Regulation of B cell function and expression of CD11c, T-bet, and FcRL5 after infection and in response to different activation pathways

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Abstract

CD11c, FcRL5, or T-bet are commonly expressed by B cells expanding during inflammation, where they can make up >30% of mature B cells. However, the association between the proteins and differentiation and function in the host response remain largely unclear. We have assessed the co-expression of CD11c, T-bet and FcRL5 over one year in patients treated for malaria. We observed dynamic co-expression profiles that changed over time, suggesting ongoing differentiation. We used an in vitro B cell culture system to determine how stimulation via the B cell receptor (BCR), toll-like receptor 9 (TLR9), and different cytokines could influence CD11c, T-bet, and FcRL5 expression. We observed different expression dynamics for all markers, but a largely overlapping regulation of CD11c and FcRL5 in response to BCR and TLR9 activation, while T-bet was strongly dependent on IFN-γ signalling. Investigating plasma cell differentiation and antigen-presenting cell functions, there was no association between marker expression and antibody secretion or T cell help, although the functions were associated with TLR9-signalling and B cell-derived IL-6 production, respectively. These results point to CD11c, FcRL5, and T-bet expression representing different activation stages and highlight the value of using multiple markers when assessing B cell differentiation.

Introduction

B cells are important contributors to long-lived immunity through their differentiation to antibody-secreting plasma cells and long-lived memory B cells. This process is initiated in secondary lymphoid organs where antigen-activated mature naïve B cells either directly differentiate to plasma cells (T-cell-independent), in response to high-valent antigen or strong and pattern-recognition-receptor (PRR)-signal, or migrate to T cell-B cell interfollicular to interact with activated cognate $CD4^+$ T cells (T-cell dependent response) [1]. In the T-cell-dependent response, B cells present peptide antigens to the cognate T cells via the major histocompatibility complex (MHC) class 2. Both the T- and B-cells then deliver additional activation and regulatory signals to each other via co-stimulatory molecules (such as CD40-CD40L, ICOS-ICOSL, CD80/86-CD28) and cytokines [2]. At this stage, B cells can undergo cytokine-dependent class-switch recombination [3], enter the B cell follicle and establish germinal centres to undergo isotype- and affinity-dependent selection [4–6], eventually producing high-affinity memory B cells and plasma cells [7], or they can directly differentiate to relatively low-affinity extrafollicular memory B cells and plasma cells [8].

For certain infectious diseases with a strong or prolonged inflammatory component, such as malaria, the process of generating long-lived B cell-mediated immunity has been suggested to be inefficient [9]. This has in part been associated with the expansion of a B cell subset lacking the expression of CD21 and CD27 while upregulating the integrin CD11c, the transcription factor T-bet and the IgG receptor FcRL5, in addition to numerous other receptors associated with cell regulation and migration [10–12]. These cells have been suggested to be exhausted or dysfunctional, in part due to reduced responsiveness to BCR-ligation and upregulation of several inhibitory receptors [12,13]. However, a recent study by Ambegaonkar et al. suggests it could have more to do with how the antigen is presented [14], and it remains unclear what function and purpose these cells play in the immune response [15]. Some studies have suggested that the cells could be primed to migrate to sites of infection or inflammation to provide localized differentiation to plasma cells or to provide antigen-presenting functions to local $CD4^+$ T cells [15–17]. In support of such functions, $CD21^-CD27^-$ B cells in malaria display an upregulation of the plasma cell-associated genes *PRDM1* and *CD38* and differentiate into antibody-secreting cells following interaction with T-follicular helper (T_{FH}) cells [18]. Additionally, mouse and human studies have indicated more efficient antigen uptake and upregulation of MHC2 and co-stimulatory molecules, potentially translating to an enhanced capacity to drive $CD4^+$ T cell responses [14,18,19].

It has become clear that $CD11c^+$ or T-bet^+ B cells are not necessarily only associated with severe inflammatory disease, as they are found at low levels during steady-state that then increase with normal ageing of the host [20–22]. These levels are, however, greatly expanded in many different infections and following vaccination [23]. Additionally, cells with a similar phenotype are also expanded in several different autoimmune diseases that have an inflammatory component [24]. Despite sharing many phenotypic and transcriptomic characteristics between different types of diseases [25], there are also differences that appear disease-specific, such as the expression of FcRL4 in HIV infection and FcRL5 in malaria [12,26]. An additional source of variation could come from how the cells are defined in different studies, where varying combinations of IgD, CD21, CD27, CD11c, CD85j, T-bet, CXCR3, and FcRL5/FcRL4 have been used. This has also led to the cells having been given many different names, such as atypical B cells, age-associated B cells, exhausted B cells, tissue-like B cells, double negative (DN) and DN2 B cells [10,12,26–32]. We have previously shown that the

80 co-expression of several of these markers is changing over time after parasitic infection [27]. It is
81 therefore possible that they could represent different stages of differentiation or alternatively that
82 the cells have received different stimuli prior to their differentiation.
83
84 In this study, we have investigated the regulation of CD11c, T-bet, and FCRL5, three of the most used
85 markers of these alternative lineage B cells. We have measured their expression patterns in malaria
86 patients from diagnosis and up to 12 months after treatment. We have also used an in vitro culture
87 system to determine how stimulation via the BCR, TLR9 or the response to different cytokines
88 influences their expression. Additionally, we have assessed their functional properties, including
89 differentiation to antibody-secreting cells and B cell-dependent $CD4^+$ T cell help.
90

Results

Co-expression of CD11c, T-bet, and FcRL5 on B cells after diagnosis with Plasmodium parasite infection

To evaluate the dynamics and co-expression of CD11c, T-bet, and FcRL5, we analysed peripheral blood mononuclear cells (PBMCs) using flow cytometry in repeated samples up to 12 months after diagnosis with *Plasmodium* parasite infections (**Figure 1A**). Samples from ten patients and six healthy controls were included. Six patients were symptomatic with *P. falciparum* parasites (parasitaemia ranging from 0.1-7%), two were symptomatic with *P. ovale* or *P. vivax*, and two were asymptomatic (one *P. malariae* and one *P. ovale*). Six individuals originated from malaria-endemic areas. From six of the patients, PBMC samples were available from all three time points tested. Cell marker expression over time and patient characteristics are available in **supplementary figure 1**.

Switched atypical memory B cells can be classified by their lack of IgD and CD27 expression and then also be referred to as double-negative (DN) B cells [31]. We observed that the percentage of DN cells was higher (approximately 30-50%) among B cells expressing CD11c, T-bet, or FcRL5 (called DN2 cells [31]) compared with those that did not express any of the three markers, where only 10-15% of the cells were IgD and CD27 negative (**Figure 1B and supplementary figure 2**). The frequency of switch memory ($IgD^{-}CD27^{+}$, approximately 20% of the cells) and unswitched memory ($IgD^{+}CD27^{+}$, approximately 15% of the cells) remained similar between CD11c, T-bet, or FcRL5 positive and negative cells, and it was mainly the naïve cells ($IgD^{+}CD27^{-}$) that had a reduced frequency compared with the DN phenotype (**Supplementary figure 2**).

The frequency of CD11c, T-bet, and FcRL5 expressing B cells among $CD38^{lo/int}CD24^{lo/int}$ mature B cells was significantly increased in individuals at diagnosis compared with healthy controls (**Figure 1C**). For CD11c and FcRL5 the expression remained significantly higher also at 10 days after diagnosis and treatment initiation but then declined over the following 12 months. At diagnosis, an average of 30.4% of the mature B cells expressed CD11c, T-bet, and/or FcRL5 (**Figure 1D**). The majority of these cells (13.9%) expressed all three markers, followed by CD11c single-positive cells (6.3%) and FcRL5 single-positive cells (4.2%). CD11c and FcRL5 double-positive cells only made up 2.6% of the mature B cells at diagnosis but then increased in frequency to 8.2% of cells at the 10-day time point. Similarly, FcRL5 single-positive cells also increase to 9.1% of the mature B cells at the 10-day time-point after which both FcRL5 single and FcRL5 and CD11c double-positive cells declined to baseline levels at 12 months. The triple-positive cells were reduced to 5.1% at 10 days and then further to 4.5% at 12 months (**Figure 1D**). Together these data show that the commonly used atypical B cell markers CD11c, T-bet, and FcRL5 are not mutually exclusive, but rather have a dynamic co-expression pattern over time following acute infection.

