Increase in annexin V-positive B cells expressing LILRB1/ILT2/CD85j in malaria

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ABSTRACT. The outcome of a *Plasmodium falciparum* infection differs greatly between patients, ranging from an asymptomatic carrier status to the most severe characteristics influenced by activating and inhibiting immune factors. The inhibitory leukocyte immunoglobulin-like receptor (LILRB1/CD85j) plays an important role in the immune response as regulator of cytotoxic T cells and of premature activation and clonal expansion of B cells. To investigate its role in malaria, we analyzed blood samples from malaria patients by cytometric analysis. We found a similar expression pattern of CD85j on PBMC in both patients and healthy children. However, malaria patients presented significantly more CD85j$^+$CD19$^+$ B cells, which also bound annexin V an indicator of early cell death. We compared the plasma levels of several cytokines, since it was speculated that CD85j expression influences cytokine release. Production of inflammatory cytokines was significantly increased in severe malaria cases. We suggest that in malaria, dying B cells contribute to the overwhelming cytokine release and the impairment of the immune memory.

Keywords: malaria, *Plasmodium falciparum*, CD85j, MHC class I, cytokine

The outcome of a *Plasmodium falciparum* infection differs between patients, particularly in young children living in malaria-endemic regions of Africa, and can range from an asymptomatic carrier status to the most severe outcome, and even death. The reason for the individual differences in the responsiveness to the infectious pathogen may be based on qualitative or quantitative changes in cell surface receptors responsible for mounting a protective immune response [1, 2].

A contributing factor could be the unbalanced expression of inhibitory receptors leading to an overwhelming immune response. One of these receptors could be the LILRB1/ILT2/CD85j (LILRB1: leukocyte immunoglobulin-like receptor B1; ILT: immunoglobulin-like transcripts; in the following we use the term CD85j). Genetically, functionally and structurally, the respective family is akin to killer cell immunoglobulin-like receptors (KIR) [3]. CD85j is broadly expressed on peripheral blood mononuclear cells (PBMC), whereas KIR are predominantly expressed on natural killer cells (NK) and on activated CD8$^+$ T cells [4, 5]. CD85j are encoded by genes that, together with KIR encoding genes, are located in the chromosomal gene cluster 19p13.4, the leukocyte receptor complex (LRC) [6-9]. The human LRC contains at least 26 genes which belong to the immunoglobulin superfamily and encode for approximately 19 receptors. The genes include two clusters of LIR loci, a cluster of KIR genes, two leukocyte-associated immunoglobulin-like receptor (LAIR) genes, as well as the Fc receptor for immunoglobulin A (IgA) and the natural cytotoxicity receptor I loci [10]. One of these immune receptors is the inhibitory immune receptor CD85j, a member of the LIR family. CD85j is expressed on most PBMC and is a receptor that recognizes MHC class I antigens and human cytomegalovirus UL18, a homologue of MHC class I antigen. It is involved in B cell maturation and suppression of T cell function [11]. The cytoplasmic tail of CD85J contains four putative immunoreceptor, tyrosine-based inhibitory motifs (ITIM) [12] that inhibit cellular responses by recruiting Src homology domain 2 (SH2)-containing tyrosine phosphatase-1 (SHP-1) [3, 13], which transfers inhibitory signals by dephosphorylating and inactivating downstream tyrosine kinases [14].

In this study, we investigated whether CD85j$^+$ cells could play a role during malaria, and analysed the expression of CD85j on various cell types in malarial infection.

METHODS

Patients

Gabonese children aged between 6 months and 6 years, with confirmed *Plasmodium falciparum* infection were recruited at the Albert Schweitzer Hospital in Lambaréné (Gabon). Children with uncomplicated malaria or severe malarial anaemia with a haemoglobin level lower than

5 g/dL, and healthy children were included in the study after informed, parental consent. This was given after explaining the nature and possible consequences of the studies. Entry criteria were fever over 37°C and *P. falciparum* parasitemia as determined by thick blood smears according to the WHO definition. Exclusion criteria were asymptomatic *P. falciparum* infection or indications of other diseases. Blood samples were taken from each child by health personnel. The ethics committees of the International Foundation for the Albert Schweitzer Hospital in Lambaréné, Gabon and the Medical Faculty of the University of Tübingen, Germany approved the study.

