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ORIGINAL ARTICLE

Increased amphiregulin expression by CD4$^+$ T cells from individuals with asymptomatic *Leishmania donovani* infection

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Abstract

Objectives. There is an urgent need to be able to identify individuals with asymptomatic *Leishmania donovani* infection, so their risk of progressing to VL and transmitting parasites can be managed. This study examined transcriptional markers expressed by CD4$^+$ T cells that could distinguish asymptomatic individuals from endemic controls and visceral leishmaniasis (VL) patients. **Methods.** CD4$^+$ T cells were isolated from individuals with asymptomatic *L. donovani* infection, endemic controls and VL patients. RNA was extracted and RNAseq employed to identify differentially expressed genes. The expression of one gene and its protein product during asymptomatic infection were evaluated. **Results.** Amphiregulin (AREG) was identified as a distinguishing gene product in CD4$^+$ T cells from individuals with asymptomatic *L. donovani* infection, compared to VL patients and healthy endemic control individuals. AREG levels in plasma and antigen-stimulated whole-blood assay cell culture supernatants were significantly elevated in asymptomatic individuals, compared to endemic controls and VL patients. Regulatory T (Treg) cells were identified as an important source of AREG amongst CD4$^+$ T-cell subsets in asymptomatic individuals. **Conclusion.** Increased Treg cell *AREG* expression was identified in individuals with asymptomatic *L. donovani* infection, suggesting the presence of an ongoing inflammatory response in these individuals required for controlling infection and that AREG may play an important role in preventing inflammation-induced tissue damage and subsequent disease in asymptomatic individuals.

Keywords: amphiregulin, CD4+ T cell, *Leishmania donovani*, regulatory T cell, visceral leishmaniasis

INTRODUCTION

The visceral leishmaniasis (VL) elimination initiative in India aims to reduce the incidence of disease to below 1 per 10 000 population per year in VL endemic areas by 2023. One of the major challenges for VL elimination is understanding the risk of disease outbreaks when a significant proportion of healthy individuals living in endemic areas with no history of VL harbour parasites, but remain asymptomatic.[1,2] The ratio of incident asymptomatic infections with *Leishmania donovani* or *L. infantum* (also known as *L. chagasi* in South America) to incident clinical cases varies from 1:2.4 in Sudan, 4:1 in Kenya, 5.6:1 in Ethiopia, 18:1 in Brazil, 50:1 in Spain, 4:1 in Bangladesh and 8.9:1 in India and Nepal.[2–9] These data indicate that the majority of individuals infected with *Leishmania* species develop an effective immune response to contain parasite growth and prevent progression to clinical disease. The role of these asymptomatic individuals in parasite transmission is not well established, but recent evidence indicates they are not a major source of parasite uptake by feeding female sand flies.[10] However, given the important role of acquired immunity, and in particular CD4+ T-cell responses for controlling parasite growth, changes to the immune status of individuals with asymptomatic infection due to other infections such as human immunodeficiency virus (HIV) or immunosuppressive treatments like steroids can result in progression to VL, and consequently, increased risk of parasite transmission. Therefore, there is a need to be able to identify asymptomatic individuals, so their risk of immune suppression can be managed to reduce the chances of progressing to VL.

The identification of individuals asymptomatically infected with *L. donovani* is difficult. The precise immune mechanisms underlying protection from VL are still not completely understood in humans.[11] An additional problem is the absence of validated markers for asymptomatic *L. donovani* infection. Most diagnostic assays are based on anti-parasite antibodies, with limitations in reliably detecting clinical disease in endemic settings. Therefore, a better test and/or set of biomarkers are needed to make the distinction between acute disease and asymptomatic infection so that individuals in the latter category can be more effectively managed to reduce their risk of progressing to clinical disease.[12]

