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Age-dependent enhancement of IFN-γ responses to *Plasmodium falciparum* liver stage antigen-1 T cell epitopes

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Abstract We assessed the cellular immunological responses to two *Plasmodium falciparum* liver-stage antigen (LSA)-1-derived T-cell epitopes in healthy Gabonese children and adults. The N-terminal peptide, designated T1, induced interferon (IFN)-γ production in peripheral blood mononuclear cells (PBMC) from a significantly lower proportion of children compared to adults, but both interleukin (IL)-10 and IL-12 were produced by similar proportions of PBMC from the two groups. The LSA-1 junction region peptide (LSA-J) also induced IFN-γ in a lower, but in this case statistically non-significant, proportion of PBMC from children compared to adults, whilst the proportions producing either IL-10 or IL-12 were again similar. Higher amounts of both IFN-γ and IL-10 were induced by LSA-J compared to T1. CD8+ T-cells were shown to be primarily responsible for the production of peptide-driven IFN-γ. The results suggest a significant age-related increase in the proportion of individuals capable of producing IFN-γ to the N-terminal T1 epitope, with a shift from a predominantly IL-10-led response in children.

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Introduction

Plasmodium falciparum affects residents and travellers in the tropics and subtropics, causing the death of more than a million children under the age of five every year in sub-Saharan Africa (World Health Organisation 1999). The development with age of a parasite-specific semi-immunity in areas of intense transmission is thought to be reflected by an increased capacity of the host's immune system to respond to parasite-specific antigens (Ramasamy 1998; Roggero et al. 1999). This non-sterile type of immunity limits parasitaemia to barely detectable levels in adults (Trape et al. 1987). Putative protective mechanisms include the generation of antibodies against the sporozoite and erythrocytic stages, as well as cellular immune responses directed to the intra-hepatic stage. Recognition of parasite-specific (and/or infection-associated) molecules in a MHC class I restricted manner by cytotoxic T lymphocytes has been proven to be protective in mice and certain HLA antigens are associated with resistance to severe malaria (Hill et al. 1992; Krzych et al. 2000; May et al. 2001).

In the intrahepatic phase of the infection, when the parasite burden is relatively low, attrition through the recognition of parasite-specific antigens will reduce the initial burden of asexually dividing parasites in the blood. A liver stage-specific antigen of *P. falciparum*, called liver stage antigen (LSA)-1, was identified some years ago (Guerin-Marchand et al. 1987). LSA-1 accumulates in the parasitophorous vacuolar space (between the inner plasmalemma and the outer parasitophorous vacuole membrane) inside the hepatocyte as well as on the surface of the developing merozoites (Hollingdale et al. 1990; Fidock et al. 1994). It is a 200 kDa protein, which is well conserved across different *P. falciparum* strains, but for which no equivalent molecule has been identified in

other plasmodia (Hollingdale et al. 1990; Yang et al. 1995; Kurtis et al. 2001). Cytokines like interferon (IFN)-γ, which promote the inflammatory arm of the immune system, have long been implicated in the control of plasmodial growth and multiplication (Ferreira et al. 1986; Seguin et al. 1994), and the production of IFN-γ in response to LSA-1 peptides is associated with protection from reinfections with *P. falciparum* (Connelly et al. 1997; Luty et al. 1999). Other studies have shown that interleukin (IL)-10 and antibody responses to LSA-1 show similar associations with protection from reinfection (Migot-Nabias et al. 2000; Kurtis et al. 2001). Recent studies in Kenya and Papua New Guinea have shown an influence of age on LSA-1-specific IFN-γ responses in particular (Bucci et al. 2000; John et al. 2000). The present study aimed to assess the evolution of LSA-1-specific immunity in a population with high-level and perennial exposure to *P. falciparum* infection by comparing cellular immunological responses in children and adults who were healthy and aparasitaemic at the time of sample collection.

