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CD14+ monocytes are the main leukocytic sources of CXCL10 in response to *Plasmodium falciparum*

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Abstract

The CXCR3 chemokine CXCL10 or IFN-γ inducible protein 10 (IP-10) has been identified as an important biomarker of cerebral malaria (CM) mortality in children. Studies in mouse malaria infection models have shown that CXCL10 blockade alleviates brain intravascular inflammation and protects infected mice from CM. Despite the key role that CXCL10 plays in the development of CM, the leukocytic sources of CXCL10 in response to human malaria are not known. Here we investigated CXCL10 responses to *Plasmodium falciparum* in peripheral blood mononuclear cells (PBMCs). We found that PBMCs from malaria-unexposed donors produce CXCL10 in response to *P. falciparum* and that this response is IFN-γ-dependent. Moreover, $CD14^+$ monocytes were identified as the main leukocytic sources of CXCL10 in peripheral blood, suggesting an important role for innate immune responses in the activation of this pathway involved in the development of symptomatic malaria.

Key words

Malaria, chemokines, pathogenesis, monocytes

Introduction

Malaria is a mosquito-borne infectious disease that is responsible for more than 200 million clinical cases and 400,000 deaths annually (WHO, 2016). Although there are five species of *Plasmodium* that can infect humans, most cases of severe malaria are caused by *P. falciparum*. Cerebral malaria is one of the most severe complications of *P. falciparum* infection, with a case fatality rate of approximately 20% even with appropriate medical treatment (Murphy & Breman, 2001). Blood-stage malaria parasites express proteins on the surface of the infected red blood cell, which allows them to bind to vascular endothelial cells and avoid clearance in the spleen. This process, known as sequestration, induces obstructions in the blood flow, resulting in hypoxia and haemorrhages (Miller *et al.*, 2002), associated with the induction of organ-specific disease syndromes (Grau *et al.*, 2003; Taylor *et al.*, 2004; Turner *et al.*, 1994; Walter *et al.*, 1982). A large body of work indicates that inflammatory responses also contribute to severe disease (Molyneux *et al.*, 1993; van der Heyde *et al.*, 2006). In addition to pRBCs, histopathological analysis of the brain of fatal CM cases revealed the presence of leukocytes in the brain microvasculature (Armah *et al.*, 2005; Patnaik *et al.*, 1994; Porta *et al.*, 1993), suggesting that recruitment of these cells to sites of parasite sequestration might result in local inflammation and contribute to disease induction.

Leukocyte trafficking is regulated by a family of chemotactic cytokines called chemokines. The observation that host cells could be found with pRBC in inflamed organs such as brain or placenta attracted interest in investigating trafficking pathways by which inflammatory cells are recruited to target organs in severe malaria. Increased levels of circulating chemokines including CCL4, CXCL4, CXCL8 and CXCL10 have been found to be associated with CM (Armah *et al.*, 2007; Jain *et al.*, 2008; John *et al.*, 2006; John *et al.*, 2008; Wilson *et al.*, 2011). Amongst these factors, the CXCR3 binding chemokine CXCL10 has been identified as the most accurate independent predictor of CM mortality in children (Armah *et al.*, 2007; Wilson *et al.*, 2011). In mice, CXCL10 blockade has been shown to protect *P.berghei* ANKA-infected mice from developing CM by reducing recruitment of $CXCR3^+$ leukocytes to the brain (Campanella *et al.*, 2008; Nie *et al.*, 2009). Furthermore, CXCL10 blockade was also shown to improve control of parasitemia by favoring the accumulation of $CXCR3^+CD4^+$ T follicular helper cells in the spleen, which enhanced antibody responses to infection (Ioannidis *et al.*, 2016).