The immune mechanisms responsible for controlling *L. donovani* growth in asymptomatic individuals are largely unknown. However, CD4+ T cells are likely to play an important role, as indicated by progression of these individuals to VL following HIV infection[13] or immunosuppressive treatments targeting CD4+ T-cell functions.[14,15] Tbet+ IFNγ-producing CD4+ T (Th1) cells are critical for activating the anti-microbial machinery in macrophages, the host cell of *L. donovani*, by inducing the generation of nitric oxide (NO) and reactive oxygen species (ROS) that kill intracellular parasites.[16] There is a marked increase in Th1 cells in some, but not all asymptomatic individuals from Ethiopia,[17] and CD4+ T cells produce IL-2, IL-5 and IFNγ in response to stimulation with *Leishmania* antigens.[18] CD4+ T cells from asymptomatic individuals also produce significantly less IL-10, compared to CD4+ T cells from VL patients.[19] Furthermore, CD4+ T cells from asymptomatic individuals exhibit enhanced antigen-specific proliferation, as well as production of cytotoxic molecules, including granzyme B and perforin.[20] Hence, there is a growing body of evidence that functional capacity, and therefore transcriptional activity, of CD4+ T cells from asymptomatic individuals is different from the same cells from VL patients.

In this study, we examined the transcriptional profiles of CD4+ T cells from the blood of asymptomatic individuals, endemic controls and VL patients. We identified a number of differentially expressed genes (DEGs) that could identify CD4+ T cells from asymptomatic individuals. We also noted different distributions of CD4+ T-cell subsets between asymptomatic individuals, endemic controls and VL patients, and found AREG expression was strongly associated with regulatory T (Treg) cells in the former group. Finally, we measured AREG in plasma and an antigen-specific whole-blood assay in asymptomatic individuals infected with *L. donovani*.

RESULTS

Identifying CD4$^+$ T-cell molecules that distinguish individuals asymptomatically infected with *Leishmania donovani* from endemic controls and visceral leishmaniasis patients

CD4$^+$ T cells play critical roles in controlling pathogens such as *L. donovani* that invade and reside in phagocytic cells.[21] Given that the majority of people infected with this parasite are asymptomatic,[22,23] it is likely that CD4$^+$ T cells from asymptomatic individuals exhibit functional properties that distinguish them from uninfected people and those with active disease. As a first step towards understanding these differences, we isolated RNA from peripheral blood CD4$^+$ T cells from asymptomatic, *L. donovani*-infected individuals, endemic controls and VL patients and employed RNAseq to identify DEGs (Figure 1). Following correction for false discovery rate (FDR), 66 DEGs were identified in CD4$^+$ T cells from asymptomatic individuals compared to endemic controls with 21 and 45 of these being significantly up- and downregulated, respectively (Supplementary table 1; Figure 1). We previously reported on DEGs in CD4$^+$ T cells from VL patients and endemic controls,[24] and next compared DEGs in CD4$^+$ T cells from asymptomatic individuals compared to endemic controls and VL patients, and found 1219 DEGs with 732 and 487 of these significantly up- and downregulated, respectively (Supplementary table 2; Figure 1). Therefore, transcriptional differences in CD4$^+$ T cells from asymptomatic *L. donovani*-infected individuals, relative to CD4$^+$ T cells from endemic controls and VL patients could be readily detected.

CD4$^+$ T-cell AREG expression marks individuals asymptomatically infected with *Leishmania donovani*

The top upregulated DEG in CD4$^+$ T cells from asymptomatic individuals compared to the other groups was *AREG,* which encodes amphiregulin (AREG), a ligand for epidermal receptor growth factor receptor (EGFR) (Figure 1). Notable downregulated DEGs in CD4$^+$ T cells from asymptomatic individuals compared to endemic controls and VL patients were *LAG3* and *IFNG* (Figure 1), both with important functional roles in VL.[25,26] The search tool for the retrieval of interacting genes and proteins (STRING): Interaction network analysis was employed to predict protein–protein interactions in DEGs associated with asymptomatic individuals (Supplementary figures 1 and 2). This revealed surprisingly few connected networks, with the exception of a tight network of downregulated DEGs involving *IFNG*, *LAG3*, *HAVCR2*, *CD38* and *CD83*, all molecules, which were previously shown to be significantly upregulated in CD4$^+$ T cells from VL patients compared to endemic controls.[26,27] Thus, the increased expression of *AREG* by CD4$^+$ T cells from asymptomatic individuals was accompanied by reduced expression of genes known to be expressed during active disease.