Flow cytometry analysis

One mL of peripheral whole blood was taken. To measure the cell surface proteins, we used blood samples from 15 healthy controls (C), 35 children with uncomplicated malaria (U) and 8 children with severe malarial anaemia (S). PBMC were isolated from EDTA whole blood by Ficoll separation and washed twice with 10 nm N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES). Two-colour immunophenotyping with monoclonal antibodies was performed as follows: 50 μL aliquots were incubated with two different antibodies for 15 min, in the dark, at room temperature. The first antibody was labelled with phycoerythrin-Cy5 (PE/Cy5) and was specific to CD4 (S3.5), CD8 (3B5), CD14 (Tük4), CD19 (SJ25-C1) (ImmunoTools, Friesoythe, Germany) or TCRγδ (5A6.E9) (Caltag Laboratories, Burlingame, CA, USA). The second antibody was R-PE-labelled and was specific to CD85j (BD PharMingen, San Jose, CA, USA). Two-colour stained PBMC were additionally stained with FITC-conjugated Annexin V (ImmunoTools, Friesoythe, Germany). AnnexinV was incubated with labelling buffer (10 mM HEPES; 0.9% NaCl; 5 mM $CaCl_2$) for 10 min, at room temperature. Samples were washed twice with HEPES and 20,000 events were acquired on a flow cytometer "Cy Flow Blue SL" (Partec, Muenster, Germany). Data were analyzed by FloMax Software©. Results were calculated using the Kruskal-Wallis-test and ANOVA-test and corrected by the Bonferroni-Dunn-method.

Cytokine measurements

Cytokine levels were measured in plasma samples from 17 patients with uncomplicated malaria (U) and 17 from patients with severe malaria (S) using a Bio-Plex cytokine multiplex assay (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions. The assay allowed simultaneous quantitation of G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte/macrophage colony-stimulating factor), interferon (IFN)-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70 and tumor necrosis factor (TNF)-α. Briefly, premixed beads (50 μL) coated with target capture antibodies were transferred to each well of a filter plate (5000 beads per well per cytokine) and washed twice with Bio-Plex wash buffer. Premixed standards or samples (50 μL) were added to each well containing washed beads and then incubated at room temperature for 30 min. After incubation, premixed detection antibodies (50 μL, final concentration of 2 μg/mL) were added to each well. After

3 washes, the beads were resuspended in 125 μL of Bio-Plex assay buffer. Beads were read on the Bio-Plex suspension array system, and the data were analyzed using Bio-Plex Manager software (v. 3.0) with 5PL curve fitting.