Consistent with its association to cerebral disease, most malaria studies investigating cellular sources of CXCL10 have focused on brain tissue. High levels of CXCL10 have been detected in the cerebrospinal fluid of children that succumbed to CM (Armah *et al.*, 2007). In mice, CXCL10 expression has been observed in brain endothelial cells (Campanella *et al.*, 2008; Miu *et al.*, 2008; Sorensen *et al.*, 2018), neurons (Campanella *et al.*, 2008), astrocytes (Hanum *et al.*, 2003; Miu *et al.*, 2008) and microglia (Ioannidis *et al.*, 2016). In addition, neutrophils and monocytes that are recruited to the brain during rodent malaria infection (Ioannidis *et al.*, 2016; Sorensen *et al.*, 2018) have been found to be sources of CXCL10 that control the recruitment of $CXCR3^+$ leukocytes involved in the development of CM, suggesting an important role for leukocyte derived CXCL10 in the induction of severe malaria disease symptoms. Consistent with this concept, PBMC-derived CXCL10 has been shown to be associated with *P. falciparum*-mediated severe malaria in children (Stanisic *et al.*, 2014). The specific cellular sources of CXCL10 in response to *P. falciparum* are still unknown. In this study, we sought to identify the leukocyte populations in the blood responsible for the induction of CXCL10 in response to *P. falciparum*. Our results show that $CD14^+$ monocytes are the main cellular source of CXCL10 in response to *P. falciparum*. CXCL10 production in response to pRBCs was also found to be stimulated via an IFN-γ-dependent mechanism.

Materials and Methods

P. falciparum culture

The 3D7 strain of *P. falciparum* was cultured as previously described (Stanisic *et al.*, 2014). Parasites were synchronized by treatment with 5% sorbitol and routinely subjected to gelatin flotation to select for knob-expressing parasites. Trophozoite/schizont stage parasitised red blood cells (pRBCs) were purified using MACS CS separation columns with a VarioMACS separator (Miltenyi Biotech).

PBMC stimulation

Peripheral blood was collected from healthy volunteers at the Volunteer Blood Donor Registry. PBMCs were then isolated by density gradient centrifugation using Lymphoprep (StemCell Technologies) and cryopreserved in liquid nitrogen until use. Cryopreserved PBMCs were thawed in complete medium (RPMI 1640 with 25mM HEPES, 10% heat-inactivated fetal bovine serum [HI-FBS; Sigma], 100U/mL streptomycin and 100 μg/mL penicillin), counted and then seeded into U-bottom 96-well plates (2×10^5 cells/well). PBMCs were stimulated with either uninfected RBCs (uRBCs; 2×10^5 RBCs/well), pRBCs (2×10^5 RBCs/well) or recombinant human IFN-γ (10ng/mL; PeproTech) for 24h at 37°C with 5% CO_2. In some experiments, PBMCs were stimulated in the presence of 20μg/mL anti-IFNγ (clone NIB42; BD Biosciences) or isotype control antibodies.

Detection of CXCL10 by ELISA

The amount of CXCL10 in PBMC culture supernatants was determined by capture ELISA. Briefly, U-bottom 96-well plates were coated with capture antibody (clone MAB266; R&D systems) in phosphate buffer pH 9.6 by overnight incubation at 4˚C. After washing, plates were blocked with 1% BSA (Sigma) in PBS for 1h at 37˚C before incubation with culture supernatants for 2h at room temperature (RT). Plates were then incubated with biotinylated anti-CXCL10 (clone BAF266; R&D systems) for 2h at RT, followed by streptavidin-conjugated HRP (Pierce) for 20 minutes at RT. After washing, bound complexes were detected by reaction with tetramethylbenzidine and H_2O_2 (KPL). Absorbance was measured at 450nm. Chemokine concentrations were calculated using recombinant CXCL10 (R&D Systems) for the preparation of standard curves.

Flow cytometry

PBMCs were stimulated with uRBCs, pRBCs or IFNγ for 24h. Brefeldin A (10μg/mL; Sigma) and monensin (2μM; BD Biosciences) were added to cells for the last 8h of stimulation. After stimulation, cells were incubated for 10 min on ice with PBS containing 10mM glucose and 3mM ethylenediaminetetraacetic acid (EDTA) to detach adherent cells. The cells were then blocked with human IgG (10μg/mL; Sigma) for 10 minutes on ice, washed and then stained with the following antibodies for 30 minutes on ice: FITC-conjugated anti-human γδTCR (clone 11F2), PerCP-Cy5.5-conjugated anti-human HLA-DR (clone L234), APC-conjugated anti-human CD123 (clone 6H6), AF700-conjugated anti-human CD19 (HIB19; Biolegend), APC-Cy7-conjugated anti-human CD14 (clone MϕP9), Pacific Blue-conjugated anti-human CD11c (clone 3.9; Biolegend), Qdot 605-conjugated anti-human CD8 (clone 3B5; Invitrogen), Brilliant Violet 650-conjugated anti-human CD4 (clone OKT4; Biolegend), BV711-conjugated anti-human CD16 (clone 3G8) PE Texas Red-conjugated anti-human CD3 (clone UCHT1; Beckman Coulter) and PE-Cy7 conjugated anti-human CD56 (clone B159). All antibodies were purchased from BD Biosciences unless otherwise stated. Aqua amine reactive dye (Life Technologies) was also included for dead cell exclusion. All washes and antibody dilutions were made in FACS buffer (PBS with 0.5% BSA [Sigma] and 2mM EDTA). Cells were then fixed in 2% paraformaldehyde and permeabilized using Perm Buffer 2 (BD Biosciences) before intracellular staining with PE-conjugated anti-human CXCL10 (6D4/D6/G2) or an isotype control for 1 hour on ice. After staining, cells were washed, re-suspended in FACS buffer and analyzed on a BD Fortessa X-1 flow cytometer. Data analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA). A positive response in pRBC-stimulated PBMCs was defined as a frequency of $CXCL10^+$ cells ≥0.1% after subtraction of the background from uRBC-stimulated cells.