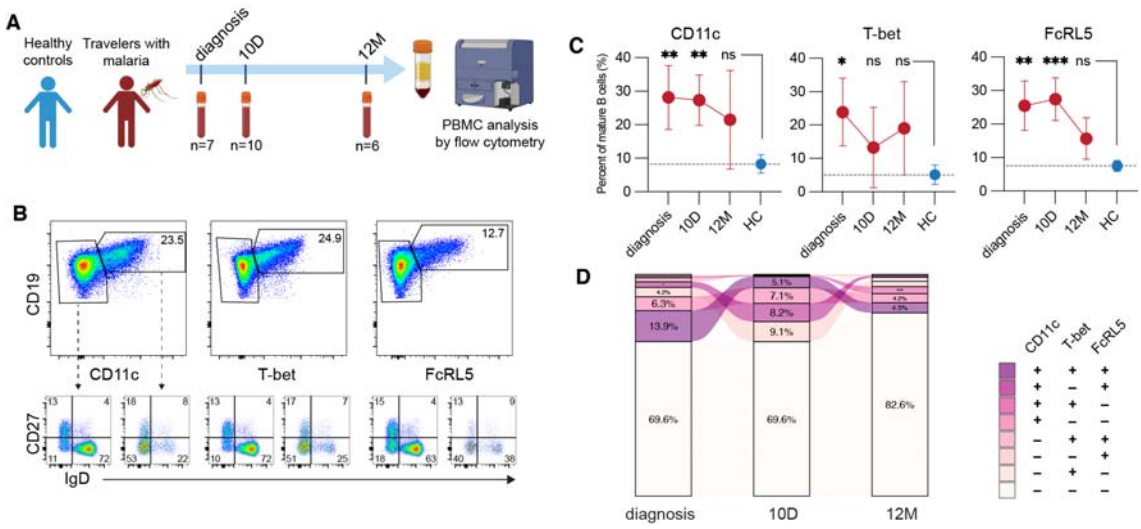

Figure 1. Expression of CD11c, T-bet, and FcRL5 on B cells following diagnosis of *Plasmodium* parasite infection. *(**A**) Schematic presentation of the donors and time points. PBMCs from healthy controls (HC, n=6), and Plasmodium parasite-infected longitudinal samples at diagnosis (n=7), 10 days after treatment initiation (n=10), and after 12 months (n=6) were assessed by flow cytometry. (**B**) Mature B cells (Live, CD19^{+}CD20^{+}CD38$^{int/low}$CD24$^{int/low}$) were analyzed for expression of CD11c, T-bet, and FcRL5 and further gated for CD27 and IgD. (**C**) CD11c+, T-bet+, and FcRL5+ B cell frequencies were determined at the diagnosis, 10-day, and 12-month time points (red) and in healthy controls (blue). The mean and 95% confidence interval are indicated. (**D**) Average frequency of co-expression of CD11c, T-bet, and FcRL5 among mature B cells at the different time points after infection. The Kruskal-Wallis test followed by Dunn's posttest was used to determine p-values between healthy controls and parasite-infected donors at different time-points after infection. *p<0.05, **p<0.01, ***p<0.001. ns indicates not significant, p>0.05.*

Differential kinetics of CD11c, T-bet, and FcRL5 after stimulation

To better understand the regulation of CD11c, T-bet, and FcRL5 in B cells, we adapted an in vitro culture system described by Ambegaonkar et al. [33] to generate B cells with an atypical phenotype. They had previously shown that both naïve and memory B cells could upregulate T-bet following in vitro stimulation via the BCR, TLR9, and by adding IFN-γ [33]. We observed a similar strong upregulation of T-bet in both naïve and memory B cells sorted from healthy blood donors, while the memory B cells were somewhat more efficient at upregulating CD11c and FcRL5 after 2 days of stimulation (**supplementary figure 3**). As both naïve and memory B cells could efficiently respond to the stimuli, and in vivo generated CD11c-, T-bet-, and FcRL5-expressing cells are likely generated from both naïve and memory B cells [23], we used total B cells isolated from healthy blood donors to further characterise the response to stimulation (**Figure 2A**). Cell marker expression was evaluated by flow cytometry after stimulation with anti-Ig (BCR ligation), CpG-C (TLR9-ligation), and the pro-inflammatory cytokine IFN-γ in comparison to unstimulated cells (**Figure 2B**). The stimulated cells adopted a CD19^{Hi} phenotype, with a significant percentage of cells having upregulated CD19 on day 2 and day 6 after stimulation compared to unstimulated controls. CD11c and FcRL5 expression was also significantly higher among stimulated cells at both time points. The T-bet expression on the other hand was highly upregulated by day 2 but then greatly reduced by day 6, although it remained significantly higher than unstimulated cells.

Given the possible difference in kinetics between the markers over time, we evaluated protein levels at 0, 2, 4, and 6 days after stimulation (**Figure 2C**). The percentage of CD11c^{+} B cells increased over time and plateaued between 4 and 6 days of stimulation. FcRL5 and CD19 on the other hand peaked at day 4 after which they started declining. T-bet was rapidly upregulated and peaked at 2 days of stimulation, after which the protein levels declined.

To assess if the changes in protein levels were associated with cell division, B cells were labelled with cell trace violet (CTV). The B cells stimulated with anti-Ig, CpG-C, and IFN-γ displayed measurable proliferation in the form of CTV dilution at day 4 after stimulation while unstimulated cells remained undivided (**Figure 2D**), clearly indicating that cell division was not a requirement for upregulation of CD19, CD11c, T-bet, or FcRL5.

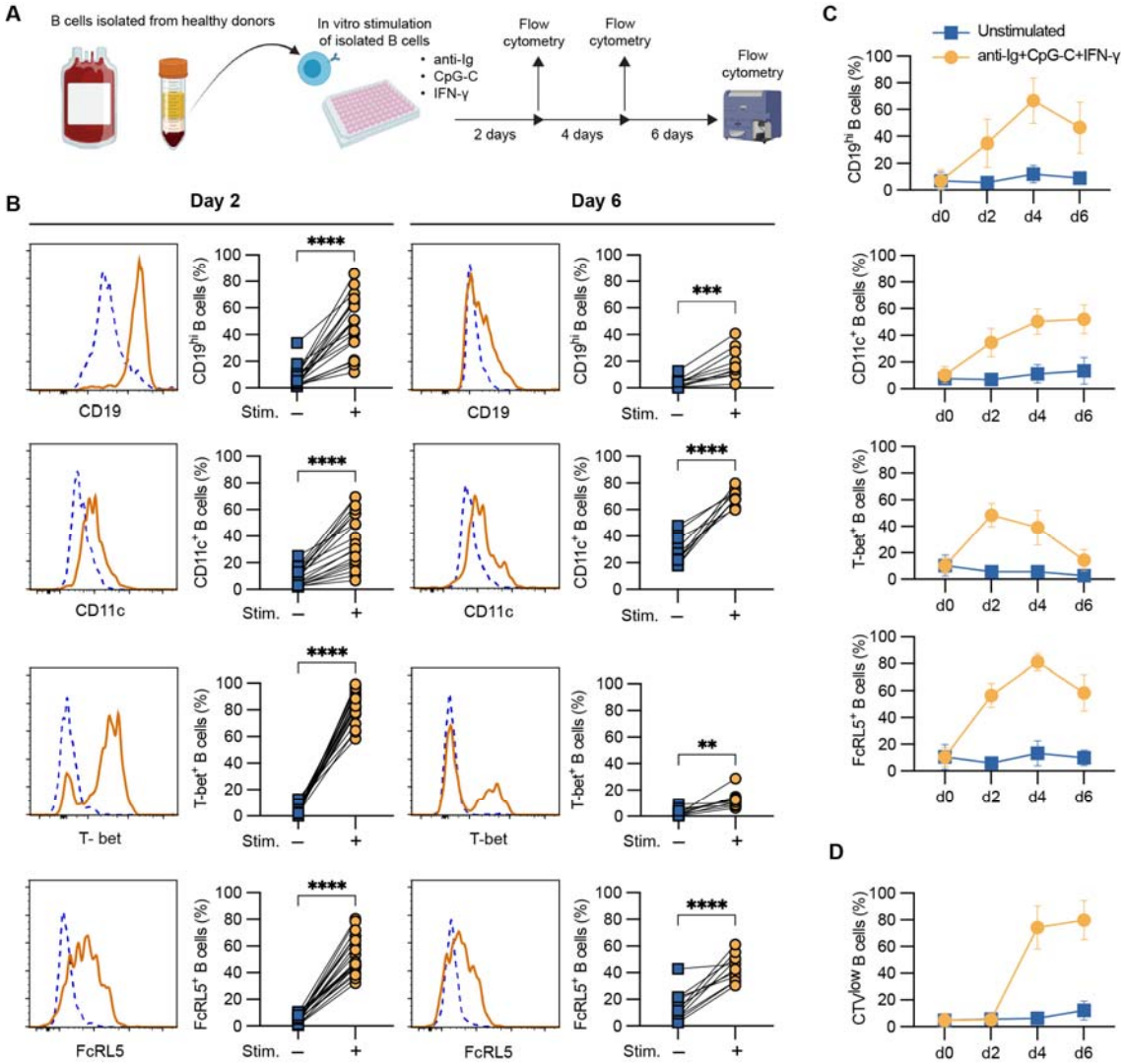

Figure 2. In vitro generation of B cells expressing high levels of CD19, CD11c, T-bet, and FcRL5. *(**A**) Schematic representation of experimental setup with B cells sorted from buffy coats using RosetteSep, followed by stimulation with anti-Ig, CpG-C, and IFN-γ for 2, 4, and 6 days. (**B**) The cells were evaluated by flow cytometry for expression of CD19, CD11c, T-bet, and FcRL5 at 2 days (n=19-20 donors) and 6 days cells (n=10-11 donors) after stimulation (+, orange solid line and circles) or left*