RESULTS

CD85J expression

In order to investigate which cell type expresses CD85j differentially during *P. falciparum* malaria, we compared various cell populations of malaria-stricken and healthy children for the expression of CD85J by flow cytometry. In accordance with previous studies, we found the protein expressed on peripheral blood cells with cell population specific differences *(figure 1)*. Only small numbers of T cells expressed CD85J on their membranes. Healthy controls showed on 2 ± 1% (range 1%-5%) of CD4+ cells CD85j expression, malaria patients had CD85j on 3 ± 1% (1%-5%) of the CD4+ cells. CD8+ cytotoxic T lymphocytes (CTL) had 15 ± 11% (7%-43%) cells with CD85j in healthy children and 20 ± 10% (5%-45%) of the CD8+ cells of the uncomplicated malaria patients expressed CD85J. Severe malaria patients expressed CD85j on 24 ± 8% (12%-34%) of CD8+ CTL. Around 56 ± 12% (27%-90%) of TCRγδ+ lymphocytes showed CD85j on their surface whether isolated from healthy or infected children. CD19+ B cells showed a protein expression of 84 ± 11% (62%-96%) among the healthy children, whereas 88 ± 9% (64%-98%) in the patient group expressed the protein, regardless to the clinical status *(figures 1, 2A)*. Nearly all CD14+ monocytes of healthy children, 99 ± 2% (96%-100%) expressed the protein. Uncomplicated cases expressed 97 ± 4% (86%-100%) and severe malaria cases expressed 98 ± 5% (88%-100%) of CD85j on their monocytes. Children expressing CD85j on a smaller proportion of T cells (CD4+ and CD8+) also expressed the protein on a smaller fraction of B cells (CD19+). A higher proportion of CD85j+ B cells correlated with a higher proportion of CD85j+CD4+ and CD85j+CD8+ lymphocytes (CD85j+/CD4+ *versus*
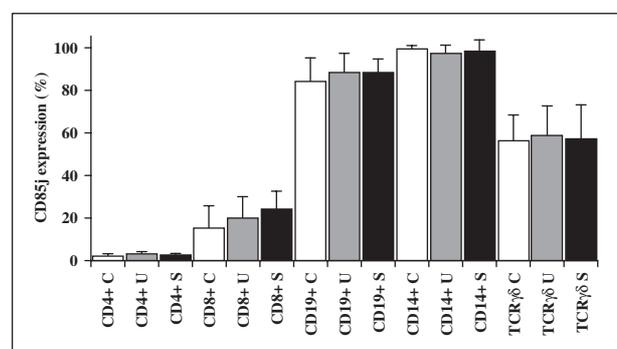

Figure 1
Cell populations of PBMC expressing CD85j (as %). Double stained cells, analysed by cytometry. Controls (C), uncomplicated malaria cases (U), severe malaria cases (S). Mean values and standard deviation are shown.
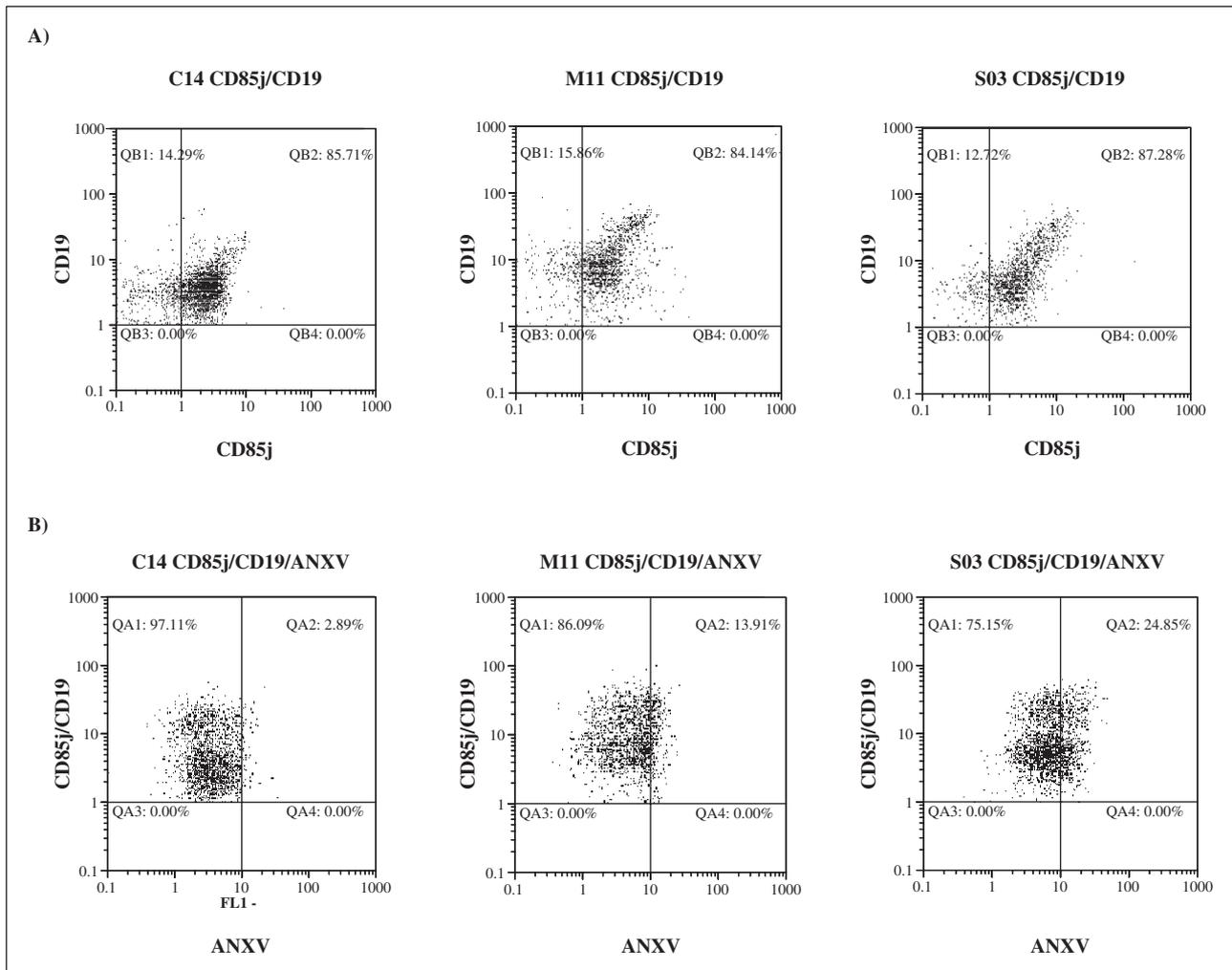
Figure 2
Results from three representative children, measured by flow cytometry. **A)** Double stained CD85j/CD19 positive cells; **B)** triple stained CD85j/CD19/ANXV positive cells.
C14: healthy control; M11: child with mild form of malaria; S03: child having severe malarial anemia.