Materials and methods

Study subjects

The study was conducted at the Albert Schweitzer Hospital in Lambaréné, Gabon, from October–November 1999, during one of the two rainy seasons. *Plasmodium falciparum* is hyperendemic and perennially transmitted in this predominantly rural, forested, equatorial area (Wildling et al. 1995; Sylla et al. 2000). Venous blood samples for the immunological assays were taken from 26 adults (age range: 15–44 years, median 24), all of whom were life-long residents in Lambaréné and will have experienced numerous malaria attacks as a consequence. Thick and thin blood smears were prepared routinely and stained with Giemsa for the detection of plasmodia. Only healthy, aparasitaemic individuals were included in the study. Samples were also taken from 40 children (age range: 4.5–10.5 years, median 7.5) who were all participants in a long-term follow-up study of malaria. They were asymptomatic, without fever or parasitaemia, at the time of sample collection, and had been aparasitaemic for at least 6 weeks prior to that time as judged by microscopical diagnostic criteria. For confirmation, the more sensitive PCR-based detection of parasites was performed with frozen whole blood samples after DNA purification (by Qiagen blood kit) at a later date. Informed consent for participation in this study was obtained, prior to inclusion, from adults and from the parent or guardian of each child. Ethical clearance was obtained for the study from the Ethics Committee of the International Foundation of the Albert Schweitzer Hospital.

Peripheral blood mononuclear cell isolation and stimulation

Peripheral blood was taken by venepuncture into sterile EDTA-containing collection tubes and processed within 2 h of collection. Peripheral blood mononuclear cells (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 1.5×10^6 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 μg/ml gentamicin (Life Technologies, Eggenstein, Germany). Aliquots of 100 μl of this suspension were distributed in the wells of flat-bottomed Costar 96-well tissue culture plates (Integra Biosciences, Fernwald, Germany), and 50 μl 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 a purified protein derivative of *Mycobacterium tuberculosis* (PPD, StatensSerumInstitut, Copenhagen, Denmark) at a final dilution of 10 μg/ml, and as mitogen, phytohemagglutinin (PHA-L, Sigma, Deisenhofen, Germany) also at 10 μg/ml. Two defined *P. falciparum* LSA-1 sequence-derived peptides were used, both at final dilutions of 10 μg/ml: the 'hinge-region'-associated LSA-J originally described by Fidock and colleagues (1994) (aa 1,613–1,636, ERRAKEKLQEQQRDLEQR KADTKK), and the N-terminal T1 originally described by Krzych and colleagues (aa 84–107, LTMSNVKNVSQTNFKSLLRNLG VS) (Krzych et al. 1995) LSA-J was synthesised by the Pasteur Institute, Paris. T1 was supplied by M. Hollingdale.

For the neutralisation of endogenous IL-10, 5 μg/ml of an anti-IL-10 antibody (clone B-S10, Diaclone, Giessen, Germany) was added to the cultures. This concentration was chosen as it has been shown to be effective in other 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 μl of MACS magnetic microbeads conjugated with monoclonal anti-human CD8 antibodies (Miltenyi) were incubated with 10^7 PBMC in a total volume of 100 μl for 15 min at 6°C. After incubation, the volume was adjusted to 500 μl 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: depleted populations contained $<1\%$ CD8+ cells.

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 μCi of tritiated thymidine (Amersham, Braunschweig, Germany). After an additional 16 h incubation, the cultures were processed for liquid scintillation counting. Stimulation indices (SI) of proliferative responses were calculated from triplicate values as previously described (Luty et al. 1994), 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-γ 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 $\rho > 0.25$ (combined with $P < 0.05$) considered significant. Contingency tables, using Fisher's exact test, were used to compare proportions within and between groups. For comparisons of continuous variables within and between groups the nonparametric Wilcoxon rank sum test and Mann-Whitney U-tests were used. The level of significance in all cases was set at a two-tailed $P < 0.05$.