AREG expression is increased on CD4$^+$ T-cell subsets from asymptomatic individuals

We next performed a validation study to confirm increased expression of *AREG* by CD4$^+$ T cells from asymptomatic individuals. We also assessed *AREG* expression by all peripheral blood mononuclear cells (PBMCs) and found significantly increased *AREG* expression by PBMCs from asymptomatic individuals, compared to endemic controls, but not VL patients (Figure 2a). Consistent with RNAseq data above, we measured significantly increased expression of *AREG* by CD4$^+$ T cells from asymptomatic individuals, compared to both endemic controls and VL patients (Figure 2b). Thus, *AREG* expression by PBMC could distinguish asymptomatic individuals from endemic controls, but not VL patients, while expression of *AREG* by CD4$^+$ T cells could distinguish asymptomatic individuals from both groups.

We next assessed the pattern of AREG expression by peripheral blood CD4$^+$ T-cell subsets using flow cytometry to determine whether changes in CD4$^+$ T-cell subset distribution and/or AREG expression associate with increased expression of *AREG* by CD4$^+$ T cells from asymptomatic individuals. We first divided CD4$^+$ T cells into regulatory T (Treg) cells and non-Treg (conventional) cells (Figure 3a). We further divided CD4$^+$ T cells into Th1, Tfh and Th9 cells (Figure 3b), due to their previous associations with AREG expression,[28] as well as central memory (TCM; CD45RA$^-$ CCR7$^+$) and effector memory (TEM; CD45RA$^{-/+}$ CCR7$^-$) cells. The most noticeable differences in CD4$^+$ T-cell subset frequencies were between asymptomatic individuals and endemic controls and/or VL patients, including decreased