Statistical analysis

Statistical analysis was performed in Prism version 8 (GraphPad Software Inc.). Wilcoxon matched-pairs signed rank tests were used to analyze matched data.

Results

$CD14^+$ monocytes are the main cellular source of CXCL10 in response to P. falciparum

The 3D7 strain of *P. falciparum* has previously been shown to induce CXCL10 production in PBMCs from severe malaria cases in an endemic area of Papua New Guinea (Stanisic *et al.*, 2014). To determine whether this parasite was also able to induce CXCL10 production in PBMCs from malaria-unexposed individuals, PBMCs from healthy donors were stimulated with pRBCs for 24 hours and CXCL10 output was determined by ELISA. PBMCs were also stimulated with autologous uRBCs as a negative control, while IFN-γ was included as a positive control. PBMCs from all donors produced CXCL10 in response to pRBC stimulation (Figure 1A). On average, pRBC-stimulated PBMCs showed a >4-fold increase in CXCL10 levels compared to either media- or uRBC-stimulated cells. Robust CXCL10 responses to IFN-γ were also observed in each donor (Figure 1B).

To identify the specific cellular sources of CXCL10 in response to *P. falciparum*, PBMCs were stimulated with pRBCs or uRBCs for 24 hours and then stained with a panel fluorescently labeled antibodies to assess CXCL10 production in monocytes, dendritic cells, NK cells B cells and various populations of T cells by flow cytometry. The gating strategy used to identify different leukocyte populations is shown in Figure 1C-D. $CD14^+$ monocytes were identified as a cellular source of CXCL10 in the majority of donors tested. In addition to $CD14^+$ monocytes, CXCL10-producing plasmacytoid DCs (pDCs) were also detected in a small proportion of donors, while CXCL10 production was observed in monocyte-derived DCs (mDCs) from one donor (Figure 1E). CXCL10 expression was not detected in PBMCs from three donors. The median fluorescence intensity (MFI) of CXCL10 was also determined in each cell population to assess the level of CXCL10 expression (Figure 1F and 1G). This showed that CXCL10 expression was significantly higher in $CD14^+$ monocytes than pDCs.

PBMC CXCL10 responses to P. falciparum are IFN-γ-dependent

Although CXCL10 was initially identified as an IFN-γ-inducible factor, it has been shown that bacterial and viral agonists stimulate its production (Asensio *et al.*, 2001; Bandow *et al.*, 2012; Brownell *et al.*, 2014; Park *et al.*, 2006), suggesting that additional pathways may also contribute to the induction of this chemokine. Consistent with this, both viral infection and the TLR 3 agonist polyI:C have been shown to induce CXCL10 production via activation of the NF-κB pathway (Brownell *et al.*, 2014; Spurrell *et al.*, 2005). Furthermore, IFN-γ-independent production of CXCL10 has also been reported (Cheeran *et al.*, 2003; Medoff *et al.*, 2002). To determine whether IFN-γ is required for CXCL10 production in PBMCs in response to *P. falciparum*, PBMCs were stimulated with pRBCs in the presence of anti-IFN-γ or isotype control antibodies and CXCL10 output was assessed by ELISA. Preliminary experiments showed that anti-IFN-γ antibodies inhibit CXCL10 responses in response to IFN-γ in a dose-dependent manner, with >80% neutralization observed at an antibody concentration of 20μg/mL (Supplementary Figure 1). Based on these data, an antibody concentration of 20μg/mL was used for subsequent experiments. As expected, CXCL10 production in response to IFN-γ was significantly reduced in the presence of anti-IFN-γ antibodies (Figure 2A). Notably, IFN-γ neutralization completely abrogated CXCL10 responses to pRBCs (Figure 2B-C). Thus, *P. falciparum* induces early/innate CXCL10 production in PBMCs from malaria-unexposed donors in an IFN-γ-dependent manner.