CD85j+/CD8+: p = 0.007; CD85j+/CD4+ *versus.* CD85j+/CD19+: p = 0.03 and CD85j+/CD8+ *versus* CD85j+/CD19+: p = o.0008; *figure 3A, B, C)*. We measured the mean peak intensity by cytometric analysis to investigated differences in the receptor amount on surface of the cells. On CD4+ and CD8+ lymphocytes, as well as on CD19+ B cells, CD14+ monocytes and on TCRγδ+ cells. We found a trend towards increased CD85j expression on the surface of blood cells in malaria patients compared to healthy children, which was statistically non-significant (data not shown).

We investigated the viability of the different CD85j cell populations by examining the ability to bind annexin V as a marker of cell death in TCRγδ+, CD4+ and CD8+ T cells, CD19+ B cells and CD14+ monocytes *(figure 4)*. We measured only $3 \pm 2\%$ (1%-8%) CD85j+/CD4+/ annexinV+ cells in blood samples of healthy children. Uncomplicated cases showed $22 \pm 10\%$ (5%-38%) and severe cases $23 \pm 7\%$ (16%-35%) of annexinV binding CD85j+/CD4+ cells. In cytotoxic T lymphocytes (CD85j+/CD8+), we detected $2 \pm 4\%$ (1%-15%) annexinV+ cells in healthy children, $19 \pm 11.0\%$ (6%-53%) in uncomplicated cases, and $13 \pm 4\%$ (6%-16%) in severe cases. Among the monocytes (CD85j+/CD14+), $5 \pm 3\%$ (3%-11%) of cells in healthy children, $26 \pm 11\%$ (8% - 51%) of them in uncomplicated malaria and $29 \pm 16\%$ (14%-70%) of them in severe malaria bound annexinV. Of the cells positive for CD85j and CD19 (B cells) in healthy children, $4 \pm 2\%$ (1%-9%) were annexinV+. In contrast to that, uncomplicated malaria patients showed $16 \pm 6\%$ (7%-29%) and severe cases $24 \pm 9\%$ (12%-37%) of B cells *(figure 2B)* positive for annexinV. In healthy children, $7 \pm 3\%$ (2%-11%) of CD85j+TCRγδ+ lymphocytes were annexinV+, in uncomplicated cases $22 \pm 9\%$ (11%-49%) and in severe cases $14 \pm 12\%$ (6%-40%). All five cell populations examined showed a significant difference in the percentage of annexinV binding between uncomplicated malaria cases and healthy controls (p < 0.0001; *figure 3*). Similar results were found for severe cases (p < 0.01). Between the two malaria groups only, a significant difference was found in the CD19+ cell population. Here we found more annexinV+ B cells in the severe malaria children than in the children having uncomplicated malaria (p = 0.003).
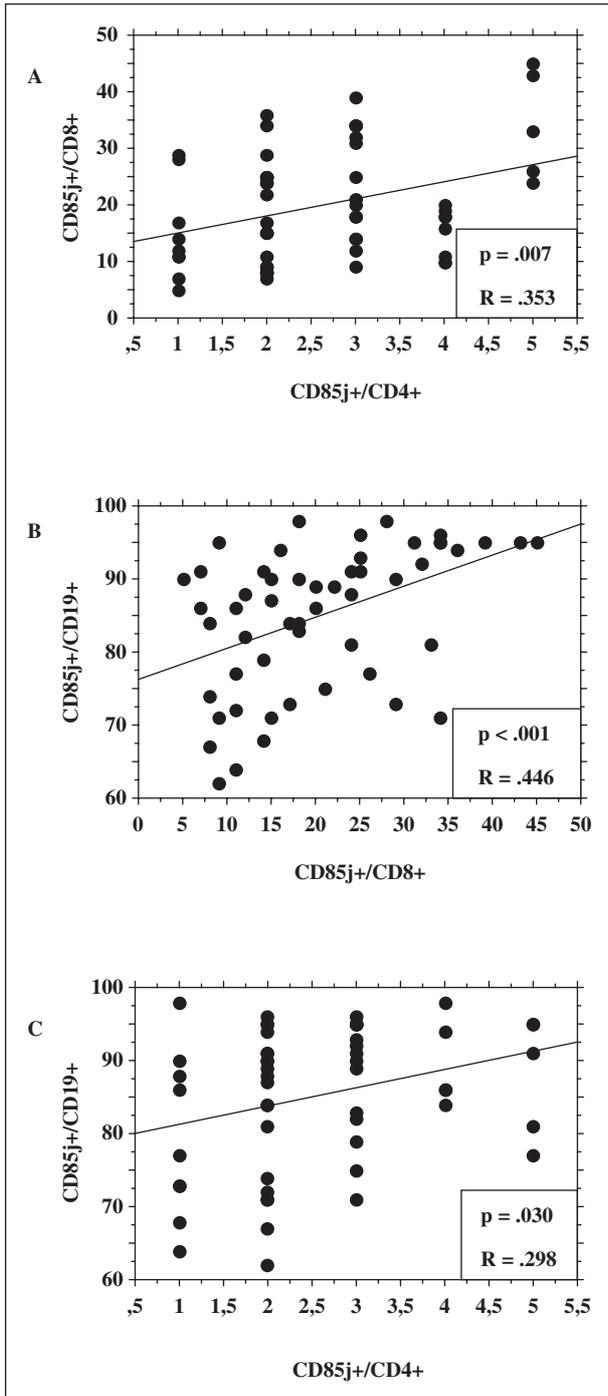
Figure 3

Correlations between the protein levels of PBMC expressing **A**) CD85j+CD4+ cells and CD85j+CD8+ cells, p = 0.007; R = 0.353, **B**) CD85j+CD4+ cells and CD85j+CD19+ cells, p = 0.03; R = 0.298 and **C**) CD85j+CD8+ cells and CD85j+CD19+ cells, p = 0.0008; R = 0.446.