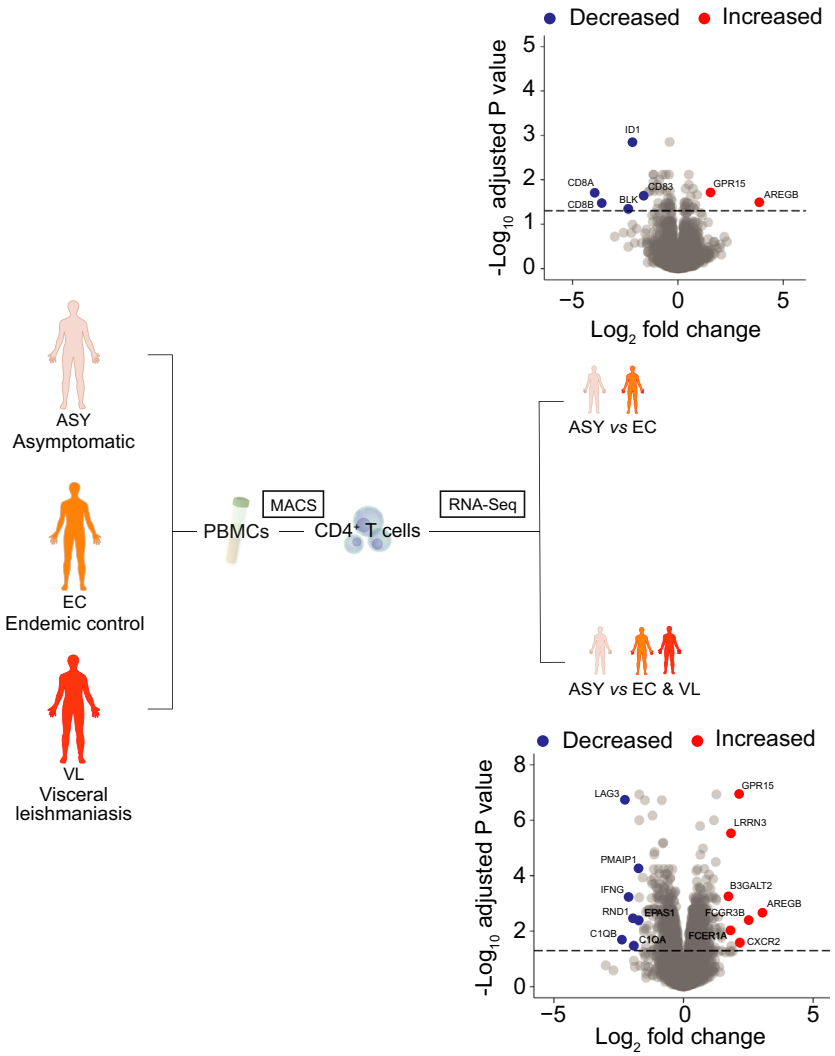

Figure 1. Defining a transcriptomic signature of peripheral blood CD4+ T cells from asymptomatic (ASY) individuals infected with *Leishmania donovani*. A schematic showing the approach taken to identify transcriptomic signatures of peripheral blood CD4+ T cells from ASY individuals, endemic controls (EC) and visceral leishmaniasis (VL) patients (n = 12 biological replicates in each group). A volcano plot of the differentially expressed genes identified in the comparison of asymptomatic versus endemic controls (upper plot) and ASY versus the average of VL patients and EC. Genes are coloured red (upregulated) or blue (downregulated) according to a false discovery rate < 0.05. Gene labels are shown for selected genes.

frequencies of naïve and TCM CD4+ T cells and increased frequencies of TEM, Tfh, Th1 and Treg cells (Figure 3c). Hence, asymptomatic *L. donovani* infection is characterised by measurable changes in peripheral blood CD4+ T-cell subset distribution.

Amphiregulin expression by CD4+ T-cell subsets from asymptomatic individuals, endemic controls and VL patients was next assessed (Figure 4a and Supplementary figure 3a). Increased AREG expression was found when all CD4+ T cells were analysed together, as well as on naïve CD4+ T cells, TCM and Treg cells from asymptomatic individuals, although the difference in AREG expression on naïve CD4+ T cells and TCM cells was only observed with VL patients and not endemic controls. Simplified presentation of incredibly complex evaluations (SPICE) was used to establish overlap in expression of AREG, the CD38 activation marker and chemokine receptors used to identify Th cell subsets (Figure 4b–e and Supplementary figure 3b–e). Again, the increased expression of AREG on Treg cells from asymptomatic individuals was especially pronounced (Figure 4d), but no strong co-expression with other cell markers was observed. Given the increased frequency of Treg

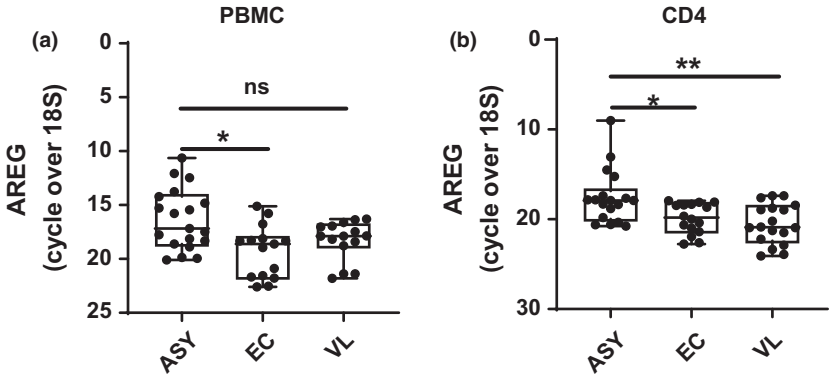

Figure 2. Amphiregulin (AREG) is elevated in CD4$^+$ T cells from asymptomatic individuals infected with *Leishmania donovani*. RNA was isolated from peripheral blood mononuclear cells (PBMCs) **(a)** and CD4$^+$ T cells **(b)** isolated from asymptomatic (ASY; $n = 19$) individuals, endemic controls (EC; $n = 15$) and visceral leishmaniasis (VL; $n = 15$) patients and subjected to qPCR to measure AREG mRNA levels. Median + minimum and maximum are shown; ns = not significant, $*P < 0.05$, $**P < 0.01$. Significance was assessed by the Kruskal–Wallis test.

cells and their expression of AREG in asymptomatic individuals, it is likely that Treg cells are an important source of the increased AREG expression associated with CD4$^+$ T cells during asymptomatic *L. donovani* infection.

