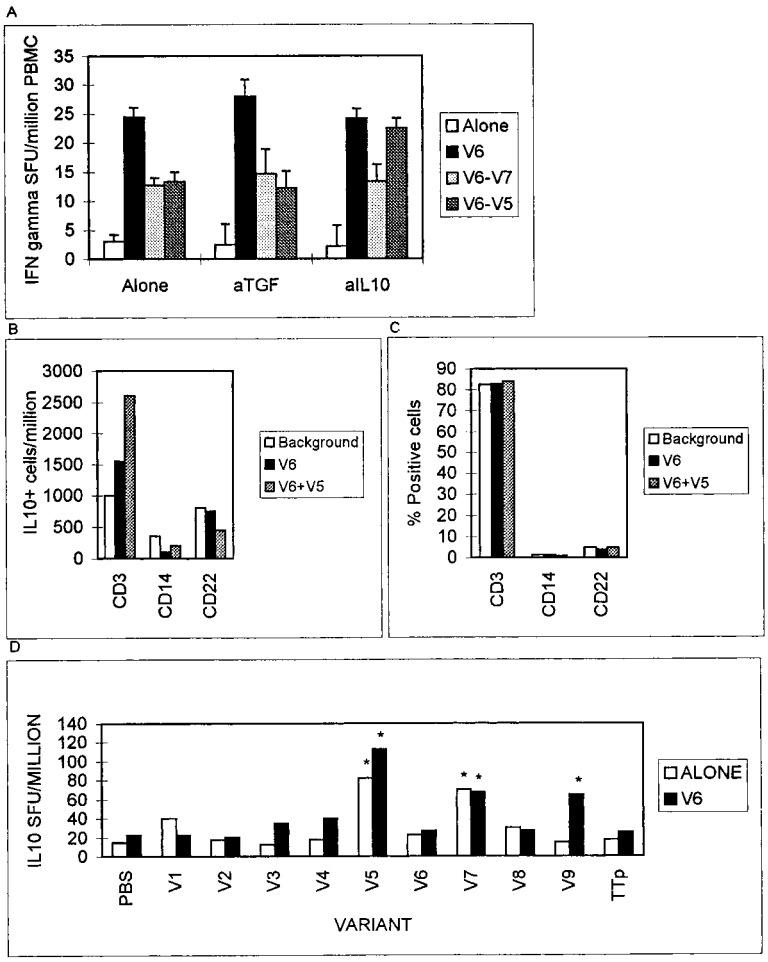

Figure 3. V5-Mediated Inhibition of V6 Responses Is Mediated by IL-10

(A) ELISPOTS were set up for antagonism assays of V5 on V6 responses in donor 57 as described in Figure 2 either alone or in the presence of neutralizing mAbs to TGFβ1 (10 ng/ml; Groux et al., 1997), interleukin 10 (10 ng/ml; Groux et al., 1997), or both. Increasing the anti-TGFβ mAb dose to 100 ng/ml failed to reverse inhibition (n = 2 assays, data not shown). A representative experiment of three is shown.

(B) The V6-specific V5 CD4 T cell line from donor 58 was stimulated with HLA-DR15-matched PBMC alone, pulsed with V6 (20 μg/ml), or pulsed first with V6 and then with V5 (40 μg/ml). The phenotype of IL-10-positive cells was analyzed by FACScan (CD3 for T cells, CD14 for monocytes-macrophages, and CD22 for activated B cells) and presented as IL-10-positive cells per million. This protocol resulted in 100% inhibition of the V6 IFNγ response by V5 in the ELISPOT assay conducted in parallel with the same samples (data not shown).

(C) The total number of CD3-, CD14-, or CD22-positive cells was unchanged under conditions with and without antagonism during this assay.