179 *unstimulated (–, dashed blue line and boxes) for unlabelled B cells and (**C**) at 0, 2, 4, and 6 days for*
180 *cell-trace violet labelled B cells (n=6-9 donors). (**D**) B cell division was determined by dilution of cell-*
181 *trace violet at 0, 2, 4, and 6 days (n=6-9 donors). Data in B indicates individual donors and is pooled*
182 *from multiple experiments. Data in C and D indicates the mean with a 95% confidence interval and is*
183 *pooled from two separate experiments. Paired t-tests were used to determine p-values between*
184 *unstimulated and anti-Ig/CpG-C/IFN-γ stimulated cells at 2 and 6 days. **p<0.01, ***p<0.001,*
185 *****p<0.0001.*
186
187 ***Role of BCR- and TLR9-ligation on B cell levels of CD19, CD11c, T-bet, and FcRL5***
188 To evaluate the specific influence of BCR- and TLR9-ligation on cell marker expression, stimulation
189 conditions without anti-Ig or CpG-C, in combination with IFN-γ, were compared to the condition with
190 all three signals in sorted B cells from healthy blood donors (**Figure 3 and Supplementary table 1**).
191 CpG-C was shown to be critical in inducing $CD19^{hi}$ cells. On day 2, the removal of either anti-Ig or
192 CpG-C led to a significant decrease in CD11c levels whereas, on day 6, only the condition without
193 anti-Ig had significantly fewer $CD11c^{+}$ cells. For T-bet levels, the addition of anti-Ig or CpG-C led to a
194 significant increase of positive cells on day 2. On day 6, however, T-bet was down-regulated to
195 baseline levels in all conditions. Both anti-Ig and CpG-C contributed to the up-regulation of FcRL5 on
196 day 2, but no significant difference between stimulated and unstimulated cells was observed on day
197 6. In summary, TLR9-ligation was required for the upregulation of all four markers assessed here,
198 while BCR-ligation led to up-regulation of CD11c, T-bet, and FcRL5, but not CD19.
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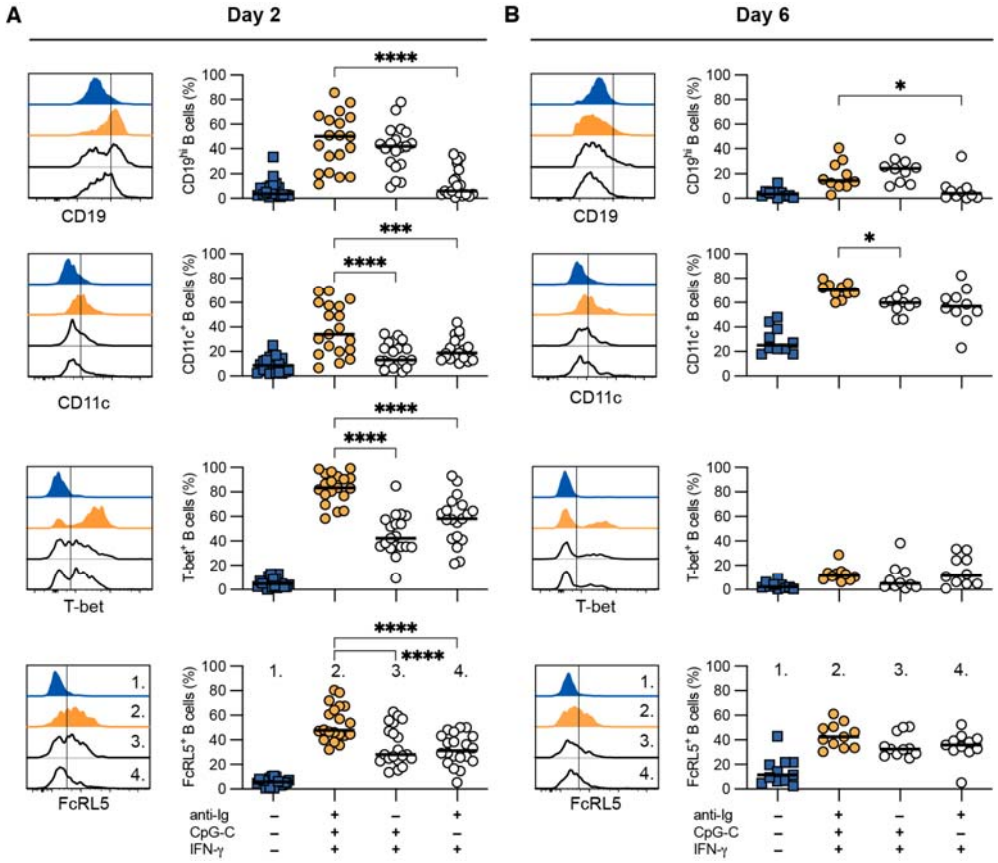


201

202
203 **Figure 3. Role of BCR- and TLR9-ligation on CD19, CD11c, T-bet, and FcRL5 expression.**
204 *Sorted total B cells from buffy coats were stimulated with combinations of anti-Ig, CpG-C, and IFN-γ*
205 *for (**A**) 2 days (n=19-20) and (**B**) 6 days (n=10-11) after which expression of CD19, CD11c, T-bet, and*
206 *FcRL5 was measured by flow cytometry. Representative histograms indicate marker expression. The*
207 *line indicates the cut-off for positive expression. Numbers indicate the stimulations (see*
208 *Supplementary table 1). P-values were calculated using matched pair one-way ANOVA with Geisser-*
209 *Greenhouse correction followed by Sidak's post hoc test. Unstimulated cells (blue boxes) were*
210 *included for visual reference. Stimulated groups (open circles, stim 3 and 4) were compared with*
211 *anti-Ig, CpG-C, IFN-γ (orange circles) stimulated cells. *p<0.05, ***p<0.001, ****p<0.0001. The*
212 *absence of * indicates p>0.05.*
213
214 ***Differential impact of cytokines on B cell CD19, CD11c, T-bet, and FcRL5 levels***
215 To further dissect the impact of the cytokines on the levels of CD19, CD11c, T-bet, and FcRL5, B cells
216 sorted from healthy blood donors were cultured with anti-Ig, CpG-C, and either IFN-γ, IL-21, or IL-10
217 (**Figure 4 and Supplementary table 1**). None of the tested cytokines led to the upregulation of CD19
218 or FcRL5. The addition of IFN-γ resulted in a significant increase in CD11c levels, but only on day 2,
219 while stimulation with IL-21 led to a significant increase on both days 2 and 6. IL-10 on the other
220 hand had no significant effect on CD11c, although the mean $CD11c^+$ frequency was somewhat higher
221 than anti-Ig and CpG-C alone on day 2. In contrast, IL-10 stimulation led to a reduction of $T\text{-}bet^+$ cells
222 compared with anti-Ig and CpG-C stimulated cells alone. Only IFN-γ out of the 3 cytokines resulted in
223 a significant increase in $T\text{-}bet^+$ B cells on day 2, and a slightly higher percentage of cells remaining on
224 day 6. This effect was not adversely affected by IL-10 (**Supplementary Figure 4**). In summary, T-bet
225 expression was highly dependent on IFN-γ, while CD11c was upregulated in response to both IFN-γ
226 and IL-21. CD19 and FcRL5 on the other hand were not influenced by the tested cytokines.
227