Results

Lymphoproliferation

Proliferation of PBMC of both adults and children was highest in response to PPD. From adults 18/22 (82%) and from children 12/22 (55%) reached a SI of 2 or more. Comparison of the SI of responders showed that there was a non-significant trend for a higher level of proliferation in PBMC from adults [median (interquartile range): 5.2(11.5) vs 3.1(4.7) adults vs children]. The parasite antigen-specific response was much less vigorous. PBMC from only 1/24 adults responded to either T1 or LSA-J with an SI > 2, whilst PBMC from 2/22 and 0/22 children responded to T1 and LSA-J, respectively. CD8 + -depletion of cultures did not affect the proportion of samples classified as responders to any stimulus, and had no effect on the level of the response to PPD (data not shown).

Cytokine responses

Cytokine production in children versus adults

PBMC from all but one individual (a child) produced IFN-γ in response to PPD and a similar proportion of children's (87%) and adults' (80%) PBMC produced IL-10 in response to this recall antigen. A lower proportion of adults' PBMC produced IL-12 after stimulation with PPD (27% vs 56%, $P = 0.024$ by Mann-Whitney U-test). A significantly higher proportion of adults' compared with children's' PBMC produced IFN-γ in response to T1, although not to LSA-J (50% vs 18%, $P = 0.007$ and 48% vs 30%, $P > 0.05$, Table 1a), but adults' PBMC produced significantly higher amounts of IFN-γ in response to either peptide (Fig. 1). Peptide-driven IL-10 or IL-12 production was similar in children's' and adults' PBMC (Table 1b, c; Fig. 2).

Since reciprocal regulatory networks exist between IL-10 and both IFN-γ and IL-12, we made more detailed assessments of the ability of individual's PBMC to respond to stimulation by comparing the ability to produce IL-10 alone or a combination of IL-10 with either IFN-γ or IL-12. In response to T1, a significantly higher proportion of samples from children compared to adults produced IL-10 alone rather than IL-10 with IFN-γ (56% vs 13%, $P = 0.023$), and a similar but non-significant trend was seen for responses to LSA-J (56% vs 23%, $P = 0.086$). Segregation according to the combination of IL10 and IL12 responses revealed no significant differences for responses to either peptide. Comparisons of the ratios of

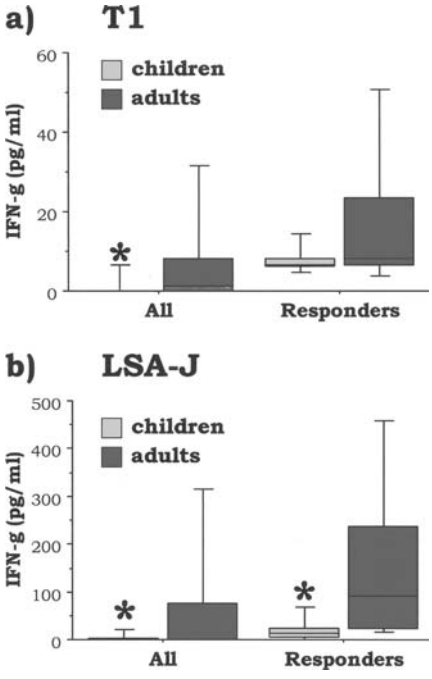

Fig. 1. The amounts of interferon-γ detected in the supernatants of children's and adults' PBMC stimulated with either T1 (**a**) or LSA-J (**b**) LSA-1-derived peptides. *Box-plots* represent medians, with 25th and 75th percentiles and *error bars* for the 10th and 90th percentiles, of responses of whole groups or of responders only. An *asterisk* indicates $P < 0.05$ for comparison of responses of children's and adults' PBMC

Table 1. The proportions of children's and adults' PBMC samples producing cytokines after stimulation with different LSA-1 peptides