(D) Early IL-10 production (24 hr) by PBMC from donor 57 in response to aa 326–347 region variants (empty bars) or in V6 antagonism assays (full bars) was tested by ELISPOT. A representative experiment of four is presented utilizing variants at 50 μg/ml. Significant IL-10 induction over control is marked by an asterisk ($p < 0.05$). Titration of responses (range 5–100 μg/ml) showed maximal IL-10 induction by V5, V6, and V7 at 25–50 μg/ml and similarly for V6 (constant at 25 μg/ml), V5, or V7 combinations in antagonism assays (n = 2). There was no significant IL-4 secretion to V6 at any concentration, alone or in combination with V5 or V7 in six experiments over a 6 month period. IL-10 and IL-4 assays used PHA and TT responses as positive controls.

when using long-term clones. Therefore, Tr1 may be an inducible phenotype. Although we induced IL-10 production by an altered peptide ligand, other stimuli may cause a similar rapid conversion. Indeed, changes in lymphokine production could be a useful part of a natural response, going from effector stages producing lymphokines that upregulate cellular to a later stage of downregulation.

Specific aa variation at predicted non-MHC-binding residues in the immunodominant CS-derived aa 326–347 epitope suggested that it may be under an unusual form of selective pressure. Altered peptide ligands of HIV and HBV have been individually shown to provide an escape strategy to these viruses (Bertoletti et al., 1994; Klenerman et al., 1994). We have recently shown how parasites bearing two epitope variants of an HLA-B35 CS epitope may gain a mutual advantage by reciprocal APL antagonism of CTL lytic activity (Gilbert et al., 1998). The family of nine naturally occurring aa 326–347 variants displayed a remarkable lack of cross-reactivity in IFNγ assays. It was difficult to understand how a large family of APLs where single variants "altruistically" minimize cross-reactivity may have been selected (Davenport, 1995). However, the existence of groups of variants with antagonistic effects on the potentially protective IFNγ response, in our case by an increase in IL-10 production, could provide a mechanism for the selective maintenance of such a family.

Interleukin 10 is a pleiotropic lymphokine that can directly or indirectly (via inhibition of accessory cell function) suppress IFNγ, TNFα, GM-CSF, and LT production by T cells and in the presence of monocyte/macrophages inhibits human T cell proliferation (Moore et al., 1993). By analyzing in detail responses to one CS variant, V6, we have observed that IL-10 rather than IFNγ secretion is promoted in response to specific APL combinations. Natural variants of V6 were found that inhibited the IFNγ and proliferative V6-induced response of donor 57. These variants alone could induce IL-10 from freshly isolated PBMC and from V6-specific T cell lines and clones from the same donor. Moreover, IL-10 production was further increased if they were used together with