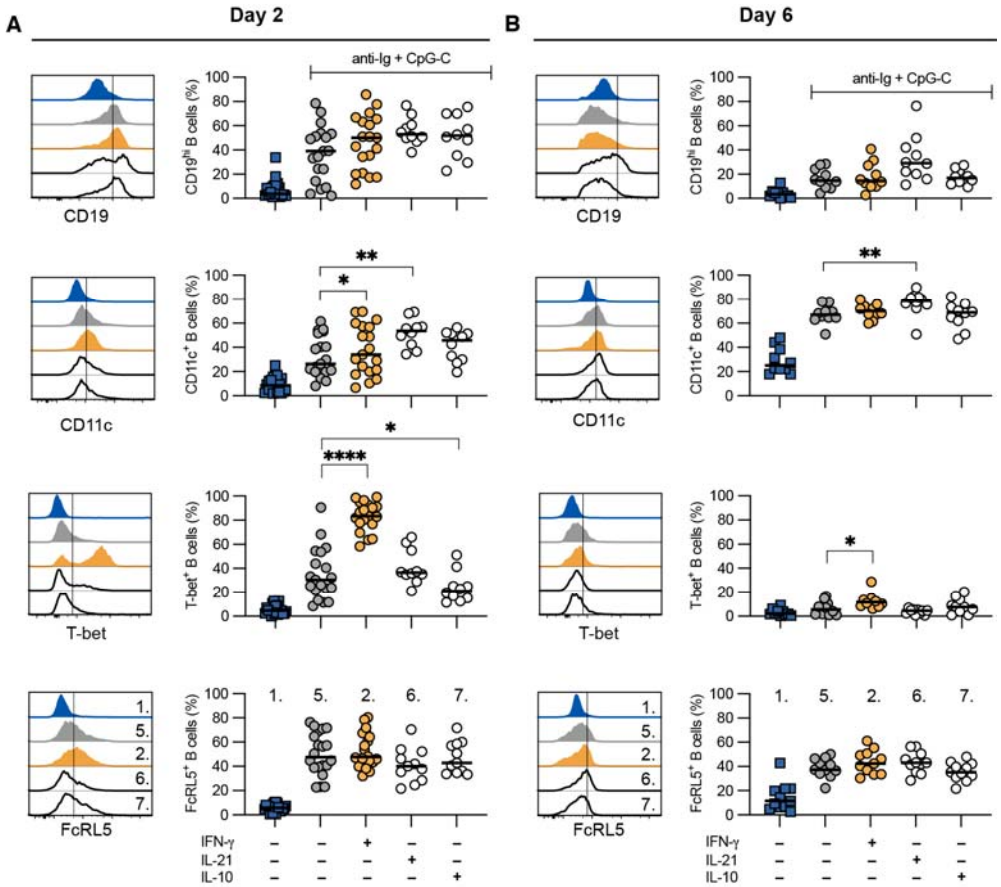


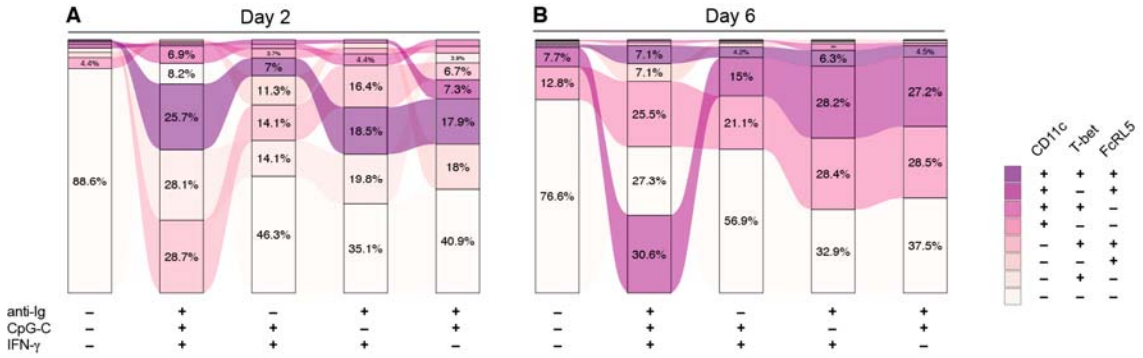
Figure 4. Role of IFN-γ, IL-21, and IL-10 on CD19, CD11c, T-bet, and FcRL5 expression.

*Sorted total B cells from buffy coats were stimulated with combinations of anti-Ig, CpG-C, IFN-γ, IL-21, and IL-10 for (**A**) 2 days (n=10-20) and (**B**) 6 days (n=10-11) after which expression of CD19, CD11c, T-bet, and FcRL5 was measured by flow cytometry. Representative histograms indicate marker expression. The line indicates the cut-off for positive expression. Numbers indicate the stimulations. P-values were calculated using matched pair one-way ANOVA with Geisser-Greenhouse correction followed by Sidak's post hoc test or in the case of missing data, by mixed-effects analysis. Unstimulated cells (blue boxes) were included for visual reference. All stimulated groups had received anti-Ig and CpG-C. Stimulations 2 (orange circles), 6, and 7 (open circles) were compared with anti-Ig and CpG-C stimulated cells alone (grey circles). *p<0.05, **p<0.01, ****p<0.0001. The absence of * indicates p>0.05.*

Dynamic CD11c, T-bet, and FcRL5 co-expression profiles in response to stimulation and time

Different combinations of markers are used to describe the B cells that expand during inflammation. Here we analysed how the co-expression of CD11c, T-bet, and FcRL5 change depending on stimulatory conditions, at two different time points (**Figure 5**). The largest proportion of triple-positive cells was generated by stimulating the B cells with anti-Ig, CpG-C, and IFN-γ for 2 days (approximately 26%). This condition also had the lowest percentage of cells not expressing any of CD11c, T-bet, or FcRL5 at both 2 and 6 days of stimulation. Compared to 2 days of stimulation, there were few cells expressing all three markers at 6 days. This was mainly explained by the rapid reduction of T-bet^{+} cells after day 2. Instead, the day 6 time-point was characterised by similar

250 frequencies of CD11c single- or CD11c and FcRL5 double-positive B cells. Most of the unstimulated
251 cells remained triple-negative at both time points (88.6% and 76.6% respectively).
252



253
254
255 **Figure 5. Stimulation-dependent co-expression of CD11c T-bet, and FcRL5.**
256 *The average frequency of CD11c, T-bet, and FcRL5 co-expression on B cells sorted from buffy coats*
257 *following stimulation for (**A**) 2 days (n=18) and (**B**) 6 days (n=9) with different combinations of anti-*
258 *Ig, CpG-C, and IFN-γ.*
259
260 ***Plasma cell differentiation and antibody production are dependent on CpG-C and B cell***
261 ***proliferation***
262 The functional properties of double negative or atypical ($CD21^{-}CD27^{-}IgG^{+}$ and $IgD^{-}CD27^{-}T\text{-}bet^{+}$) B
263 cells remain unclear. It has been suggested that the cells contribute to the circulating antibody pool
264 [34] and that they could represent an intermediate plasma cell differentiation stage [35]. We,
265 therefore, assessed plasma cell differentiation and antibody production in the context of anti-Ig,
266 CpG-C, and IFN-γ stimulation and CD11c, T-bet, and FcRL5 expression. We cultured CTV-labelled and
267 unlabelled B cells sorted from healthy blood donors over 6 days with different combinations of anti-
268 Ig, CpG-C, IFN-γ, and IL-21. We then assessed CTV dilution (indicating proliferation), plasma cell
269 differentiation at 0, 2, 4, and 6 days of culture, and antibody production at 6 days of culture (**Figure**
270 **6A**). After subtracting media controls, we compared pooled log2 transformed antibody
271 concentrations (**Figure 6B**). anti-Ig, CpG-C, and IFN-γ stimulated cells (stim. 2) produced significantly
272 more antibodies compared to unstimulated cells (stim. 1). Exchanging IFN-γ with IL-21 (stim. 7) led
273 to further increased antibody levels, although IFN-γ did not have a detrimental effect on the
274 antibody levels (stim. 7 vs stim. 9). Antibody production was however completely abolished in the
275 absence of CpG-C, independent on other stimuli (stim. 4 and 11), indicating that CpG-C was required
276 to plasma cell differentiation.
277
278 We next assessed plasma cell differentiation by flow cytometry. Plasma cells were identified by high
279 levels of CD27, which was only observed in CTV^{low} proliferating cells (**Figure 6C**). Quantifying
280 $CTV^{low}CD27^{hi}$ plasma cells after stimulation showed that plasma cells became detectable and
281 reached peak levels at day 4 or stimulation. Plasma cells were, however, only observed after
282 stimulation with anti-Ig, CpG-C, and IFN-γ (stim. 2) and not anti-Ig and IFN-γ alone (stim. 4), or in
283 unstimulated cells. This corroborates the antibody data and shows that CpG-C was critical for B cell
284 proliferation and plasma cell differentiation (**Figure 6D**).
285

Since it has been indicated that T-bet contributes to plasma cell differentiation [35], we assessed if CD11c, T-bet, or FcRL5 were specifically enriched in plasma cells compared with total live B cells at day 4 of culture, when all markers were expressed and at peak of plasma cell frequencies (**Figure 6E**). Cells expressing either CD11c, T-bet, or FcRL5 were similarly enriched among the two compartments, indicating that none of the proteins was specifically required for plasma cell differentiation (**Figure 6E**).