Discussion

The CXCR3 chemokine CXCL10 has been shown to be a strong independent predictor of *P. falciparum*-mediated CM mortality in children (Armah *et al.*, 2007; Wilson *et al.*, 2011) and to promote the development of CM in mice (Campanella *et al.*, 2008; Nie *et al.*, 2009). The identity of the cell types responsible for the production of CXCL10 in response to *P. falciparum* in human blood are still unknown. Here we have shown that $CD14^+$ monocytes are the main leukocytic source of CXCL10 in the blood in response to the parasite. As CD16 expression on CD14$^+$ monocytes was not assessed, it remains to be determined which specific monocyte population (i.e. classical, intermediate or non-classical) is responsible for the production of CXCL10 in response to *P. falciparum* (Ziegler-Heitbrock *et al.*, 2010).

Although CXCL10 was initially identified as an IFN-γ-inducible chemokine, it has also been reported that bacterial and viral agonists can stimulate its production from astrocytes (Asensio *et al.*, 2001) and macrophages (Kopydlowski *et al.*, 1999). Our results show that CXCL10 production by PBMCs in response to *P. falciparum* is IFN-γ-dependent. This finding is consistent with previous studies showing that CXCL10 production in human brain endothelial cell-PBMC co-cultures is largely IFN-γ-dependent (Khaw *et al.*, 2013). Previous studies have identified γδT cells, NK cells and conventional αβT cells as sources of IFN-γ in *P. falciparum*-stimulated PBMCs (D'Ombrain *et al.*, 2007a; D'Ombrain *et al.*, 2007b), suggesting that IFN-γ production from these cells facilitates down-stream CXCL10 production from CD14$^+$ monocytes in the blood. These studies also found that the IFN-γ response to *P. falciparum* was highly heterogeneous among individuals. Thus, the inability of some donors to produce CXCL10 (Figure 1E) may reflect poor IFN-γ responsiveness to *P. falciparum* in these individuals.

IFN-γ has been shown to have a dual role in malaria, contributing to both the control of infection and the development of severe disease (King & Lamb, 2015). During the early stages of infection, IFN-γ can help to activate macrophages and enhance their phagocytic activity thereby promoting parasite clearance. IFN-γ also enhances monocyte-mediated antibody-dependent cellular cytotoxicity (Bouharoun-Tayoun *et al.*, 1995). Systemic production of IFN-γ during infection has also been shown to up-regulate ICAM-1 expression on vascular endothelial cells, which promotes parasite sequestration and the development of severe disease (Amani *et al.*, 2000). Here we found that *P. falciparum*-induced IFN-γ results in the production of CXC10, described to be one of strongest biomarkers of severe malaria, suggesting that in addition to its direct effects triggered by IFN-γ-receptor mediated signalling, down-stream effects activated via IFN-γ–mediated chemokine cascades, are also important in the induction of symptomatic malaria. In addition to its well-defined role in leukocyte recruitment, CXCL10 has also been shown to exert anti-angiogenic function (Bodnar *et al.*, 2006; Yates-Binder *et al.*, 2012) and inhibit endothelial cell proliferation (Campanella *et al.*, 2010). Thus, IFN-γ-mediated production of CXCL10 during infection may also inhibit vascular growth and repair required to maintain oxygen and nutrient supply to hypoxic tissues.

Consistent with our findings here, monocytes have also been identified as a major leukocytic source of CXCL10 in CM-susceptible infected mice (Ioannidis *et al.*, 2016; Sorensen *et al.*, 2018). Microglial cells and brain endothelial cells have also been identified as cellular sources of CXCL10 and stable interactions between brain endothelial cells and $CD8^+$ T cells and are thought to be required for the induction of CM in mice (Ioannidis *et al.*, 2014; Sorensen *et al.*, 2018). CXCL10 production by brain-recruited monocytes induces trafficking of $CXCR3^+$ T cells to this site during infection, thereby promoting the development of CM (Ioannidis *et al.*, 2016). Interestingly, monocyte accumulation has been observed in the brain microvasculature of fatal CM cases (Hochman *et al.*, 2015), raising the possibility that a similar process might takes place in human CM. Consistent with this hypothesis, high levels of CXCL10 in cerebrospinal fluid have been associated with CM mortality in children (Armah *et al.*, 2007).

