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Plasmodium falciparum liver-stage antigen-1 peptide-specific interferon-gamma responses are not suppressed during uncomplicated malaria in African children

Abstract

Liver-stage antigen (LSA)-1 is a candidate vaccine molecule for Plasmodium falciparum malaria, but knowledge of the evolution of naturally acquired immune responses to LSA-1 in African children is lacking. We therefore assessed cellular immune responses to two defined T cell epitopes of LSA-1, during and after

uncomplicated P. falciparum malaria in a group of Gabonese children. In terms of their prevalence, interferon (IFN)-gamma responses of peripheral blood mononuclear cells (PBMC) to an LSA-1 N-terminal peptide, T1, were significantly higher when measured during the acute phase compared with convalescence. IFN-gamma responses to the LSA-J (hinge region) peptide showed a similar profile, but at a lower prevalence. Depletion experiments confirmed that CD8+ T cells are a major source of peptide-driven IFN-gamma, but both lymphoproliferation and the production of IL-10 in response to either of the peptides was low in all children at all times. PBMC from 25% of the children failed to produce IFN-gamma in response to either peptide at any time-point. The results suggest that lymphocytes producing IFN-gamma in response to at least one T cell epitope of LSA-1 are most frequent in the peripheral circulation during the acute phase of P. falciparum malaria. Thus, in this case, the generalised suppression of cell-mediated responses which characterises acute malaria does not affect liver-stage antigen-specific IFN-gamma production. These findings imply that measurements of the frequency of parasite antigen-specific cellular immune responses in clinically healthy individuals may represent significant underestimations, which has important implications for the design of field-based vaccine antigen-related studies.

Full text

INTRODUCTION

The emergence of multi-drug resistant *Plasmodium falciparum* is a rapidly increasing problem world-wide [1], making the need for an effective vaccine more urgent than ever. One of the main prerequisites for the development of a successful vaccine is a good understanding of the naturally acquired immune responses directed against the parasite, and the lack of such knowledge is currently one of the many obstacles impeding research in this field [2]. Protective immune mechanisms that have been identified to date include both antibody-mediated responses, principally directed at the parasite's erythrocytic stages, as well as cellular immune responses directed at both pre-erythrocytic and erythrocytic stages [3-6]. There is to date, nevertheless, relatively little detailed information concerning the precise targets, in terms of parasite antigens or epitopes thereof, of these responses, although recent results gave some insights into these issues in the context of a sporozoite antigen-based vaccine [7].

The sterile protection conferred to both humans and animals by immunisation with irradiated sporozoites is thought to be the result of the recognition of antigens expressed during intra-hepatocytic development. Following successful penetration of a hepatocyte by a sporozoite, liver stage parasites grow and mature inside a parasitophorous vacuole within the infected cell. The latter are therefore assumed to be able to process and present parasite antigen-derived peptides together with HLA class I and possibly also class II molecules, providing potential targets for, respectively, $CD8^+$ or $CD4^+$ T cell recognition [8]. One source of such peptides is *P. falciparum* liver stage antigen (LSA)-1, which is expressed uniquely during the parasite's intra-hepatocytic phase, where it accumulates as a flocculent mass inside the parasitophorous vacuole before being released along with mature merozoites when the liver schizont ruptures [9-11]. LSA-1 has proven immunogenicity in individuals exposed to infection either artificially or naturally [12]. Recently, a substantial body of evidence has accumulated which points to a pivotal role for LSA-1 in the acquisition of both cell- and antibody-mediated responses mediating protection from infection [12-14]. Since, in addition, LSA-1 is well conserved across *P. falciparum* strains [9, 15, 16], it is now recognised as one of the principal candidates for inclusion in a vaccine [12, 15, 17].

Interferon (IFN)-gamma has been shown to inhibit the intrahepatocytic development of plasmodia *in vitro*, and field data demonstrate that IFN-gamma responses to LSA-1 are associated with protection from reinfection with *P. falciparum* [18-20]. Field studies have also shown that $CD8^+$ cells represent a primary source of LSA-1-induced IFN-gamma [18, 21]. There is, however, only a limited amount of data concerning such responses in African children. We therefore designed the study presented here with the aim of comparing the prevalence and specificity of cellular immune responses directed to two different epitopes of LSA-1 in African children during acute *P. falciparum* malaria as well as during the convalescent period, two and four weeks after anti-malarial treatment.