Increased AREG in plasma and antigen-stimulated whole-blood assays from individuals with asymptomatic *Leishmania donovani* infection

Finally, we sought to determine whether AREG could be readily detected in asymptomatic individuals. We first measured AREG in plasma samples and found AREG levels were significantly elevated in asymptomatic individuals compared to endemic controls and VL patients (Figure 5a). Next, we employed a parasite antigen-driven whole blood assay[26–29] and measured significantly elevated levels of AREG in antigen-stimulated asymptomatic individuals blood samples, but not endemic control and VL patient samples (Figure 5b). The increased antigen-specific production of AREG correlated with increased IFNγ production in asymptomatic individuals to levels similar or even higher than found in VL patients (Figure 5c). Together, these results indicate that elevated AREG levels could be readily detected in plasma or induced in an antigen-specific whole-blood assay from individuals with asymptomatic *L. donovani* infection.

DISCUSSION

In this study, we identified a CD4$^+$ T-cell transcriptional signature for asymptomatic individuals infected with *L. donovani*. AREG was the most upregulated gene in these cells, while several genes with known roles in VL patients were found to be downregulated in asymptomatic individuals. Accompanying these transcriptional changes were alterations in the distribution of CD4$^+$ T-cell subsets, including increased frequencies of TEM, Tfh, Th1 and Treg cells in asymptomatic individuals compared to endemic controls and VL patients. However, of these CD4$^+$ T-cell subsets, Treg cells showed the largest difference in AREG expression between the three groups, indicating they were an important source of AREG in asymptomatic individuals. We also established that elevated AREG protein levels were readily detected in individuals with asymptomatic *L. donovani* infection.

Amphiregulin, which was first recognised as a Th2 cell cytokine, is a member of epidermal growth factor family. It interacts with EGFR to activate essential intracellular signalling cascades that govern cellular metabolism, inflammation and cell cycle progression.[30–32] It is secreted by various activated immune cells from the innate and adaptive arms of the immune system.[28] Immune-mediated resistance and tolerance mechanism are critical components of host immune responses. In this context, AREG has been identified as a key regulatory factor, which not only promotes host resistance to pathogens but also assists in tissue repair and wound healing under different inflammatory conditions.[28] Our findings support a similar conclusion, whereby elevated expression of AREG is associated with individuals able to control parasite growth without inducing an inflammatory response that