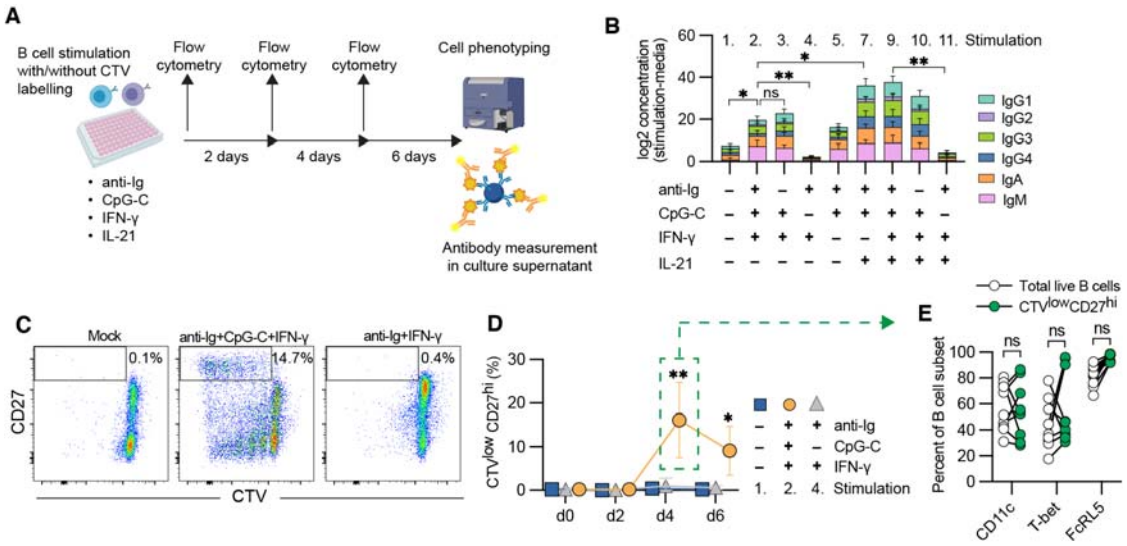


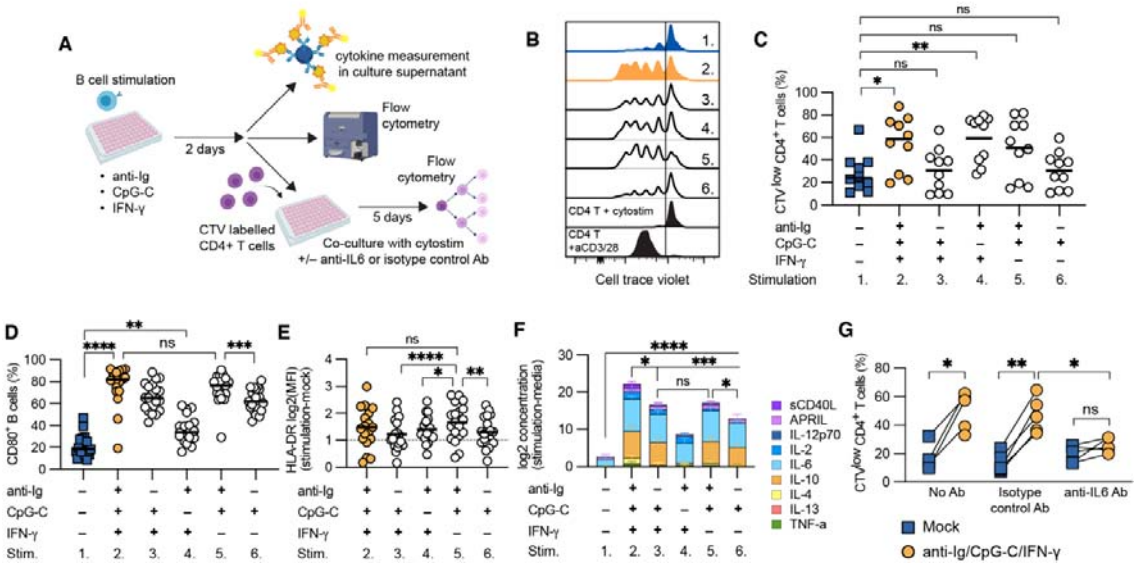
Figure 6. CpG-C is required for plasma cell differentiation and antibody production.
*(**A**) Schematic illustration of B cell cultures with cell-trace violet labelled and unlabelled B cells sorted from buffy coats, stimulated for 0, 2, 4, and 6 days before (**B**) measuring IgG1, 2, 3, 4, IgA, and IgM antibody levels in culture supernatants associated with different combinations of stimulation with anti-Ig, CpG-C, IFN-γ, and IL-21 as indicated (n=10). (**C**) Representative flow cytometry plots from day 6 and (**D**) summary graph indicating the frequency of cell-trace violet low and CD27 high plasma blasts at 0, 2, 4, and 6 days with no stimulation (blue boxes), anti-Ig+CpG-C+IFN-γ(orange circles), or anti-Ig+IFN-γ stimulation (grey triangles) (n=9). (**E**) Percent CD11c, T-bet, or FcRL5 expressing B cells within total live B cells (empty circle) or CTV^{low}CD27^{hi} cells (green circle) at day 4 after culture with anti-Ig, CpG-C, and IFN-γ. P-values were calculated using repeated-measures one-way ANOVA with Geisser-Greenhouse correction followed by Holm-Sidak's post hoc test. ns=not significant p>0.05, *p<0.05, ***p<0.001.*

B cell stimulation leads to the upregulation of co-stimulatory molecules and cytokine secretion resulting in increased T cell help

In addition to plasma cell differentiation, it has been proposed that CD11c^{+} B cells can provide enhanced antigen-presenting functions to CD4^{+} T cells in mice and humans [19,21]. To assess to what extent B cells stimulated with different combinations of anti-Ig, CpG-C, and IFN-γ could drive B cell-dependent CD4^{+} T cell help, we first stimulated B cells from healthy blood donors for 2 days. We then phenotyped the cells, measured cytokines in the supernatants and washed, counted, and co-cultured the B cells at a 1:5 ratio with autologous purified CD4^{+} T cells in the presence of cytostim, crosslinking MHC2 and the TCR (**Figure 7A**). To measure proliferation, the CD4^{+} T cells were stained with cell trace violet (CTV) (**Figure 7B**). As a negative control, CD4^{+} T cells were cultured with

318 cytostim in the absence of B cells, and as a positive control $CD4^+$ T cells were cultured with beads
319 coated with anti-CD3 and anti-CD28. Unstimulated B cells co-cultured with $CD4^+$ T cells had a similar
320 frequency of CTV low cells as the negative control, while > 90% of the cells had divided in response
321 to the positive control (**Figure 7B**). B cells pre-stimulated with anti-Ig, CpG-C and IFN-γ (stim. 2)
322 induced significantly more T cell proliferation (CTV^{low} cells) compared with co-cultures with
323 unstimulated B cells. There were also significantly more dividing T cells in the co-cultures with B cells
324 pre-stimulated with anti-Ig and IFN-γ (stim. 4) when compared with unstimulated cells (**Figure 7C**).
325
326 In vivo, B cells and $CD4^+$ T cells interact via MHC2 and the TCR, with additional survival and polarising
327 signals provided via cytokine secretion and co-stimulatory molecules, such as CD80 and CD86 on the
328 B cells [36,37]. To better understand what factors were responsible for the increased B cell-
329 dependent $CD4^+$ T cell proliferation, we assessed the levels of CD80 and HLA-DR after stimulation
330 (**Figure 7D**). CD80 was strongly upregulated by anti-Ig, CpG-C, and IFN-γ (stim. 2). The upregulation
331 was independent of IFN-γ as stimulation with anti-Ig and CpG (stim. 5) reached a similar frequency of
332 $CD80^+$ cells, while anti-Ig and CpG-C seemed to have an additive effect (**Figure 7D**). All B cells express
333 HLA-DR, so therefore we assessed the surface levels (median fluorescent intensity, MFI) following
334 stimulation after subtracting with the MFI of unstimulated cells (**Figure 7E**). All stimulations that we
335 tested had an average HLA-DR $\log2(MFI^{stim}–MFI^{unstim})>0$, indicating upregulation of HLA-DR in
336 response to stimulation. Cells stimulated with anti-Ig and CpG (stim. 5) had a similar HLA-DR MFI
337 level as triple-stimulated cells, while the condition gave significantly more upregulation compared
338 with CpG and IFN-γ (stim. 3), anti-Ig and IFN-γ (stim. 4), or CpG-C alone (stim. 6) (**Figure 7E**).
339
340 We next assessed a panel of 13 cytokines in the culture supernatants from day 2 of the B cell culture
341 with the rationale that this could indicate which cytokines were produced by the B cells at the time
342 of co-culture with the T cells. Cytokines that were detected above media background levels
343 ($\log2[pg/ml^{stim}–pg/ml^{media}]>0$) were pooled together to give a bulk measure of B cell cytokine
344 production (**Figure 7F**). IFN-γ was omitted from the cytokine pool data, since it was spiked in for
345 some stimulations, and is shown as relative concentration (MFI) in **Supplementary Figure 5** together
346 with the other cytokines measured. All stimulations had significantly more cytokines in the culture
347 supernatant compared with unstimulated cells ($p<0.0001$). anti-Ig, CpG-C, and IFN-γ (stim. 2)
348 stimulated cells had significantly more cytokines than all other conditions (stim. 2 vs stim. 3 $p<0.05$,
349 stim. 2 vs stim. 4-6 $p<0.001$). CpG-C + IFN-γ (stim. 3) and anti-Ig + CpG-C (stim. 5) had similar levels of
350 cytokines ($p>0.05$) and anti-Ig + CpG-C (stim. 5) significantly more than CpG-C alone (stim. 6 $p<0.05$),
351 indicating that anti-Ig, CpG-C, and IFN-γ all contributed to increasing overall cytokine production
352 (**Figure 7F**). Cells stimulated with anti-Ig and IFN-γ (stim. 4) had the least total cytokine levels of the
353 stimulated cells. This was mainly due to no detectable production of IL-10, which made up 33-39 %
354 of the measured cytokines in the other stimulations, where CpG-C was present. IL-6 was the other
355 main cytokine produced by the B cells, making up 39-64 % of the measured cytokines for the
356 stimulated cells (**Figure 7F**).
357
358 To test if IL-6 was potentially important for the improved $CD4^+$ T cell help, IL-6 blocking antibodies or
359 isotype control antibodies were added to co-cultures of pre-stimulated B cells and CTV-labelled $CD4^+$
360 T cells. Both the isotype antibody-treated and untreated co-cultures had a significantly higher
361 frequency of dividing CTV^{low} T cells when cultured with anti-Ig, CpG-C, and IFN-γ pre-stimulated B