STUDY SUBJECTS AND METHODS

Study subjects

The study was conducted at the Albert Schweitzer Hospital in Lambaréné, Gabon. *Plasmodium falciparum* transmission in this area is perennial and

hyperendemic, with an entomological inoculation rate of ~50 [22, 23]. Venous blood samples for the immunological assays were taken from 21 children (age range: 1.5-10 years, median 7 years) on three separate occasions corresponding to pre-treatment (day 0) and post-treatment (days 14 and 28), treatment comprising a standard dose of sulfadoxine-pyrimethamine. Thick and thin blood smears were routinely prepared and stained with Giemsa. Individuals with uncomplicated malaria and a parasitemia of at least 3,000/mul (range: 3,000-91,000/mul) were included in the study. Informed consent for participation in this study was obtained, prior to inclusion, from the parent or guardian of each individual. Ethical clearance was obtained for the study from the Ethical Committee of the International Albert Schweitzer Foundation.

Peripheral blood mononuclear cell (PBMC) isolation and stimulation

Peripheral blood was taken by venepuncture into sterile EDTA-containing collection tubes and processed within 2 hours of collection. PBMC were separated by standard density gradient centrifugation (Biochrom, Berlin, Germany) using Leucosep® separation tubes (Greiner, Frickenhausen, Germany), spin-washed in RPMI 1640 (Biochrom) and resuspended at a concentration of 1.5 **x** 10^6 cells/ml in complete culture medium comprising RPMI 1640 (Biochrom) containing 25 mM HEPES, 10% decomplemented normal human AB^+ serum (PAA, Cölbe, Germany) and 50 mug/ml gentamicin (Life Technologies, Eggenstein, Germany); 100 mul aliquots of this suspension were distributed in the wells of flat-bottomed Costar® 96-well tissue culture plates (Integra Biosciences, Fernwald, Germany), and 50 mul volumes of either medium alone, as a control, or antigens/peptides diluted appropriately in medium, were added to the PBMC in triplicate wells. The plates were then incubated at 37° C in a humidified atmosphere containing 5% CO_2. As recall antigen, we used purified protein derivative of *Mycobacterium tuberculosis* (PPD, Statens Serum Institut, Copenhagen, Denmark), at a final dilution of 10 mug/ml, and as mitogen, phytohemagglutinin (PHA-L, SIGMA, Deisenhofen, Germany), also at 10 mug/ml. Two defined *P. falciparum* LSA-1 sequence-derived peptides were used, both at final dilutions of 10 mug/ml: the "hinge-region"-associated LSA-J originally described by Fidock *et al.* (aa 1613-1636, ERRAKEKLQEQQRDLEQRKADTKK) [9], and the N-terminal T1 originally described by Krzych *et al.* (aa 84-107, LTMSNVKNVSQTNFKSLLRNLGVS) [24]. LSA-J was synthesised by the Pasteur Institute, Paris. T1 was supplied by Professor M. Hollingdale.

For neutralisation of endogenous IL-10, 5 mug/ml of an anti-IL-10 antibody (clone B-S10, Diaclone, Giessen, Germany) was added to the cultures. This concentration was chosen as it was shown to be effective in preliminary studies. An isotype-matched, mouse anti-human control antibody (clone B-Z1, Diaclone) was used at the same concentration in control wells.

$CD8^+$ T-lymphocytes were depleted by a standard sterile magnetic bead separation technique using MACS® reagents and equipment (Miltenyi Biotech, Bergisch-Gladbach, Germany). Briefly, 20 mul of MACS® magnetic microbeads conjugated with monoclonal anti-human CD8 antibodies (Miltenyi) were incubated with 10^7 PBMC in a total volume of 100 mul for 15 min at 6° C. After incubation, the volume was adjusted to 500 mul and applied first to a MACS® pre-filter and then to the pre-filled and washed MACS® separation column (both Miltenyi). The flow-through from the column was collected in a 2 ml volume of MACS® elution buffer, washed and diluted in medium to the appropriate concentration and subsequently referred to as the CD8-depleted population. A MACS® fluorescein isothiocyanate-labelled anti-CD8 antibody (Miltenyi) was used to confirm that only $CD8^+$ cells were depleted from the PBMC.