This study identified CXCL10 production from unexposed donors suggesting an important role for innate responses in the activation of this pathway. However, it remains to be determined whether additional parasite-specific adaptive responses also contribute to the

CXCL10 response to *P. falciparum* in malaria-exposed individuals. Further work with cells from *P. falciparum*-infected individuals will be required to establish if CXCL10 production and its association with the development of severe malaria is further enhanced after activation of other cell populations that have been previously exposed to malaria parasites.

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Conflicts of Interest

The authors declare no conflicting interests.

Ethical Standards

All experiments were conducted in accordance with the requirements of the Walter and Eliza Hall Institute Human Research Ethics Committee, the National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research and the Helsinki Declaration of 1975, as revised in 2008.

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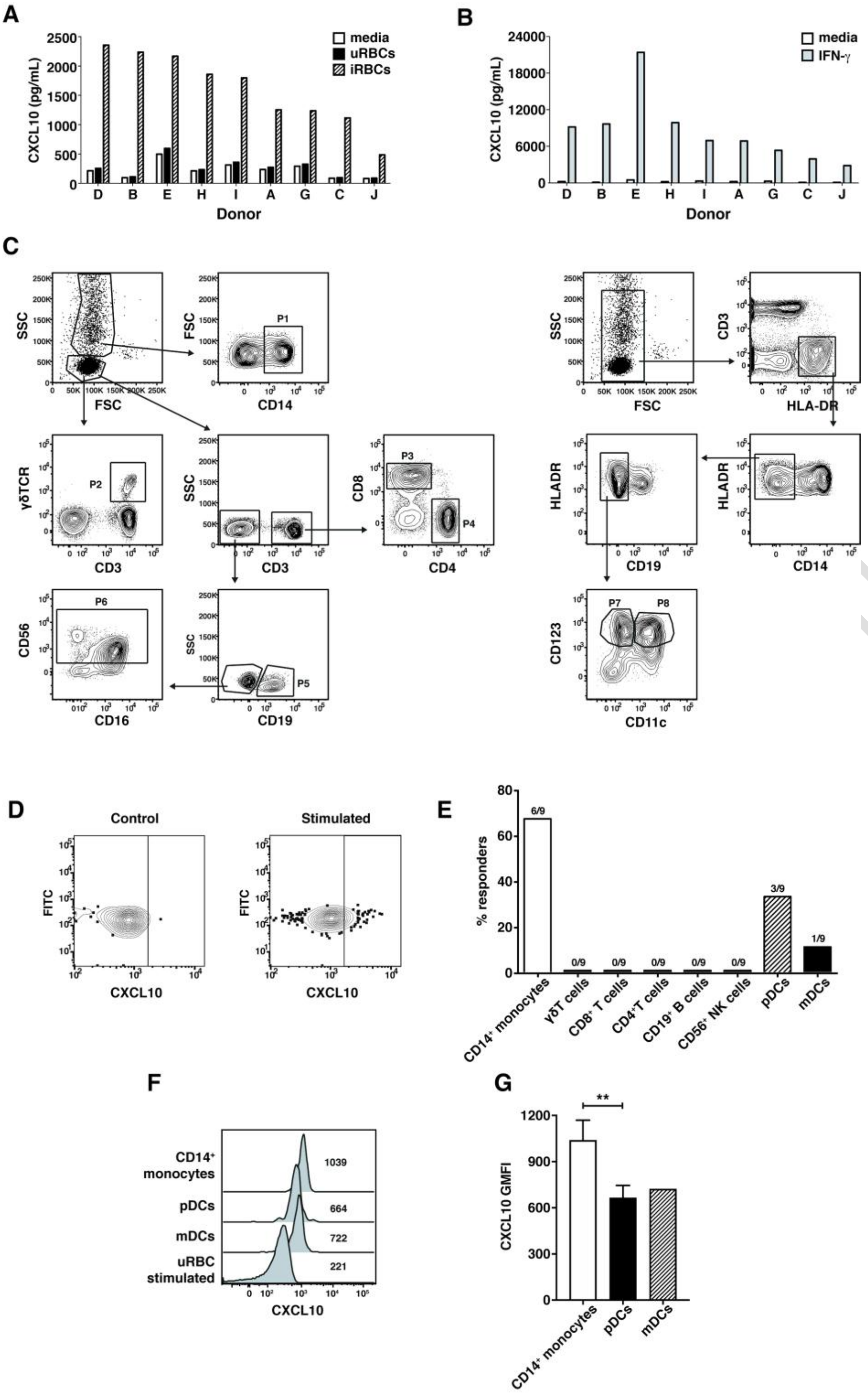