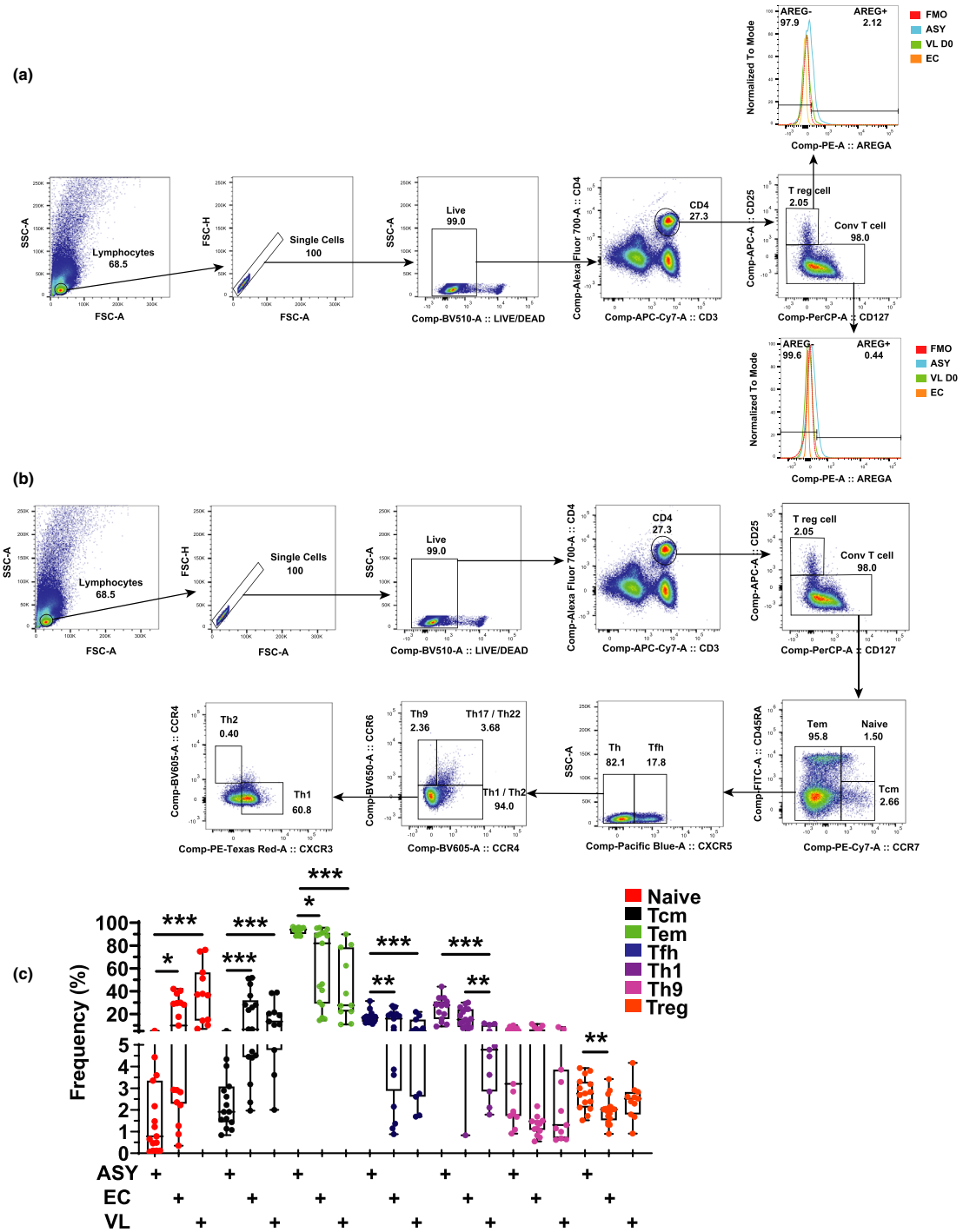

Figure 3. Changes in CD4+ T-cell subset frequencies and expression of AREG. CD4+ T cells were identified by CD3ε and CD4 expression prior to being divided into regulatory T (Treg) cells and conventional T cells, based on CD25 and CD127 expression, before assessing AREG expression **(a)**. CD4+ T cells were divided into T helper cell subsets based on chemokine receptor expression **(b)** and frequencies measured in peripheral blood from asymptomatic (ASY; $n = 15$) individuals, endemic controls (EC; $n = 17$) visceral leishmaniasis patients (VL; $n = 11$). The box shows the extent of the lower and upper quartiles plus the median, while the whiskers indicate the minimum and maximum data points **(c)**. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Significance was assessed by the Kruskal–Wallis test.