Pooled culture supernatants from triplicate wells, taken after 3 or 6 days' incubation, were frozen at - 80° C until used for cytokine analysis (see below). On the sixth day, supernatants were replaced with an equal volume of medium containing 1 muCi of tritiated thymidine (Amersham, Braunschweig, Germany). After an additional 16 hours incubation, the cultures were processed for liquid scintillation counting. Stimulation indices (SI) of proliferative responses were calculated from triplicate values as previously described [25], and PBMC samples resulting in a SI > 2 were considered to be "responders".

Cytokine assessments

The concentrations of IL-10 (human, not viral) and IL-12 (p40/p70) were measured in supernatants taken on day 3, and of IFN-gamma in those taken on day 6, using commercial ELISA kits (Flexia™, Medgenix-Biosource, Ratingen, Germany), with detection limits of 4.6, 4.5 and 5.7 pg/ml, respectively. After adjustment for sensitivity, cytokine concentrations in the supernatants of unstimulated cultures were subtracted from the values obtained for supernatants of (peptide-) stimulated cultures.

Statistical methods

The correlation between continuous variables was assessed with the Spearman rank test, with a value of rhô > 0.25 (combined with $P < 0.05$) considered significant. Contingency tables, using Fisher's exact test, were used to compare proportions within and between groups. For paired and unpaired analyses, respectively, the nonparametric Wilcoxon rank, Mann-Whitney U- and Kruskal-Wallis tests were used to determine the significance of differences between continuous variables. The level of significance in all cases was set at a two-tailed $P < 0.05$.

RESULTS

Lymphoproliferation

Proliferative activity was consistently highest after stimulation with PPD, and the proportion of responders (SI > 2) increased during convalescence: PBMC from 7/21 (33%) of the children showed proliferative responses in the acute phase, rising to 70 and 90% on days 14 and 28 respectively. The corresponding LSA-1 peptide-specific response was negligible: after stimulation with T1, PBMC from only one child had a SI > 2 (day 28), and only two after stimulation with LSA-J (on days 0 and 14). In CD8-depleted cultures of PBMC from the same children, the proliferative responses to both the plasmodial and mycobacterial stimuli were markedly reduced (data not shown).

Cytokine responses

The median level of IFN-gamma produced by PBMC in response to PPD was highest on day 14 post-treatment, and was significantly higher at this time than on day 0 (Table 1a). PBMC from all but two individuals produced IFN-gamma in response to PPD on all three occasions.

The amounts of IFN-gamma produced after stimulation with the two LSA-1 peptides were comparable both for the group as a whole (ranges: T1: 0-311 pg/ml; LSA-J: 0-422 pg/ml, $P > 0.05$ by Wilcoxon test for all time points), as well as when considering only those designated as responders (Table 1b, c). On day 0, the proportion of IFN-gamma responders to T1 was maximal, and was significantly higher than the proportion responding to LSA-J (0.60 *versus* 0.32, *P*

= 0.018 by khi^2 test, Table 1b, c). All six individuals with responses to LSA-J at this time also responded to T1, producing similar amounts of IFN-gamma to the two peptides (medians [interquartile ranges]: LSA-J: 36 [55]; T1: 10 [10], $P > 0.05$ by the Wilcoxon test). The proportion of IFN-gamma responders to the T1 peptide declined significantly from day 0 to day 14/day 28 (0.60 *versus* 0.21, $P = 0.023$ by khi^2 test, Table 1c). The proportion of IFN-gamma responders to LSA-J declined, although not significantly so, after 28 days of convalescence (Table 1b). Cumulated IFN-gamma-production data show that PBMC from 70% of the children produced IFN-gamma after stimulation with T1, PBMC from 55% produced IFN-gamma in response to both T1 and LSA-J, 15% responded to T1 alone, and 5% to LSA-J alone. PBMC from 25% of the children failed to produce IFN-gamma in response to either of the two peptides at any time point. Of the total of fourteen individuals responding to T1 stimulation with IFN-gamma, only two were non-responders on day 0. Individuals whose PBMC produced IFN-gamma in response to LSA-J on day 0 were not the same as those responding during the convalescent period. There were no significant differences in the ages of peptide-specific IFN-gamma responders at the different time-points (*e.g.* for T1: day 0 (median age in years ± interquartile range) 6.25 ± 4.37; day 14 6.62 ± 4.62; day 28 8.5 ± 3.13; P = NS by the Kruskal-Wallis test).