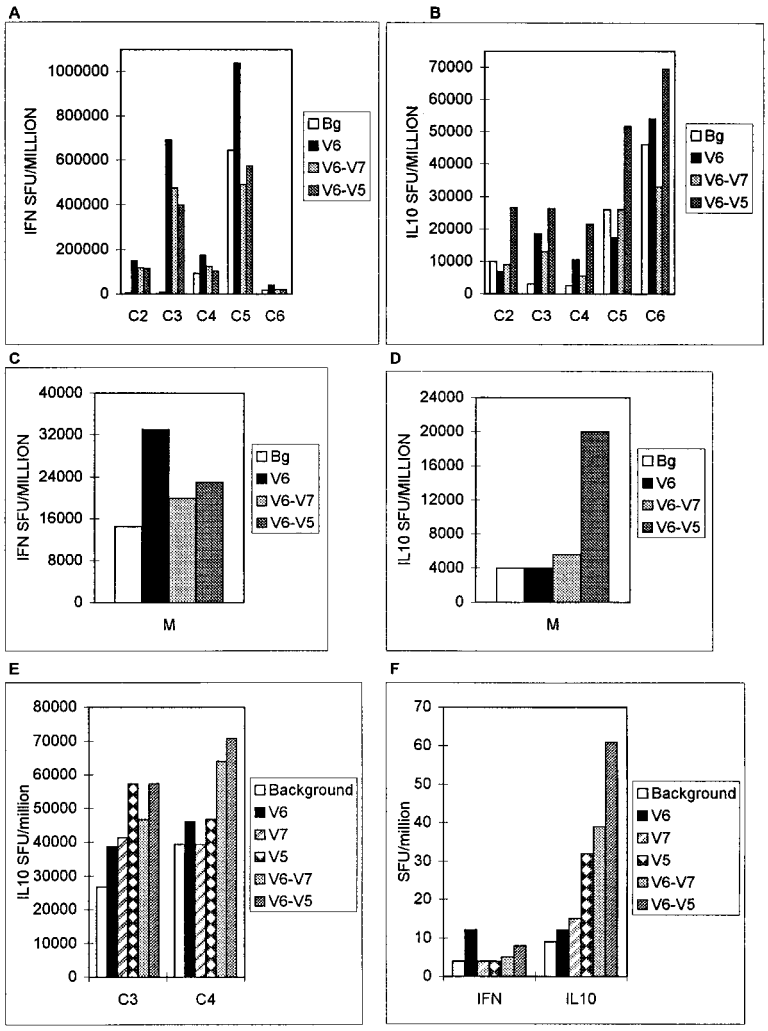

Figure 4. Change in the IFNγ to IL-10 Production Ratio for V6-Specific Responses by Costimulation with V5

(A–D) V6-specific T cell clones generated from donor 57 were tested in antagonism ELISPOT assays. IFNγ, IL-4, and IL-10 production were tested in parallel. Clones (C2, C3, C4, C5, and C6) were utilized at 500 cells/well with either HLA-matched PBMC or an autologous Bcl line (at 50,000/well) as presenters. Oligoclonal responses were assessed by mixing all clones (M). IFNγ responses shown in (A) and (C) and IL-10 responses in (B) and (D) are all from a single experiment performed in duplicate and are representative of four separate experiments for clones C3 and C4, two for C5 and C6, and one for C2. No significant IL-4 production was detected, and presenters alone did not generate any lymphokine in response to any of the peptides tested (data not shown). Significant IFNγ responses over background to V6 and inhibition of V6-induced responses by V5 were seen for all clones ($p < 0.05$). C3 and C4 could give significant IL-10 responses to V6 alone ($p < 0.05$). All clones showed significant IL-10 responses to V6/V5 ($p < 0.05$), and for C2, C4, C5, and C6 these were significantly increased ($p < 0.05$) over V6 alone. For clone C3, the increase in IL-10 for V6/V5 compared to V6 alone was consistently observed ($n = 4$ experiments) but did not reach significance in any single experiment. (E) Clones C3 and C4 were further tested for their ability to respond by IL-10 production to V5 or V7 alone compared to V5 and V7 in the presence of V6. The results shown are from a single experiment performed in triplicate. Clone C3 showed weak IL-10 reactivity over background to V6 or V7 alone or V6/V7 ($p < 0.05$) and strong reactivity to V5 alone or V6/V5 ($p < 0.001$). Clone C4, by contrast, failed here to respond significantly to V5, V6, V7, or V6/V7 by IL-10 production but did so to V6/V5 together ($p < 0.05$). Both clones responded to V6 but not V5 or V7 by IFNγ production (data not shown). (F) Freshly isolated PBMC from donor 57 were set up in parallel agonism and antagonism IL-10 and IFN ELISPOT assays. One of two representative assays is shown.

V6. Thus, IL-10 production and suppressive effects were dominant over the IFNγ response.

Two nonexclusive hypotheses may account for the strong inhibitory effect in polyclonal responses of APL downregulation of proliferation and IFNγ production by IL-10. The first hypothesis is that separate T cell populations react to V6 (by IFNγ production) or V5 (by IL-10 production). When both are induced in the same culture, the IL-10 induced by V5 may nonspecifically downregulate V6 IFNγ responses. However, IFNγ ELISPOT PPD responses in the same donor were not inhibited by V5 costimulation in parallel assays. Thus, IL-10 production induced by V5 alone may not suffice to explain the observed inhibition of V6 responses. Alternatively, T cells bearing the same TCR recognizing both V5 and V6 may be susceptible to differential lymphokine regulation by APL antagonism. In support of this hypothesis, T cell clones did respond differentially by IFNγ and IL-10 production to V6 alone compared to V6 copresented with V5. However, it is likely that, once the IFNγ/IL-10 ratio decreases for a single clone, the IL-10 produced may also play a positive feedback role in promoting an IL-10 response bias.