362 cells compared with unstimulated B cells (**Figure 7G**). Treatment with IL-6 blocking antibodies,
363 however, abolished this effect by significantly reducing the frequency of CTV^{low} T cells to baseline
364 levels, comparable to T cells co-cultured with unstimulated B cells (**Figure 7G**). This indicates that IL-
365 6 was a key factor in the B cell-driven $CD4^+$ T cell proliferation.
366



367
368
369 **Figure 7. B cell-dependent $CD4^+$ T cell proliferation is mediated via IL-6.**
370 *(**A**) Schematic illustration of B cell-T cell co-culture experiments of cells sorted from buffy coats. (**B**)*
371 *representative histograms and (**C**) data for cell-trace violet dilution among sorted $CD4^+$ T cells co-*
372 *cultured for 5 days with autologous pre-stimulated B cells in the presence of cytostim (n=10). B cells*
373 *were pre-stimulated for 2 days as indicated, followed by washing, counting, and re-seeding at a 1:5*
374 *B:T ratio. (**D**) Frequency of CD80-positive B cells after two days of stimulation (n=19). (**E**) B cell*
375 *surface levels of HLA-DR, calculated as $log2[MFI^{stim}]–log2[MFI^{unstim}]$, after 2 days of culture (n=19). (**F**)*
376 *Cytokine levels for sCD40L, APRIL, IL-12p70, IL-2, IL-6, IL-10, IL-4, IL-13, and TNF-α in culture*
377 *supernatants calculated as log2[pg/mL in stimulated]–log2[pg/mL in culture media] following 2 days*
378 *of culture (n=10). (**G**) The same co-culture setup as in (C) with no stimulation (blue boxes) or anti-*
379 *Ig+CpG-C+IFN-γ(orange circles) pre-stimulated B cells in the presence of autologous $CD4^+$ T cells and*
380 *No antibody, isotype control antibody, or IL-6-blocking antibody (n=5). P-values were calculated*
381 *using repeated-measures one-way ANOVA with Geisser-Greenhouse correction followed by Sidak's*
382 *post hoc test. For (C), all comparisons were against unstimulated cells. ns=not significant p>0.05,*
383 **p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.*
384

385 **Discussion**

386 In this study, we have described how the cell surface proteins CD11c and FcRL5 and the intracellular
387 transcription factor T-bet are dynamically regulated after infection and following in vitro stimulation
388 via different activation pathways. These proteins are commonly used in different combinations
389 together with CD21, CD27, and IgD to identify a subset of B cells expanding during inflammatory
390 conditions [23]. In a previous study of CD11c$^+$ B cells in malaria, we observed that these cells
391 displayed different co-expression with FcRL5 and CXCR3 over time [27]. This led us to hypothesize
392 that the expression of these markers could be regulated via different activation pathways, leading to
393 different patterns of expression over time.

394

395 We first investigated the co-expression during and up to 12 months after diagnosis with *Plasmodium*
396 parasite infection. The frequency of cells expressing either CD11c, T-bet, or FcRL5 was the same at
397 approximately 30% of the mature B cells at diagnosis and at 10 days after treatment initiation. This
398 was then reduced to 18% at 12 months. However, despite a similar frequency of B cells with these
399 markers at diagnosis and the 10-day time-point, there was a substantial shift in the co-expression
400 pattern with mostly triple-positive cells at diagnosis followed by CD11c and FcRL5-double positive
401 cells at the 10-day time-point. This observation could have several different explanations, such as a
402 more transient expression of T-bet compared with CD11c and FcRL5, that cells expressing T-bet
403 leave the circulation, that T-bet-expressing cells preferentially differentiate to plasma cells, or that
404 they rapidly die.

405

406 We have previously shown that many of the dividing B cells during and after acute malaria express
407 CD11c and are enriched for parasite-specific reactivities, indicating a BCR-dependent upregulation of
408 CD11c [27]. Similar observations of recently activated B cells being enriched for CD11c$^+$ vaccine or
409 infection-specific B cells have also been made with antigen-specific tetramer staining [28,38,39]. It is
410 further also consistent with stimulation of the BCR in vitro leading to CD11c upregulation [21]. FcRL5
411 has been shown to be transiently upregulated via BCR-ligation where it upon co-stimulation via the
412 BCR and TLR9 led to a strong proliferation of naïve B cells [40]. FcRL5 is an IgG-receptor, enabling B
413 cells to respond to immune complexes [41]. In conjunction with CD21 (complement receptor 2),
414 which binds C3-fragments this effect is further enhanced [42], suggesting that early upregulation of
415 FcRL5 could be important to prime B cells for more efficient uptake and subsequent responses to
416 opsonized antigens. CD11c is an integrin and potentially has complementary roles to FcRL5 in
417 enhancing phagocytosis and cell migration [43] that could improve the early B cell response to
418 infection.

419

420 During malaria, the high abundance of parasites and subsequent pathogen-associated molecular
421 patterns (PAMPs), such as parasite DNA, will stimulate innate immune cells, including monocytes,
422 DCs, and B cells via the expression of endosomal TLR9 and other pattern-recognition receptors [44–
423 47]. Signaling via TLR9 is important for parasite control in both mice [48] and humans, where
424 different polymorphisms in human TLR9 greatly influence symptomatic disease [49]. Binding to the
425 BCR, followed by subsequent antigen uptake into endosomes and signaling via TLR9 constitutes early
426 cellular events that the B cells can do independently of other cellular interactions. In this study, we
427 found that these events were the primary influence on B cell expression of CD19, FcRL5, CD11c, HLA-
428 DR, CD80, and B cell-derived cytokine production, indicating a role for all these effects in the early B
429 cell response to infection. BCR-ligation also leads to the upregulation of CCR7 and EBI2, which will

430 guide the activated B cells to the B cell-T cell interfollicular region in secondary lymphoid organs
431 [50,51], where they can interact with cognate $CD4^+$ T cells [51,52].
432
433 Prior to their migration to the B cell-T cell interfollicular region, $CD4^+$ T cells are primed by activated
434 DCs that have taken up and recognized PAMPs. In response to intracellular pathogens, such as
435 *Plasmodium* parasites, DCs produce IL-12, which in turn will polarize the $CD4^+$ T cells to become IFN-
436 γ secreting T_H1 cells [53]. IL-12 together with IFN-γ can then further generate $T_{FH}1$ cells producing
437 both IFN-γ and IL-21 [54]. During malaria, circulating $T_{FH}1$ cells, identified by CXCR3-expression are
438 expanded in peripheral blood and are shown to provide suboptimal B cell help compared with
439 conventional CXCR3-negative cT_{FH} cells [55]. Especially in children where $T_{FH}1$ cells made up a larger
440 fraction of circulating T_{FH} cells [56]. IFN-γ has previously been shown to be important for T-bet
441 expression in human B cells [11] and $T_{FH}1$ cells likely constitute an important driver for the expansion
442 of T-bet^+ B cells in malaria and other inflammatory diseases [57]. Mouse models have in addition to
443 IFN-γ also indicated a role for IL-21 in the generation of $CD11c^+$T-bet^+ B cells [58–60]. We similarly
444 show that IFN-γ is critical for optimal T-bet expression but that it is not induced by IL-21 in the
445 absence of IFN-γ. CD11c on the other hand was more strongly upregulated in response to IL-21 than
446 IFN-γ, indicating separate roles of the cytokines and consistent with different regulations of CD11c,
447 FcRL5, and T-bet. In support of this, other studies in mice have identified a population of $CD11c^+$ B
448 cells in the absence of T-bet [60,61], and recently, Gao at al. and Gu et al. identified CD11c to likely
449 be regulated via the transcription factor Zeb2 in humans and mice, respectively [62,63].
450
451 IL-21 is a key cytokine in germinal centre B cell survival and plasma cell differentiation [64]. It is
452 mainly produced by $CD4^+$ T_{FH} cells and its induction is dependent on interaction with cognate B cells
453 [65]. It was shown that $CD11c^+$ B cells can become plasma cells in the presence of IL-21 and T cell
454 help [18,21,57,66]. Other studies have indicated a contribution of $CD21^-CD27^-$ or $CD11c^+$T-bet^+ B
455 cells to the circulating antibody pool [34,67,68], indicating either active plasma cell differentiation or
456 that the cells are coming from the same lineage as antibody-secreting cells. Further supporting an
457 active plasma cell differentiation, it has been suggested that T-bet^+ B cells in systemic lupus
458 erythematosus (SLE) could correspond to intermediate-stage plasma cells and that regulation of the
459 IFN-γR by T-bet is important for subsequent full plasma cell differentiation [35]. We observed an
460 enhanced effect of IL-21 on antibody production that was unaffected by the presence or absence of
461 IFN-γ in the stimulation and subsequent varying levels of T-bet (lower in the absence of IFN-γ).
462 Additionally, when measuring the expression of CD11c, T-bet, and FcRL5 in plasma cells or other
463 dividing cells responding to the stimulations, there was no enrichment of the markers among plasma
464 cells, suggesting that none of the proteins was actively required for plasma cell differentiation in
465 vitro. However, *in vivo* it is possible that co-expression of these markers is a common occurrence
466 during inflammatory conditions as antigen-specific B cells would upregulate CD11c and FcRL5, after
467 which they would migrate to the interfollicular region where they would encounter T cells producing
468 IFN-γ and IL-21, driving upregulation of T-bet and potentially leading to extrafollicular plasma cell
469 differentiation.
470
471 In response to the proinflammatory environment generated by the malaria parasites, there is also an
472 expanded anti-inflammatory response characterized by regulatory cells [69,70], modulation of
473 innate immune responses [71–73] and secretion of soluble anti-inflammatory proteins, such as IL-10