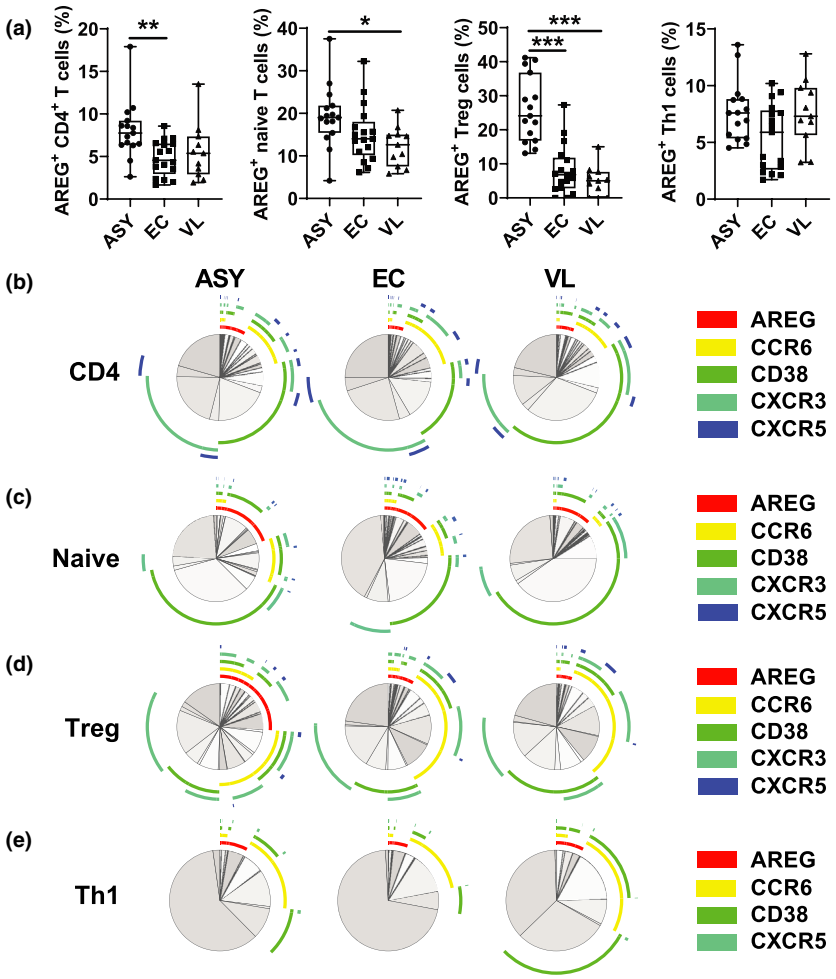

Figure 4. Amphiregulin (AREG) expression by CD4$^+$ T-cell subsets. The frequency of AREG$^+$ CD4$^+$ T-cell subsets was measured by FACS. The box shows the extent of the lower and upper quartiles plus the median, while the whiskers indicate the minimum and maximum data points **(a)**. A simplified presentation of incredibly complex evaluations (SPICE) was used to establish overlap in expression of AREG, CD38 and indicated chemokine receptors by all CD4$^+$ T cells **(b)**, naive CD4$^+$ T cells **(c)**, regulatory T (Treg) cells **(d)** and Th1 cells **(e)** from asymptomatic (ASY; $n = 15$) individuals, endemic controls (EC; $n = 17$) and visceral leishmaniasis (V; $n = 11$) patients, as indicated. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Significance was assessed by a one-way ANOVA with a Tukey's multiple comparisons test **(a)**.

causes disease. Although *L. donovani* infection stimulates a potent Th1 cell response,[16] similar roles for AREG balancing pathogen-specific immune responses and pathology have been reported in broad range of infections. For example, in C57BL/6 mice infected with *Trichuris muris*, AREG provided resistance against this parasite by inducing the proliferation of gut epithelia and facilitated its clearance.[33] Similarly in mice infected with *N. brasiliensis,* AREG promoted tissue repair after *N. brasiliensis*-induced lung injury.[34] In RAG$^{-/-}$ mice infected with influenza virus, AREG not only promoted the regeneration of bronchial epithelium but also enhanced tissue integrity and survival of infected mice.[35] Also, in mice infected with influenza virus and bacterial coinfection, exogenous AREG administration promoted tissue repair and improved survival in co-infected animals without affecting pathogen burden.[36] In another study, *C. albicans* infected, dectin-1 deficient mice had severe damage to pulmonary epithelium compared to wild-type mice despite similar pathogen burden, and treatment with recombinant AREG not only promoted epithelial repair but also increased survival of the dectin-1-deficient mice.[37] The role of AREG in tissue repair appears to be an important aspect of its functions during infection, supported by the observation that mice lacking functional AREG have reduced

intestinal regeneration capacity compared to wild-type mice following radiation-induced injury.[38] Together, these data suggest that AREG is an important factor that promotes host tolerance by sustaining tissue integrity and homeostasis following infection and inflammation. Thus, it is possible that the pro-inflammatory environment that develops to control parasite growth in asymptomatic individuals infected with *L. donovani* is finely balanced to prevent parasite expansion, but also avoid tissue damage, and AREG may be an important component of this process.

$CD4^+$ T cells play an important role in controlling *Leishmania* parasite replication.[26] Changes in the frequency of $CD4^+$ T cells and their subsets in VL patients before and after treatment, compared to endemic controls and individuals with asymptomatic infection, have been previously reported.[39,40] Here, we provide data showing alterations in $CD4^+$ T-cell subset composition in asymptomatic individuals on a larger scale. Decreased frequencies of naïve and TCM $CD4^+$ T cells and increased frequencies of TEM, Tfh and Th1 cells were observed in the peripheral blood of asymptomatic individuals, compared to VL patients and endemic controls. Naïve and TCM cells re-circulate through secondary lymphoid organs, while TEM cells are mostly located within non-lymphoid tissues or remain in circulation and rapidly produce anti-parasitic effector molecules upon antigen encounter.[41,42] Thus, the above-mentioned increase in the frequency of TEM $CD4^+$ T cells in the peripheral blood of asymptomatic individuals, relative to other groups, suggest it is likely that these $CD4^+$ T cells were actively engaged in anti-parasitic defence.