These results suggest that naturally occuring aa 326–347-specific CD4 T cell responses may change qualitatively by costimulation with APL-bearing parasites. The in vivo consequences of a change in the ratio of IFNγ to IL-10 production are extensive. IL-10 downreglates MHC class II molecules on antigen-presenting cells, inhibits nitric oxide production, and prevents the priming of Th1 T cells (Moore et al., 1993; Groux et al., 1997). Thus, not only the specific IL-10-inducing parasite strain but all malaria parasites in the host it infects may secondarily benefit from this immune evasion strategy. This is in contrast to antagonism at the effector phase of CD8 T cell responses where effects may be localized to APL-infected cells (Davenport, 1995). In support of a *trans*-effect of IL-10 secretion, V5 inhibited V6 responses even when presented by separate APCs. The mechanism we observe for V5 is thus a suppressive-dominant form of "partial agonism" rather than antagonism in its strict definition, although the latter term has been used to cover several related phenomena (Jameson and Bevan, 1995). The same APL negatively affected both rapid IFNγ secretion and proliferation in parallel assays in donor 57. This may make this immune evasion

mechanism singularly powerful, since these two effector functions may be mediated by different memory T cell subsets (K. L. F. and M. P., unpublished data). V7 also induced IL-10 from PBMC, but IL-10 neutralization did not reverse V6 inhibition, and additional suppressive mechanisms might be operating.

IFNγ-secreting cells capable of changing toward increased IL-10 secretion may not be restricted to the aa 326–347 epitope region in malaria and may indeed be a common occurrence. Thus, a recent study of malaria-infected children and adults in Gabon observed that about half of the IL-10 producing CD4 and CD8 T cells (induced nonspecifically) also coexpressed IFNγ. The authors speculate that this might reflect an indirect feedback mechanism for inhibition of IFNγ expression (Winkler et al., 1999). We further suggest that such cells may represent transitory populations capable of the IFNγ to IL-10 secretion change that we describe and thus may provide a fertile ground for parasite immune evasion. Preliminary studies of rapid IL-10 secretion in adult Gambian donors suggested that the majority (10/12 tested) present high background levels of IL-10 secretion. How these high background levels may relate to malaria exposure remains to be determined (Plebanski, unpublished data). Utilizing a highly sensitive PCR technique, some CD4 T cells capable of early IFNγ secretion that also made IL-10 were detected in murine viral bronchiolitis (Hussell et al., 1996). Moreover, T cell lines to *Borrelia burgdoferi* capable of simultaneous IL-10 and IFNγ secretion are greatly increased in patients with Lyme disease compared to controls. Further analysis of this T cell phenotype in a variety of disease states should provide insights into its possible role in homeostatic immunoregulation and immune evasion. Rapid IFNγ/IL-10 modulation is likely to play a pivotal role in autoimmune (O'Garra et al., 1997; Cobbold and Waldmann, 1998; Mason and Powrie, 1998) and infectious (Mahanty and Nutman, 1995; Trinchieri, 1997; Hill, 1998) disease progression.

IL-10 induction by agonist/antagonist aa 326–347 CD4 epitopes is likely to prevent the priming of IFNγ-producing cells (Moore et al., 1993; Groux et al., 1997) and may thus extend in time the benefit to the parasite population by generating a population of susceptible hosts. Indeed, unusually low levels of reactivity characterize T cell responses to *P. falciparum* (Doolan et al., 1993, 1994; Plebanski et al., 1997). Several alternative mechanisms have been suggested for the induction of malaria immunosuppression, such as inhibition of protein synthesis in hepatocytes and macrophages during the preerythrocytic stage (Frevert et al., 1998) and downregulation of macrophage activity by blood stage parasites (Wangoo et al., 1990; Schwarzer et al., 1992) but their relevance to evasion of specific T cell protective responses is still unclear. But these do not explain why in malaria extensive polymorphism is focused on CS T cell epitopes. The mechanism described here, which permits rapid suppression of potentially protective responses, may explain through inefficient priming in an IL-10-rich milieu both general low levels of reactivity and the selection and maintainance of a family of allelic immunodominant epitopes.