Figure 1. CD14^{+} monocytes are the main cellular source of CXCL10 in response to *P. falciparum*. PBMCs were stimulated with uRBCs, pRBCs (**A**) or IFN-γ (**B**) for 24 hours. Cell culture supernatants were then collected and CXCL10 levels were determined by ELISA. Each bar represents the mean of duplicate wells. Letters indicate individual donors.

(**C**) PBMC were stained with fluorescent antibodies for identification of $CD14^{+}$ monocytes ($CD14^{+}$; P1), γδ T cells ($CD3^{+}$γδ TCR^{+}; P2), $CD8^{+}$ T cells ($CD3^{+}CD8^{+}CD4^{-}$; P3), $CD4^{+}$ T cells ($CD3^{+}CD8^{-}CD4^{+}$; P4), $CD19^{+}$ B cells ($CD3^{-}CD19^{+}$; P5), $CD56^{+}$ NK cells ($CD3^{-}CD19^{-}$ $CD16^{+/-}CD56^{+}$; P6), pDCs ($CD3^{-}$HLA-$DR^{+}CD14^{-}CD19^{-}CD11c^{-}CD123^{+}$; P7) and mDCs ($CD3^{-}$HLA-$DR^{+}CD14^{-}CD19^{-}CD11c^{+}CD127^{+}$; P8) by flow cytometry. CXCL10 intracellular staining was evaluated within each gated population as shown in the representative contours (**D**). The proportion of CXCL10 responders was determined for each cell population (**E**). A positive response in pRBC-stimulated PBMCs was defined as a frequency of $CXCL10^{+}$ cells ≥0.1% after subtraction of the background from uRBC-stimulated cells. (**F**) Representative histograms showing the level of CXCL10 expression in response to pRBC by $CD14^{+}$ monocytes, pDCs and mDCs. uRBC-stimulated lymphocytes are included as a background control. Numbers represent the average geometric mean fluorescence intensity (GMFI) for each cell population. The average GMFI of CXCL10 was also determined among all responder donors for each population (**G**). Bars represent the mean ± SD, ** $p<0.01$ unpaired t-test.

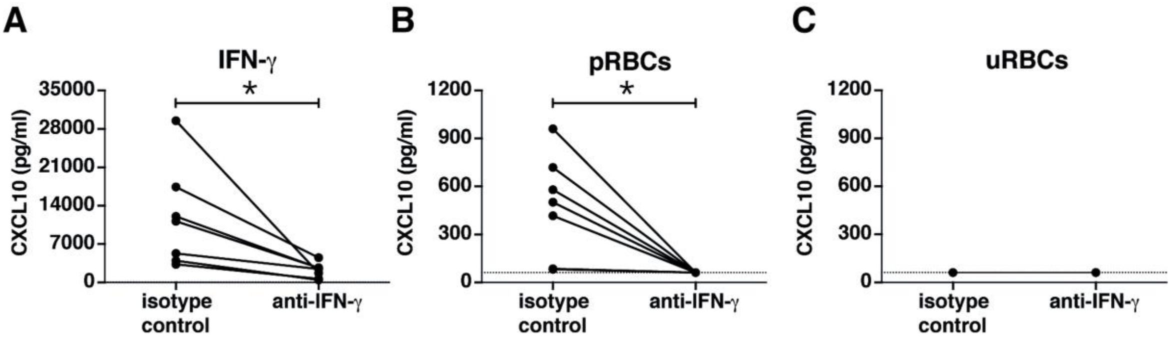

Figure 2. CXCL10 production in response to *P. falciparum* is IFN-γ-dependent. PBMCs were stimulated with IFN-γ (**A**), pRBCs (**B**) or uRBCs (**C**), or for 24 hours in the presence of 20 μg/mL anti-IFN-γ or isotype control antibodies. Cell culture supernatants were then collected and CXCL10 levels were determined by ELISA. Lines connect the response of individuals to each stimulus in the presence of anti-IFN-γ or isotype control antibodies. Dotted line represents the detection limit of the ELISA. *p<0.05 Wilcoxon matched-pairs signed rank tests.