474 [71,74]. In mice, an increased level of IL-10 is shown to inhibit T-cell derived IFN-γ, leading to
475 reduced T-bet$^+$ B cells [75,76], indicating a potentially dynamic regulation of T-bet$^+$ B cells *in vivo*. We
476 also observed a reduced T-bet induction in B cells stimulated with IL-10, but not when co-stimulated
477 with IFN-γ. It has been suggested in mouse and human studies of malaria that both B cells and T cells
478 are major sources of IL-10 and are needed for disease control [69,77,78]. We observed a strong
479 induction of IL-10 in all stimulations containing CpG-C, consistent with TLR-ligands being major
480 inducers of IL-10 production in B cells [79,80]. Polymorphisms in both TLR9 and IL-10 are associated
481 with clinical presentation in malaria, highlighting the importance of this pathway in malaria disease
482 control [49,81]. In addition to IL-10, we also observed a large production of IL-6 from the stimulated
483 B cells. The IL-6 levels were also higher with CpG-C stimulation, but they were not completely CpG-
484 dependent, as observed for IL-10. IL-6 is a pleiotropic cytokine that affects both hematopoietic and
485 non-haematopoietic cells. It is an important survival and differentiation factor for B cells [82] and
486 CD4+ T cells, where it enhances proliferation, and influences migration and polarization [83]. By
487 blocking IL-6, we completely abrogated the B cell-mediated CD4$^+$ T cell proliferation, indicating that
488 IL-6 was a key mediator of how the B cells provided T cell help in our in vitro system.
489
490 It has been shown that CD11c$^+$ B cells can provide enhanced APC functions in mice [19]. We also
491 found that in vitro-stimulated B cells expressing CD11c, T-bet, and FcRL5 could support improved
492 CD4$^+$ T cell survival and proliferation via the production of IL-6. However, there was no obvious
493 association with T-bet, as stimulations lacking IFN-γ, and therefore were relatively low for T-bet,
494 provided T cell help equally well as stimulation with IFN-γ and high T-bet levels. Instead, the
495 improved T cell proliferation was mainly associated with B cells pre-stimulated via the BCR. This was
496 not clearly associated with specific up-regulation of CD11c, FcRL5 or specific levels of IL-6 either. It is
497 therefore likely that additional factors, driven by BCR-signalling and not identified here, can provide
498 further T cell help in conjunction with IL-6.
499
500 B cells expressing CD11c, T-bet, and or FcRL5 remain an enigmatic part of the B cell response. In this
501 study, we have evaluated co-expression after infection and triggered different activation pathways
502 *in vitro* to learn about how the markers are dynamically regulated over time. We found that the
503 stimuli leading to the upregulation of CD11c and FcRL5 were relatively overlapping, with BCR and
504 TLR9-ligation being the most important, while T-bet was dependent on IFN-γ. We also investigated
505 the stimulated B cells functionally and observed a clear dependency of plasma cell differentiation on
506 proliferation, while T cell help was dependent on the production of IL-6. However, the effector
507 functions were not clearly associated with given patterns of CD11c, T-bet, or FcRL5 expression,
508 potentially indicating that the upregulation of these markers is part of a normal response and not a
509 specific B cell lineage. However, to better understand how these cells behave in humans *in vivo*,
510 further studies are needed where B cells with different receptor expressions, potentially indicating
511 different stages of differentiation or time after antigen encounter, are characterised for specificity,
512 phenotype, and function.
513

514 **Materials and Methods**

515

516 **Clinical samples**

517 Adult travellers returning to Sweden and presenting with malaria at the emergency department at
518 Karolinska University Hospital and migrants identified through malaria screening were enrolled in a
519 prospective study. Malaria parasite species and parasitaemia were determined by microscopy
520 and/or qPCR by the Karolinska University Hospital lab. Blood samples were collected at the time of
521 diagnosis, after 10 days (10D), and again after 12 months (12M). In this study, samples from 10
522 patients, at three different time points, and 6 healthy controls were included for analysis. Venous
523 blood was collected in EDTA vacutainer tubes (BD) and peripheral blood mononuclear cells (PBMCs)
524 were isolated by density gradient centrifugation using Ficoll–Paque Plus (Cytiva). Briefly, the blood
525 was transferred to 50 ml tubes and diluted 1:1 with phosphate-buffered saline (PBS) supplemented
526 with 2% heat-inactivated fetal bovine serum (FBS), both from Thermo Fisher Scientific. 15 ml of
527 Ficoll-Paque was added to 50 ml Leucosep tubes and centrifuged at 1000 *g* for 1 minute at room
528 temperature (RT). The diluted blood was poured into the Leucosep tubes on top of the Leucosep
529 filter and centrifuged at 800 *g* for 15 minutes at RT without breaking at the end. The mononuclear
530 cell layer at the plasma-Ficoll interface was removed using a Pasteur pipette. The cells were diluted
531 to 50 ml with PBS + 2% FBS and centrifuged at 300 *g* for 8 min at RT. Supernatants were discarded
532 and cell pellets were resuspended in 30 ml PBS + 2% FBS and centrifuged again at 250 *g* for 10
533 minutes at RT. After the final wash, the cells were resuspended in PBS + 2% FBS and counted using a
534 Countess II (Invitrogen) following a 1:1 dilution with trypan blue. The cells were centrifuged again
535 and resuspended at 5×10^6 cells/ml in freezing medium (FBS + 10% DMSO) and placed inside a
536 CoolCell at –80°C overnight, after which the cells were transferred to liquid nitrogen for long-term
537 storage.

538

539 **Samples – in vitro cultures**

540 For the in vitro parts of the study, cells were sorted from buffy coats from healthy donors. Buffy
541 coats were obtained from the Karolinska University Hospital blood bank. Complete media that was
542 used in all cultures consisted of RPMI-medium supplemented with 10% heat-inactivated FBS, 2mM
543 L-glutamine, 100 U/ml of penicillin, 100 µg/ml of streptomycin, 10 mM HEPES and 0.05 mM 2-
544 Mercaptoethanol (all from ThermoFisher Scientific).

545

546 **Total B cell and $CD4^+$ T cell isolation**

547 B cells and $CD4^+$ T cells were isolated from buffy coats by negative selection with the RosetteSep
548 Human B Cell Enrichment Cocktail or RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail
549 respectively, using 50 ml SepMate tubes (all from Stemcell technologies). Briefly, 25 µl of the Cell
550 Enrichment Cocktail was added for each ml of blood and incubated for 20 minutes at RT. Two
551 volumes of PBS was then added to the sample. 15 ml Ficoll–Paque Plus (GE Healthcare) was added
552 to SepMate tubes and centrifuged at 1000 *g* for 1 minute (RT). Samples were poured into the
553 SepMate tubes and centrifuged at 1200 *g* for 10 min with the break on. The cell fraction was
554 transferred to new tubes and washed twice with PBS + 2% FBS (300 *g*, 10 min, brake at 5, RT). For
555 two donors the B cells were isolated using MACS cell sorting with anti-human CD19 microbeads and
556 MS columns from Miltenyi Biotec, according to the manufacturer's instructions. Isolated cells were
557 frozen and stored as previously described. B cell and $CD4^+$ T cell purity was assessed by flow
558 cytometry and were on average±SD 90.8±5.0% and 90.5±4.8%, respectively.