Experimental Procedures

Antigens

Peptides representing variant epitopes in aa 326–347 of the preerythrocytic antigen circumsporozoite protein (CS) of *P. falciparum* were from Research Genetics (V1-V9) (Figure 1). Tetanus toxoid (TT) (Evans Biomedical, UK), purified protein derivative from *Mycobacterium bovis* (PPD) (Serumstatens Institut, Denmark), and the mitogen phytohaemoglutinin (PHA) (PHA-L, Wellcome Pharmaceuticals, UK) were positive controls. Unless otherwise stated, antigens were utilized at 25 μg/ml and PHA at 1 μg/ml.

HLA-DR Binding Assays

Binding assays were carried out blind to all cellular results using the principle of competitive binding with a promiscuously binding invariant chain [Ii-(96-114)] biotinylated CLIP peptide (I*) as described (Davenport et al., 1995a, 1995b). In brief, test peptides in DMSO were serially diluted 1:10 in pH 5 buffer (0.02 M 2-N-morpholinoethaqne-sulphonic acid in 0.1 M NaCl and 0.02% azide) and incubated at 37°C for 24 hr with I* (0.1 mg) and class II protein (0.15 mg). Solutions were neutralized with Tris-HCl (pH 7.5) before transfer to 96-well BSA-blocked immunoplates (Nunc) precoated with L-243 antibody. After 2 hr, plates were washed 3× with PBS 0.1% Tween (PBS-T) and 3× with PBS. Plates were developed with avidin-horseradish peroxidase (ExtrAvidin; Sigma)/biotinylated anti-avidin (Sigma)/ExtrAvidin and developed with o-phenylene diamine (0.4 mg/ml) in phosphate citrate buffer. The reaction was monitored every 5 min at 495 nm and peak preequilibrium values used to calculate the concentration of unlabeled peptide required to inhibit 50% binding of I* (IC50). The data was fitted to the binding algorithm $Y = (-(Ymax^* X/(ic50 + X) + background$ using GraphPad Prism 2.0. Binding assays were repeated in triplicate at least twice and the mean calculated.

Cells and Culture Conditions

Blood donors were adult informed consent volunteers living in the villages of Brefet, Dampha Kunda, or Bakau, The Gambia, or volunteers with a known history of malaria exposure working at the John Radcliffe Hospital, Oxford. Heparinized blood was separated on Ficoll and the peripheral blood mononuclear cell (PMBC) fraction collected. Cells were resuspended in RPMI medium supplemented with 10% heat-inactivated human AB serum and 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulphate (RN10) (all Gibco, UK) for immediate use in early ELISPOT assays or for restimulation and expansion in culture. For the latter, cells were cultured for 14 days with 25 μg/ml of the corresponding peptide at 1–2 × 10^6/ml in 1 ml well flat-bottom plates at 37°C in a humid CO_2 incubator, and interleukin 2 (10 U/ml, recombinant human IL-2, Lymphocult T-HP, Biotest, UK) was added on days 5 and 10. Cells recovered on day 14 were washed in RPMI and used at 0.1 million cells/well in ELISPOT as described below.

Assay of Early Lymphokine Production

Unless otherwise stated, for early ELISPOT assays 0.4 million PBMC/well were cultured at 37°C in a humid CO_2 incubator with antigens for 16–24 hr in 96-well flat-bottom nitrocellulose plates (MAIP 45S, Millipore, UK) precoated with anti-IFNγ, anti-IL-4, or anti-IL-10 mAbs according to the manufacturer's instructions (Mabtech, Norway). In assays of antagonism, a suboptimal concentration index peptide (25 μg/ml) was added to the assay 1 hr before the variants (5–100 μg/ml). The next day, cells were removed and the plates washed 4× with phosphate-buffered saline (PBS) with 0.05% Tween 20 (Sigma) followed by 2× in PBS, after which they were incubated at room temperature for 1 hr with the corresponding biotinylated second antibodies, followed by a washing as above and a 1 hr incubation with a streptavidin-alkaline phosphatase conjugate. After washing, a precipitable alkaline phosphatase (AP) substrate was added (Biorad). Data is expressed as the number of spot-forming units (sfu) corresponding to areas of lymphokine secretion counted with the aid of a dissection microscope per million PBMC. To assess lymphokine production from specific T cell clones, HLA-matched PBMC or lymphoblastoma Epstein-Barr transformed B cells (Bcl)

were utilized at 0.05 million cells/well as presenters and the clones at 100–500 cells per well.