Cytokine release

We examined cytokines involved in the acute phase of inflammation (IL-1β, IL-6, TNF-α, G-CSF and GM-CSF), as well as cytokines involved in the chronic phase of inflammation (IL-2, IL-10, IL-12 and IFN-γ) during *P. falciparum* infection *(table 1)*. The pro-inflammatory cytokine IL-1β is able to induce fever and haematopoiesis. Expression was four times higher in severe cases than in uncomplicated cases. IL-6 is a classical pro-inflammatory
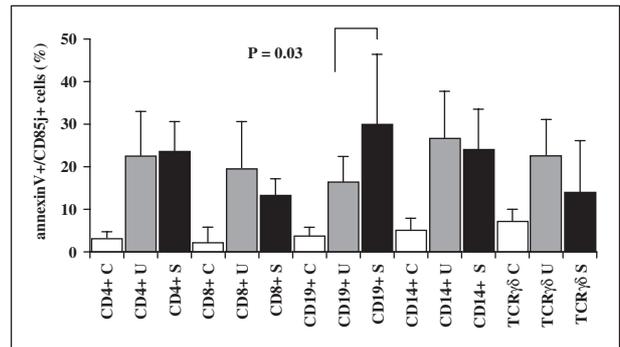

Figure 4

AnnexinV+/CD85j-expressing cell populations of PBMC (as %). Triple stained cells, analysed by flow cytometry. Controls (C, white bars), uncomplicated malaria cases (U, grey bars), severe malaria cases (S, black bars). All C *versus* U comparisons were significant (p < 0.0001), as well as all C *versus* S comparisons (p < 0.01). Of the S *versus* U comparisons only CD19+CD85j+ was significant (p = 0.003). Mean values and standard deviation are shown.

cytokine and was expressed twice as strongly in severe cases compared to uncomplicated cases. TNF-α showed five-fold higher expression in severe cases compared to uncomplicated cases. The growth factors G-CSF and GM-CSF are involved in stimulation of myelopoesis. G-CSF levels were four times higher in blood samples from severe compared to uncomplicated. GM-CSF levels were five times higher in severe compared to uncomplicated case cells. The Th1 cytokine IL-2 is a well known cell activator of T and B cells, NK cells, granulocytes and monocytes and it is able to induce apoptosis in target cells; we measured four times more IL-2 in severe than in uncomplicated cases. The cytokine serum level of IL-12(p70) was six-fold higher in the blood samples from severe compared to uncomplicated cases. IL-12 is able to induce a Th1 immune response and can induce the production of IFN-γ. Levels of IFN-γ, which is known to inhibit the proliferation of Th2 cells and in particular B cells, were three-fold higher in the severe cases. However, IL-10 expression was twice as high in cells from children having uncomplicated malaria than in severely affected cases.

DISCUSSION

CD85j is an inhibitory receptor that recognizes MHC class I antigens and human cytomegalovirus UL18. Since CD85j is also able to control cytokine release from PBMC, its receptor was the focus of our research into malaria. We found CD85j expression on virtually all CD19+ and CD14+ cells; in addition, about half of the TCRγδ+ cells carry this receptor. Only minor expression is observed on CD4+ and CD8+ cells; these results are in accordance with other studies [15]. Also, during malaria the expression level of CD85j does not change dramatically in the cell populations examined. The same is observed in most melanoma patients when looking at CD8+ T cells [16]. In HIV positive individuals, CD8+ cells carry significantly more CD85j, and these cells are more prone to apoptosis and are therefore hampered in their functionality [17]. By inhibiting important T cell responses it is thought that the virus gains the upper hand during chronic infection. It has been shown that in murine malaria, the main complex for

Table 1
Plasma levels (pg/mL) of cytokines measured in blood samples of children having uncomplicated or severe malaria