559

560 **Naïve and memory B cell isolation**

561 Memory B cells and naïve B cells were isolated from RosetteSep pre-sorted total B cells via positive
562 selection using the EasySep™ Human Memory B Cell Isolation Kit (STEMCELL™ Technologies), and
563 via negative selection using the EasySep™ Human Naive B Cell Isolation Kit (STEMCELL™
564 Technologies), following the manufacturer's protocol. Briefly, total B cells were prepared as
565 previously described and resuspended at a concentration of 1×10^8 cells/mL. CD27 Positive Selection
566 Cocktail was then added at 50 µL/mL and incubated for 5 minutes, after which 100 µL/mL of
567 Releasable RapidSpheres were added. Total B cells were then incubated in an EasySep magnet for 5
568 min. The negative fraction was poured into a new tube and kept for naïve B cell isolation. The tube
569 containing isolated memory B cells was incubated with 100 µL/mL of Release buffer and 50 µL/mL of
570 Dextran RapidSphere for 5 minutes in an Easysep magnet. The isolated memory B cells released
571 from the selection beads were poured into a new tube and directly used for stimulation. The cells in
572 the negative fraction (from the memory B cell isolation) were resuspended at 5×10^7 cells/mL and 50
573 µL/mL of Cocktail Enhancer and Isolation Cocktail were added. After 5 minutes of incubation, 50
574 µL/mL of RapidSpheres was added and tubes were placed in an Easysep magnet for 5 minutes.
575 Negatively selected Naïve B cells were poured into new tubes and were directly used for stimulation.
576 Memory and Naïve B cell purity was assessed by flow cytometry and were on average±SD 86.4±5.2
577 % and 92.7±6.9 %, respectively. The buffer used during both isolations was PBS + 2% FBS.

578

579 **B cell stimulation**

580 Previously sorted total B cells were thawed, moved to a 15 ml Falcon tube and 13 ml of complete
581 media was added dropwise before mixing and resting the cells on ice for 20 min. The sorted B cells
582 were then centrifuged at 300 *g* for 5 min, washed once in complete media (300 *g* 5 min),
583 resuspended in 1 ml complete media and counted. Stimulations of purified B cells were done in a 96-
584 well round-bottom tissue-culture plate (Techno Plastic Products). The stimuli included α-Ig
585 (IgA+IgG+IgM [H+L] AffiniPureF(ab')2 Fragment goat-anti-human [Jackson ImmunoResearch
586 Laboratories]) at 1.25 µg/ml, CpG-C (Invivogen) at 2.5 µg/ml, IFN-γ (Peprotech) at 25 ng/ml, IL-21
587 (Peprotech) at 10 ng/ml and IL-10 (Peprotech) at 10 ng/ml. The stimuli were added in different
588 combinations (**Supplementary Table 1**) to wells seeded with 1×10^5 cells in a total volume of 200 µl
589 complete media. Unstimulated cells were cultured in complete media. Plates were incubated at 37°C
590 and 5% CO_2.

591

592 **Flow cytometry**

593 For flow cytometry, the cells were washed twice in PBS and then incubated with Live/Dead stain
594 (ThermoFisher Scientific), diluted 1:1200 in PBS, for 20 min on ice. Cells were then washed twice in
595 PBS + 2% FBS before incubation for 20 min on ice with an antibody mix targeting cell surface
596 markers. For intracellular markers, the cells were washed once after surface staining, then incubated
597 for 30 min at RT with the FoxP3 Fix/Perm buffer (eBioscience), washed once in FoxP3 Wash/perm
598 buffer, and incubated for 30 min at RT with an antibody mix targeting intracellular markers. The cells
599 were then washed again in Wash/perm buffer followed by one wash in PBS + 2% FBS. Samples were
600 then resuspended in 300 µl PBS + 2% FBS together with 5 µl CountBright™ Absolute Counting Beads
601 (ThermoFisher Scientific) and run on a 5-laser BD Fortessa (BD Biosciences). See **Supplementary**
602 **Tables 2-7** for the different antibody panels.

603

604
605
606 **Cell Trace Violet**
607 For tracking cell division, the cells were stained with Cell Trace Violet (CTV) (Thermo Fisher
608 Scientific). The cells were suspended at a density of 2×10^6 cells per ml in PBS. CTV was added to the
609 cell suspension at 1 mM and incubated for 7 min at 37°C in a ventilated incubator. Staining was
610 stopped with FBS (3 times the staining volume) and PBS was added up to 15 ml. The cells were then
611 centrifuged (300 *g* 5 min) and washed once in PBS (300 *g* 5 min) and once in complete media. The
612 cells were then rested for 1 h in complete media at 37°C 5% CO_2 before further simulation or
613 analysis.
614
615 **Antibody measurement**
616 Antibody levels in the supernatants from B cells that had been stimulated for 6 days were measured
617 using the LEGENDplex™ Human Immunoglobulin Isotyping Panel (6-plex) (Biolegend), a bead-base
618 immunoassay, according to the manufacturer's protocol with some modifications. The assay was
619 performed in a U-bottom 96 well plate, the plate was wrapped in aluminium foil during all
620 incubations. Samples were diluted 1:100 in PBS + 2% FBS. 25 µl diluted sample was mixed with 25 µl
621 assay buffer and 12.5 µl pre-mixed beads. A standard 1:4 serial dilutions were prepared, and 25 µl
622 standard was mixed with 25 µl assay buffer and 12.5 µl pre-mixed beads. The plates were incubated
623 on a plate shaker at 700 rpm for 2 h, RT. Plates were then centrifuged at 650 *g*, 1 min and washed
624 with 200 µl wash buffer. A detection antibody was added at 1:1 in PBS, 25 µl/well. The plates were
625 then incubated on a plate shaker at 700 rpm for 1 h, RT. Streptatividin-PE was then added at 1:1 in
626 PBS, 25 µl/well (no wash in between), and again incubated on a plate shaker at 700 rpm for 30 min,
627 RT. The plates were then washed twice, as before, and resuspended in 200 µl PBS. The fluorescent
628 signal was measured on a BD 4-laser Fortessa using the 488 nm laser (forward scatter vs side scatter)
629 to separate beads based on size and granularity and the 640 nm laser (780/60 filter) to separate
630 beads based on cytokine specificity. The 561 nm laser (582/16) filter was then used to detect the
631 signal, translating to the amount of cytokine. The data was analyzed using the LEGENDplex™ data
632 analysis software.
633
634 **Cytokine measurement**
635 Cytokine levels in the supernatants from B cells that had been stimulated for 48 hr were measured
636 using the LEGENDplex™ Human B cell Panel (13-plex) (Biolegend), using the same procedure as
637 described for the antibody measurements, but with the addition of 12.5 µl Matrix B7 to each
638 sample. Supernatants were not diluted prior to being added to the plate and mixed with pre-mixed
639 beads, assay buffer and Matrix B7.
640
641 **Co-cultures – in vitro stimulated B cells and $CD4^+$ T cells**
642 In vitro stimulated B cells were co-cultured with autologous $CD4^+$ T cells and a superantigen to
643 explore the capacity of in vitro stimulated B cells to induce $CD4^+$ T cell proliferation. B cells were
644 stimulated in vitro (as previously described) for 48 h. The cells were then washed twice in fresh
645 complete media and counted. 20'000 B cells from each condition were cultured for five days with
646 100'000 CTV-labelled $CD4^+$ T cells along with 1 µl CytoStim™ (200X dilution, Miltenyi Biotec).
647 TransAct (300X dilution, Miltenyi Biotec) was used as a positive control for $CD4^+$ T cell proliferation.
648 To test the hypothesis that IL-6 signalling influences $CD4^+$ T cell proliferation, anti-hIL-6-IgG

(Invivogen) or mouse IgG1 kappa Isotype Control (eBioscience) were added at 1 µg/ml to the co-culture system at the start, and another 1 µg/ml of anti-hIL-6-IgG (Invivogen) or mouse IgG1 kappa Isotype Control were added to the wells after 2 days of co-culture.

Data analysis

Statistical analyses and graphs were done using GraphPad Prism (version 9). Data is generally presented as individual data points or mean with error bars indicating the 95% confidence interval. Groups were compared using repeated-measures one-way ANOVA with Geisser-Green house correction of unequal variance or a mixed-effects model if including missing data, followed by Sidak's test for multiple comparisons, or a Kruskal-Wallis or Friedman test followed by Dunn's post hoc test for non-parametric data. A p-value < 0.05 was considered significant. Analysis of flow cytometry data was done with FlowJo (version 10.8.1, BD). Alluvial plots were generated using the *ggalluvial* package [84] in R (version 4.1.1, www.r-project.org).

Data availability statement

Data is available upon reasonable request to the corresponding author (christopher.sundling@ki.se).

Conflict of interest disclosure

The authors declare no competing interests.

Ethics approval statement

This study was performed in accordance with the declaration of Helsinki and following ethical approval (2006/893-31/4 with addendum 2019-03436) by the Swedish Ethical Review Authority.

Patient consent statement

All study participants were included following informed consent.

Author contributions

LK planned and performed experiments, performed analysis, and wrote the first draft. ADCG planned and performed experiments and wrote parts of the first draft. MJL performed analysis and wrote parts of the first draft. AF was responsible for the malaria cohort and supervised students. CS planned the study, performed analysis, supervised students, and wrote parts of the first draft. All authors contributed to editing the first draft.

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