	Responder to T1 only	Responder to T1 and LSA-J	Responder to LSA-J only
a) IFN-γ			
Children	10%	8%	21%
	18% T1 responder		30% LSA-J responder
Adults	39%	14%	33%
	53% T1 responder		47% LSA-J responder
b) IL-10			
Children	7%	18%	32%
	25% T1 responder		50% LSA-J responder
Adults	5%	18%	27%
	23% T1 responder		46% LSA-J responder
c) IL-12			
Children	19%	25%	14%
	44% T1 responder		39% LSA-J responder
Adults	13%	25%	21%
	38% T1 responder		46% LSA-J responder

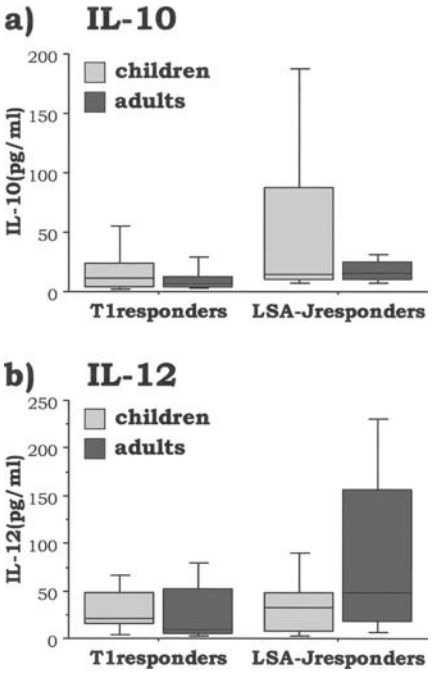

Fig. 2. The amounts of IL-10 (**a**) or IL-12 (**b**) detected in the supernatants of children's and adults' PBMC stimulated with either T1 or LSA-J LSA-1-derived peptides. *Box-plots* represent medians, with 25th and 75th percentiles and *error bars* for the 10th and 90th percentiles, of responses of responders only

cytokine concentrations showed that the IL10:IFN-γ ratio decreased markedly from children to adults in response to both T1 and LSA-J, and similar but statistically non-significant changes were observed for the IL10:IL12 ratio (Table 2).

Effects of CD8+ cell depletion or neutralization of IL-10

Depletion of CD8+ cells caused varying levels of reduction in the proportion of responders to the two peptides: in both children and adults a similar proportion (9/13 and 5/7, i.e. ~70%) of PBMC with T1-driven IFN-γ responses converted to IFN-γ non-responders after the removal of CD8+ cells, whereas those with IFN-γ responses to LSA-J were reduced by 46% (5/11) and by 40% (4/10) in children and adults, respectively, after CD8+ cell depletion. The amount of IFN-γ produced by PBMC samples of donors that persisted as responders after CD8+ cell depletion was unchanged (data not shown).

Depletion of CD8+ cells had no effect on either the proportions of either IL-10 or IL-12 responders to either peptide, or on the amounts of either IL-10 or IL-12 produced in response to either peptide. There was no difference in the amount of IL-10 or IL-12 produced by PBMC samples following segregation according to an increase or decrease of either IFN-γ or IL-10 production after removal of CD8+ cells (data not shown).

The efficiency of neutralization of IL-10 was confirmed by the absence of detectable IL-10 in the supernatants of cultures treated with anti-IL-10 antibody. Stimulation with T1 induced PBMC from nine children to produce IL-10 without IFN-γ. Two of these PBMC samples did produce IFN-γ after neutralization of IL-10. No such changes were seen with PBMC samples of adults.

Comparison of responses to T1 and LSA-J peptides

T1 and LSA-J stimulated IFN-γ production by PBMC from similar proportions of adults (50% vs 48%), and from 18% and 30% of children's' PBMC, but the difference between the latter proportions was not statistically significant (Table 1a). The proportions of samples producing IFN-γ to both peptides simultaneously did not differ significantly between the groups (8% vs 12% in children and adults, respectively, Table 1a). LSA-J stimulated significantly higher amounts of IFN-γ compared to T1 whether comparing all individuals ($P=0.046$ by Wilcoxon rank sum test) or only the amounts of IFN-γ produced by PBMC of responders ($P=0.043$ by Mann-Whitney U-test, Fig. 1). The amounts of IFN-γ produced in response to the two peptides were not correlated ($\rho=0.149$, $P=0.371$ in children, $\rho=-0.232$, $P=0.300$ in adults, by Spearman rank test).