IL-1β (pg/mL)	IL-2 (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)		IL-12(pg/mL)	G-CSF (pg/mL)	GM-CSF (pg/mL)	TNF-α (pg/mL)	IFN-γ (pg/mL)
Severe cases (S)	20.6	528.2	341.3	127.2	163.7	161.3	5928.8	167.4	404.0
STDEV	15.8	325.3	210.3	133.3	123.9	102.5	4254.2	110.4	254.3
Uncompl. cases (U)	5.3	124.3	191.6	296.9	25.6	43.7	1172.6	32.2	125.5
STDEV	5.4	151.0	363.0	807.8	36.4	42.9	1556.9	43.6	123.7
Ratio (S/U)	3.90	4.25	1.78	0.43	6.39	3.69	5.06	5.20	3.22
p-value	0.012	>0.001	0.002	0.028	>0.001	>0.001	>0.001	>0.001	0.002

The mean and standard deviation, the ratio between severe and uncomplicated malaria cases (S/U), and the p-values as calculated by Mann-Whitney for non-normal distributions are shown.

antigen presentation is MHC class II [18]. CD85j however, interacts with MHC class I, which presents parasite antigen only in minor amounts; this could explain why CD85j protein expression is not enhanced by parasite exposure. The range of CD85j-expressing cells in each group examined is very broad, regardless of the state of health of the proband. An explanation for the observed individual variability of receptor expression might be the haplotypes, as it is known that different haplotypes express different amounts of CD85j depending on a range of polymorphisms. Individuals carrying the LILRB1.PE01 haplotype exhibit significantly lower protein on lymphocytes and monocytes compared to LILRB1.PE01 negative individuals (LILRB1.PE02, LILRB1.PE03) [19]. This explains the correlations of CD85j expression between the three different subpopulations of CD4+, CD8+ and CD19+ lymphocytes in the samples examined here. So far, no association between the different haplotypes and infectious diseases have been conducted. Since the number of patients is limited in this study, a genetic analysis of the highly polymorphic locus would not be meaningful and so we abandoned this undertaking.

In malaria, the rate of annexinV +/CD85j+ cells increases in all populations examined compared to the healthy control cohort, indicating a severely affected function. Comparing malaria patients with severe or uncomplicated malaria, the only significant difference was observed in CD85j+/CD19+/annexinV+ B cells. Since CD85j is a marker for B cell maturation, the higher percentage of dying B cells may explain why memory B cells cannot be produced, which explains on one hand, the weak immune memory [20] and the observed, more frequent re-infection rate of children having had severe malaria [21].

It is noteworthy that malaria patients often suffer as a result of an overwhelming immune response driven by pro-inflammatory cytokines. In addition to its role in suppressing cytotoxic T cell function, CD85j prevents the release of pro-inflammatory cytokines, resulting in aggravation of infection. This was also shown in previous studies; when CD85j was blocked, higher cytokine release was measured [13]. The reduced function of the inhibitory CD85j leads to less inhibition of inflammatory cytokines such as IL-1β, TNF-α, G-CSF and GM-CSF. The release of smaller quantities of IL-10 leads to a further overwhelming immune response. Since the above mentioned cytokines are mainly expressed by MHC class I antigen-presenting monocytes, which are recognized by CD85j, apoptotic CD85j+ cells are functionally disabled. Thus, all pro-inflammatory cytokines are enhanced in severe malaria cases, whereas expression of the malaria-protective cytokine IL-10 was found to be two-fold less in the severe cases and this has been shown in many studies [22, 23]. It is known that IL-10 is a cytokine that is able to limit inflammation, inhibit the IL-12 immune response and regulate growth and differentiation of T, B and NK cells as well as of mast cells and granulocytes. This important cytokine can limit the severe outcome of malaria. Therefore, it was not surprising to find that release of the protecting IL-10 cytokine was inhibited in severe malaria cases, whereas it was strongly enhanced in uncomplicated cases.

We conclude that CD85j therefore could influence malarial infection at the B cell level, by inhibiting maturation, and at the T cell level, by leading to an increased inflammatory response. Further studies are needed to examine whether other cells express CD85j and whether other pathogens affect regulation of its receptor.

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