Depletion of $CD8^+$ cells resulted in a 50% reduction in the number of samples showing T1-driven IFN-gamma responses at day 0, and a 100% reduction in samples taken subsequently (Table 1c). Pairwise comparison of the amounts of T1-induced IFN-gamma detected in the acute phase (day 0) by PBMC samples unaffected by the removal of the $CD8^+$ population, showed that they were similar to the amounts produced in the undepleted PBMC samples ($P > 0.05$ by the Wilcoxon test, Table 1c). In day 0 samples, ELISA results showed that the anti-IL-10 monoclonal antibody effectively neutralised all IL-10 activity, but this affected neither the proportion of PBMC that responded to T1 peptide stimulation with IFN-gamma production, nor the amount of IFN-gamma produced (data not shown).

The amount of IL-12 produced by responders' PBMC after PPD or LSA-1 peptide stimulation did not change significantly with time for either PPD or the LSA-1 peptides, although the proportion of individuals responding to all three molecules did decline from day 0 to day 14/28, significantly so in the cases of PPD and the T1 peptide ($P < 0.001$ and $P = 0.006$, respectively, by khi^2 test, Table 2a, c). Half of the individuals with IFN-gamma responses to T1 on day 0 also had

IL-12 responses to the same peptide at that time, as did 5/11 with IFN-gamma responses to LSA-J, but the amounts of IFN-gamma and IL-12 produced in response to each peptide were not correlated ($P > 0.05$ by the Spearman Rank test).

Production of IL-10 in response to PPD increased significantly over the time course of 4 weeks, accompanied by an increase in the proportion of those responding with production of this cytokine from day 0 to day 14 (Table 3a). Following stimulation with the LSA-1 peptides the number of responders producing IL-10 was low (~20%) throughout, as were the amounts of IL-10 produced to T1 in particular (LSA-J: range: 0-92 pg/ml; T1: range: 0-65 pg/ml, Table 3b, c).

DISCUSSION

This study was designed to compare the cellular immunological responses to two different peptide epitopes of liver stage antigen-1 during and after uncomplicated *P. falciparum* malaria in African children. Subsidiary aims included a determination of the effects on these peptide-specific responses of the immunosuppression which characterises acute *P. falciparum* infections, as well as elucidating the role of $CD8^+$ T cell populations and cytokines in generating and controlling peptide-specific IFN-gamma responses. The principal findings concerned firstly the proportion of individuals capable of mounting IFN-gamma responses during the acute phase of the infection, which was markedly higher for the N-terminal T1 compared with the hinge region LSA-J peptide. Secondly, we found that Th1-type cytokine responses, IL-12 and IFN-gamma, to the two LSA-1 peptides were unaffected by the immunosuppressive effects which demonstrably affected both lymphoproliferative and cytokine responses to an unrelated mycobacterial antigen. We also found that IFN-gamma responses to the two peptides depend to a large extent on a $CD8^+$ T cell population, but that IL-10 does not regulate IFN-gamma responses to the N-terminal T1 peptide.

The prevalence of lymphoproliferative responses to the LSA-J peptide in a cohort of Gabonese children with mild *P. falciparum* in an earlier study at the same site was low (< 20%, A.J.F. Luty, unpublished observations). The low prevalence we report here (in a group of children with an older mean age compared with those in the earlier study) is thus consistent with that finding and with those of Fidock *et al.* [9] in their Madagascan study. A contrastingly

high prevalence (> 80%) of lymphoproliferative responses to the T1 peptide was found with cells from adult Papua New Guineans [18], although their prevalence was lower (59%) in a recent study with a group of adolescent/adult Indians [26]. In a parallel study of healthy, aparasitaemic (blood-smear negative) Gabonese children and adults, we found that the prevalence of lymphoproliferative responses to either peptide was also low (M. Bongartz, unpublished observations), whilst similarly low frequencies of such responses to LSA-1 peptides were seen in a separate study of Gabonese schoolchildren [14].