The proportion of children's' PBMC that produced IL-10 was significantly higher after stimulation with LSA-J compared with T1 (50% vs 25%, $P=0.034$, Table 1b), and a similar but non-significant trend was seen with adults' PBMC (46% vs 19%, $P=0.066$, Table 1b). LSA-J stimulated significantly higher amounts of IL-10 ($P<0.001$ by Wilcoxon rank sum test). The amounts of IL-10 produced to the two peptides were positively correlated in adults ($\rho=0.536$, $P=0.014$) but not in children ($\rho=0.238$, $P=0.147$).

In terms of their ability to produce IL-12 in response to either T1 or LSA-J, the proportions of PBMC from adults and children were similar (Table 1c). The amounts of IL-12 produced in response to the two peptides did not differ significantly but showed a significant positive correlation in children ($\rho=0.349$,

Table 2. Comparison of the ratios of anti- to pro-inflammatory cytokines produced by children's and adults' PBMC in response to stimulation with different LSA-1 peptides. Ratios are of cytokine concentrations in pg/ml

	Response to T1				Response to LSA-J			
	Ratio IL-10:IFN-γ	*P*	Ratio IL-10:IL-12	*P*	Ratio IL-10:IFN-γ	*P*	Ratio IL-10:IL-12	*P*
Children	5.4	0.010	2.4	NS	37.7	0.065	14.6	NS
Adults	0.9		1.1		3.7		3.6	

$P = 0.039$), and a similar but non-significant trend in adults ($\rho = 0.374$, $P = 0.073$).

The amounts of IL-10 and IL-12 produced to each peptide showed weak positive correlations (T1: $\rho = 0.252$, $P = 0.042$; LSA-J: $\rho = 0.282$, $P = 0.034$), but there was no correlation in either case with the amounts of IFN-γ produced.

Discussion

The main aim of this study was to compare the cellular immunological responses to two different peptide epitopes of *P. falciparum* liver stage antigen-1 in groups of healthy, parasite-free children and adults living under conditions of constant exposure to infection. We focussed our attention on cytokine responses, especially IFN-γ and IL-10, since our own and others' studies have shown that production of these two cytokines in response to LSA-1 is associated with protection from infection (Luty et al. 1999; Kurtis et al. 2001).

The low prevalence of lymphoproliferation in response to LSA-1 peptides which we detected here is consistent with our own and others' studies in African populations, but not with the higher prevalences of such responses to the T1 peptide seen in non-African adults (Hollingdale et al. 1990; Connelly et al. 1997; Luty et al. 1999; Joshi et al. 2000; Migot-Nabias et al. 2000). In terms of the profile of peptide-specific cytokine responses which we observed, that of IFN-γ was particularly notable for the age-dependent increase both in the frequency of responders and in the amount of this pro-inflammatory cytokine produced relative to the amount of anti-inflammatory IL-10. These findings accord well with the age-dependent enhancement of LSA-1 peptide-induced IFN-γ responses reported by others, and further substantiate the idea that LSA-1-specific IFN-γ responses correlate with increasing protection from infection with *P. falciparum* (Connelly et al. 1997; Luty et al. 1999; Bucci et al. 2000; John et al. 2000). It is nevertheless of note, that the prevalence of T1 peptide-specific IFN-γ responses is enhanced in African children during an acute malaria attack, and to a level (60%) even greater than that observed here in the group of aparasitaemic adults (Luty et al. 2001). In contrast, the frequency of LSA-J-specific IFN-γ responses shows no such marked infection-related change (Luty et al. 2001).