Assay of Proliferation

Peripheral blood mononuclear cells were suspended at 1–2 million/ml in RN10 and cultured at 37°C in a humid CO_2 incubator in flat-bottom 1 ml culture plates with the corresponding antigens. Two antagonism assays were performed: the index peptide was added to the culture 1 hr before the variant peptides; or half of the PBMC were irradiated and either incubated for 3 hr with the index peptide (25 μg/ml), washed (3× RPMI), incubated for 3 hr with the variants (50 μg/ml), washed, and then added 1:1 to untreated PBMC as responders and set up at a final concentration of 2 million/well (sequential pulsing), or the irradiated PBMC were prepulsed for 3 hr separately with 50 μg/ml of index or variant peptides, washed, mixed 1:1, and added to untreated responder PBMC (1:1). Triplicate 50 μl samples from each well were transferred between days 5–10 to 96-well U-bottom plates and 1 μCi of ^{3}H-thymidine per well added for 16 hr. The incorporated radioactivity was counted using conventional scintillation methods. Data are expressed as the mean counts per minute (cpm) of triplicate samples ± standard error (SE).

Generation of T Cell Clones

Peripheral blood mononuclear cells from donor 57 (HLA-DR15,4; -DQ6,8;-DP52,53) were cultured with 25 μg/ml V6 as above. On day 7, T cell blasts were isolated by density on a Percoll gradient (30%–40% interfase after 20 min centrifugation at 600 g) and cultured with 10 U/ml IL-2 for 7 days. The blast cell line was V6 specific and HLA-DR15 restricted in IFNγ ELISPOT assays utilizing a panel of class II matched/mismatched PBMC as presenters (data not shown). Cells from this blast line were cloned by limiting dilution onto V6 prepulsed autologous irradiated (2000 rads) PBMC (1 million/ml) in RN10 supplemented with 10 U/ml IL-2. Six clones were isolated from 600 initial wells (C1-6). Clone C1 failed to survive for retesting and has been excluded from the present study. The T cell receptor (TCR) β chain from clones C3 and C4 was sequenced (Moss et al., 1995), confirming for each the existence of a single dominant β-chain TCR product (data not shown). The T cell clones were CD4 positive by FACSCAN analysis (data not shown).

Intracellular Cytokine Staining

The CD4 T cell line (2×10^6) derived from Percoll isolated blast cells (see above) was stimulated in culture with HLA-DR15 matched irradiated PBMC (2×10^6) either alone, with V6 at 20 μg/ml, with V6 at 20 μg/ml first and V5 at 40 μg/ml added after 1 hr. After incubation in flat-bottom 1 ml wells for 18 hr at 5% CO_2 at 37°C, 2 μM monesin was added to cultures for 6 hr. Intracellular cytokine staining was performed to manufacturer's instructions (IL-10 intracelluar staining mAb, Biosource International). In brief, cells were incubated for 30 min on ice with anti-CD3, CD14, CD22, or isotype-matched control FITC-conjugated antibodies (Serotec, UK) in 1% normal human serum, 0.1% (w/v) sodium azide in phosphate-buffered saline (PBS-NHS1az). Cells were washed in PBS-NHS1az and fixed with 4% (w/v) paraformaldehyde in PBS. Fixed cells were permabilized in PBS-NHS1az with 0.1% (w/v) saponin. Anti-IL-10 RPE-conjugated mAb was then added to the cells and incubated for 30 min on ice. Cells were washed twice in the PBS-NHS1az 0.1% saponin (w/v) and suspended in a required volume of isoton buffer (Becton Dickinson) for FACScan analysis.

Acknowledgments

We thank Kirsty Thomson for her generous help during these studies and Miles Davenport for assistance. For comments and encouragement, we thank Ita Askonas, Andrew McMicheal, Fiona Powrie, Hilton Whittle, and Keith MacAdam. C. G. is a Wellcome Trust Senior Fellow and A. H. a Wellcome Trust Principal Research Fellow. This work was funded by the Wellcome Trust.

Received February 12, 1999; revised April 23, 1999.

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