We have previously observed a dissociation of cytokine responses from lymphoproliferative responses to parasite antigens/peptides during acute *P. falciparum* infections, as have others [20, 27, 28]. Thus, the presence of cytokine responses in the absence of a proliferative response that we observed in the present study is consistent with those findings. In this context, the high frequency (60%) of IFN-gamma responses to the T1 peptide are of particular interest. A number of other studies have investigated IFN-gamma responses either to the same peptide, or to shorter (9-mer) peptides corresponding to HLA class I-restricted epitopes within the T1 sequence. None are strictly comparable with our own, as the majority used cell samples exclusively from adults, and none examined responses during an acute malaria episode. Nevertheless, in no case was a 60% frequency of IFN-gamma responses found. In those studies which used the same T1 peptide, 45% of an adult Indian population responded with IFN-gamma, whereas 33% of an adult Papua New Guinean population and ~20% of a mixed-age Kenyan population did so [18, 21, 26]. In one Kenyan study which used shorter T1-sequence peptides, cells from < 10% of adults produced peptide-specific IFN-gamma, whilst in another Papua New Guinean study, ~10% of children aged less than ten, but > 40% of adults aged greater than thirty did so [29, 30]. The frequency of peptide-specific IFN-gamma responses we observed in the present study during the convalescent period, ranging from 21-39% for T1 & LSA-J, is in closer accordance with these other studies.

The high frequency of peptide-specific IFN-gamma responses we observed in the acute phase of the infection was paralleled by a suppression of such responses to the non-parasite antigen PPD. *P. falciparum* malaria is accompanied by a reduction in the absolute numbers and perhaps also the functional capacity of lymphocytes in the peripheral blood [31-33]. In addition, it has been shown that IFN-gamma responses to a mixture of soluble asexual stage *P. falciparum* antigens are reduced in young children during acute *P.*

falciparum malaria, but elevated during convalescence [28]. This is the same profile we observed in an earlier study with a 15-mer peptide from a conserved region of the *P. falciparum* merozoite surface antigen-1 (A.J.F. Luty, unpublished observations), but clearly the converse of our observations here with LSA-1 peptide-stimulated responses. We speculate that the release of LSA-1 directly into the bloodstream during the rupture of infected hepatocytes [9, 34] leads to a systemic activation, clonal expansion and distribution of LSA-1-specific $CD4^+$ and $CD8^+$ T cells. The frequency of such cells in the peripheral circulation would not be restricted due to sequestration or reallocation to deep organs in response to the asexual blood stage infection. As a result, they could be present at a relatively higher frequency during the acute phase of the infection when both the number and frequency of asexual parasite antigen-specific populations is reduced due to reallocation [32]. The reduction in the frequency of LSA-1-specific cells during convalescence could result from a combination of a decline in their rate of expansion, as well as the rapid overpopulation of the peripheral circulation with re-emergent cells after treatment, which would have a diluting effect on any pre-existing populations [32].

A predominantly $CD8^+$-driven IFN-gamma response to LSA-1 peptides was first reported by Connelly *et al.* in their study of adult Papua New Guineans, and subsequently by John *et al.* in their Kenyan study [18, 21]. Our results confirm these earlier findings, but show, in addition, that there is a separate source of IFN-gamma during acute *P. falciparum* malaria in a proportion of individuals. Further studies are necessary to determine the cellular sources of this non-$CD8^+$ T cell-derived IFN-gamma. Both LSA-1-specific $CD4^+$ T cells and/or non-specific NK cells, for example, could be present in the PBMC populations we used. IFN-gamma-producing $CD4^+$ T cells are recognised as important effectors in pre-erythrocytic stage immunity [35], and there is strong evidence for the existence of HLA class II-restricted LSA-1-specific $CD4^+$ T cells [13]. Also, a population of IFN-gamma-producing $CD4^-/CD8^-$ cells, which may include NK cells, is present in the peripheral circulation of children with *P. falciparum* malaria [36], and both NK and NKT cells have been identified as effector cells in experimental models of malaria [37-39]. Induction of IL-12, as we observed in ~50% of acute phase samples in this study, would be expected to upregulate IFN-gamma production, especially from pre-activated NK cells.

The low frequency of peptide-driven IL-10 responses we observed, in particular for the T1 peptide, accords well with the findings of other studies which also

used LSA-1 peptides [14, 21]. Abrogation of IL-10 using an anti-IL-10 antibody has previously been shown to enhance proinflammatory cytokine (TNF, IL-1beta, IL-6) production from malaria antigen-stimulated PBMC of individuals with *P. falciparum* [27, 40]. Our results are not consistent with a regulatory role for IL-10 in the peptide-driven proinflammatory responses we assessed during the acute phase here, although it should be stressed that we measured only IFN-gamma and IL-12 production. Production of the bioactive heterodimer (p70) of IL-12 is controlled at least partly by the presence of its p40 homodimer, which is normally produced in excess [41]. This could explain the decreased IL-12 activity we detected in samples taken during convalescence. As we have shown in a separate study, heterodimeric IL-12 is detectable during acute *P. falciparum* malaria [42].