The lack of any age-dependency of the profile of LSA-1 peptide-induced IL-10 responses confirms the findings of John and colleagues (2000) in a Kenyan population, but, as mentioned above, the more detailed consideration of the relative amounts of peptide-specific pro-(IFN-γ, IL-12) and anti-(IL-10) inflammatory cytokines which we performed here shows a clear age-dependent decline in the influence of the latter. Perhaps surprisingly, therefore, we were unable to demonstrate a marked regulatory effect of IL-10 on the production of peptide-specific IFN-γ. Others have concluded that IL-10 probably does not influence LSA-1-driven IFN-γ production, however the cytokine regulatory networks operating during acute malaria may differ from those detectable in parasite-free individuals (Ho et al. 1995; John et al. 2000). Other molecules, such as nitric oxide and transforming growth factor-β, which we and others have not considered in this context, probably also have a regulatory function in malaria (Omer et al. 2000; Perkins et al. 2000).

An aspect of this study which is of potential interest in the context of vaccine development concerns the differential immunogenicity of the T1 and LSA-J peptides. LSA-1 is a strong candidate for development as a *P. falciparum* vaccine, either as a single component construct or as a constituent part of a multicomponent construct, but there is currently no consensus on the optimum configuration of any LSA-1-based molecule (Kurtis et al. 2001). As we observed here, and as others have shown, a consistent finding with the N-terminal T1 peptide compared with any of a range of C-terminal peptides is the lower frequency of IL-10 versus IFN-γ responses, regardless of the age-group studied (John et al. 2000). Our own recent study showed an association between protection from reinfection and the IFN-γ response to the C-terminal LSA-J peptide, whereas a study in Papua New Guinea showed a similar association for IFN-γ responses to T1 but not for responses to C-terminal peptides (Connelly et al. 1997; Luty et al. 1999). The fact that the participating Papua New Guinean population carries, at 67.5% prevalence, an HLA-A allele that specifically binds a 9-mer peptide in the T1 sequence may form the basis for an explanation of the latter observation (Bucci et al. 2000). Murine models of malaria do indicate that both CD8+ T cells and IFN-γ responses are essential for effective pre-erythrocytic-stage immunity, and that a population of liver-resident CD8+ memory T cells may play a pivotal role in this (Schneider et al. 1998; Guebre-Xabier et al. 1999; Doolan et al. 2000). As we have shown here, IFN-γ production in response to T1 by PBMC of Gabonese depends to a relatively greater extent on a CD8+ population compared with LSA-J, which is consistent with a predominantly HLA Class 1-mediated response to the T1 peptide, although LSA-J did induce the production of significantly higher amounts of this cytokine. Studies with a Kenyan population showed a similar CD8+ cell dependency of LSA-1 peptide-driven IFN-γ responses (John et al. 2000). Since the current goal of pre-erythrocytic-stage malaria vaccine development is to optimise the generation of IFN-γ responses, a consideration both of the contrasting cytokine profiles seen with different peptides, and of the profile of their HLA-binding specificities, will be valuable in guiding the choice of LSA-1 epitopes for inclusion in any epitope-based vaccine construct.

In conclusion, this study has shed further light on questions concerning the age-related evolution of cellular immunological responses to LSA-1 in populations exposed to perennial and high-level transmission of

P. falciparum. The most striking feature of this evolution is the apparently enhanced capacity for LSA-1 peptide-specific IFN-γ production in older age-groups, which contrasts with the reported diminution of anti-parasite IFN-γ responses in semi-immune individuals (Chizzolini et al. 1990). In this and other studies, we have emphasized the need to evaluate responses to specific parasite antigens in groups of individuals with a broad age range and at different times with respect to acute infection in the same individuals, since, in our opinion, such an approach optimises the chances of elucidating the regulatory mechanisms influencing these responses.

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