CONCLUSION

In summary, the principal findings of this study imply that estimates of individuals cells' ability to produce cytokines, and in particular IFN-gamma, in response to candidate vaccine antigens, may be marked underestimations if assessed only in healthy and/or asymptomatic individuals. The study also shows that stimulation with the T1 peptide of LSA-1 results in the induction of IFN-gamma production by mononuclear cells from a majority of young African children residing in an area where *P. falciparum* is perennially transmitted, and that a $CD8^+$ T cell subset present in peripheral blood represents a major source of this cytokine. More detailed studies will be necessary to define the identity of other cell types involved in generating specific cytokine responses to LSA-1 in this setting.

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Figures

Table 1
IFN-γ responses of childrens' PBMC during and after acute *P. falciparum* infection

	Number of responders*	IFN-γ production (pg/ml)**	Number of non-responders	Number of responders after CD8+ depletion	IFN-γ production (pg/ml)** CD8+ depleted
a) PPD stimulation					
Day 0	18	358 (169)[1]	2	15	358 (201)
Day 14	19	547 (379)	0	19	421 (538)
Day 28	18	519 (537)	1	18	517 (653)
b) LSA-J stimulation					
Day 0	6	36 (55)	13	4	40 (216)
Day 14	7	43 (228)	11	3	38 (31)
Day 28	4	5 (29)	14	2	16 (20)
c) T1 stimulation					
Day 0	12	11 (107)	8	6	22 (44)
Day 14	4	54 (154)	15	0	–
Day 28	4	7 (4)	15	0	–

* responders are defined by PBMC cytokine production of at least 1 pg/ml greater than the assay sensitivity threshold and 1 pg/ml over the medium (unstimulated) control value.
** values given are median (interquartile range) of responders' PBMC only.
[1] $P = 0.031$ for comparison of IFN-γ level with that on day 14 (Mann-Whitney U-test); levels did not differ significantly between any of the time-points for either LSA-1 peptide (Kruskal-Wallis test).
In this and following Tables, insufficient numbers of cells precluded stimulations with PBMC from all 21 study participants at all times.

Table 2
IL-12 responses of childrens' PBMC during and after acute *P. falciparum* infection

	Number of responders*	IL-12 production (pg/ml)**	Number of non-responders
a) PPD stimulation			
Day 0	12	41 (77)	8
Day 14	0	0	19
Day 28	6	27 (40)	13
b) LSA-J stimulation			
Day 0	10	39 (175)	9
Day 14	5	24 (170)	14
Day 28	5	27 (25)	14
c) T1 stimulation			
Day 0	11	53 (73)	9
Day 14	2	82 (70)	17
Day 28	3	22 (29)	16

* responders are defined by PBMC cytokine production of at least 1 pg/ml greater than the assay sensitivity threshold and 1 pg/ml over the medium (unstimulated) control value.
** values given are median (interquartile range) of responders' PBMC only, which did not differ significantly between any of the time-points for PPD or for either of the LSA-1 peptides (Mann-Whitney/Kruskal-Wallis tests).

Table 3
IL-10 responses of childrens' PBMC during and after acute *P. falciparum* infection

	Number of responders*	IL-10 production (pg/ml)**	Number of non-responders
a) PPD stimulation			
Day 0	15	41 (172)'	5
Day 14	19	146 (184)'	0
Day 28	18	418 (439)	0
b) LSA-J stimulation			
Day 0	4	39 (175)	15
Day 14	4	24 (170)	15
Day 28	3	27 (25)	16
c) T1 stimulation			
Day 0	2	38 (54)	18
Day 14	1	3 (0)	18
Day 28	3	5 (8)	16

* responders are defined by PBMC cytokine production of at least 1 pg/ml greater than the assay sensitivity threshold and 1 pg/ml over the medium (unstimulated) control value.
** values given are median (interquartile range) of responders' PBMC only, which did not differ significantly between any of the time-points for either of the LSA-1 peptides (Kruskal-Wallis test).
' $P < 0.02$ for comparison of IL-10 level with that on day 28 (Mann-Whitney U-test).

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