Contrary to the findings in VL patients where PBMC Treg cell frequencies were unchanged, compared to endemic controls,[40] we found increased Treg cell frequencies in the blood of asymptomatic individuals compared to endemic controls and/or VL patients (Figure 3c). Tregs cells, which are critical for mediating peripheral tolerance under different inflammatory conditions, also secrete AREG. Accumulation of a transcriptionally distinct Treg cell population overexpressing AREG with enhanced muscle regeneration capacity has been reported in mouse model of acute skeletal muscles injury.[30] AREG expressed by muscle-resident Treg cells has been shown to improve muscle repair *in vivo* and also act directly on muscle satellite cells *in vitro* to mediate tissue repair.[30,36] The accumulation of AREG-producing Treg cells has also been reported in the brains of mice after ischaemic stroke, which suppressed neurotoxic astrogliosis and provided neuronal protection.[43] Since Treg cells are critical in preventing immune dysregulation and we observed increased Treg cell numbers, as well as increased expression of AREG by Treg cells from asymptomatic individuals, it is possible that increased AREG production by Treg cells is a response to prevent tissue damage caused by anti-parasitic control mechanisms during asymptomatic *L. donovani* infection. However, it is not clear whether activation of Treg cells in these individuals is mediated by *L. donovani* infection or other co-infections prevalent in the VL endemic region under investigation, and this will require further investigation. Because helminth infections are common in the resource-poor settings of VL endemic areas, it will be important to establish their impact on AREG expression by Treg cells to help resolve this issue.[44] Individuals infected with helminths in endemic areas have a polarised immune responses,[45] and along with the genetic makeup of individuals, co-infection with *L. donovani* is likely to have a significant impact on progression to active disease.

Amphiregulin has previously been identified as a biomarker for several types of cancer,[46–51] as well as in other inflammatory conditions such as rheumatoid arthritis,[52] asthma,[53] intestinal acute GVHD[54] and cholesteatoma.[55] It has also been identified as a predictive biomarker for resistance or sensitivity to anti-EGFR mAb therapy in various cancers. The elevated AREG plasma levels in asymptomatic individuals, compared to those with active diseases and endemic controls, and enhanced AREG production upon *Leishmania* antigen stimulation in whole-blood assays support a potential role for AREG as a biomarker for asymptomatic *L. donovani* infection. However, based on the relative variability of responses between individuals and dynamic range of responses detected in both assays, it is likely that AREG would have to be combined with additional biomarkers to maximise sensitivity and accuracy for a diagnostic assay. This approach has been successful in other infectious diseases, such as leprosy.[56,57]

In conclusion, we report increased AREG expression associated with increased frequency of Treg cells in seropositive asymptomatic individuals living in a VL endemic area of India. We also

observed increased AREG plasma levels, as well as increased secretion of antigen-specific AREG in the whole blood of these individuals. Taken together, these findings identify AREG as an immunological determinant for asymptomatic *L. donovani* infection.

METHODS

Human subjects and ethical clearance

Blood samples were collected from (n = 46) asymptomatic, (n = 48) symptomatic VL patients and (n = 59) endemic control individuals at the Kala-azar Medical Research Center (Muzaffarpur, Bihar, India). Active VL cases were confirmed based on clinical signs, including fever (> 2 weeks), splenomegaly, positive serology for rK39 rapid diagnostic test and by microscopic demonstration of *Leishmania* amastigotes in splenic or bone marrow aspirate smears. Clinical data from these patients are summarised in Table 1. Asymptomatic healthy subjects living in endemic regions were tested for anti-leishmania antibodies by direct agglutination test (DAT) and rK39 ELISA. A positive and negative control (filter paper pooled eluates from VL patients and non-endemic healthy controls, NEHC) were run in each rK39-ELISA, and the positive control was used as a reference to calculate a relative value of positivity of each sample, expressed as percentage positivity (PP). In the first sero-survey, subjects positive by both DAT and rK39-ELISA (≥ 1:1600 and ≥ 14PP, respectively), and highly seropositive by one or both assays (≥ 1:25 600 and ≥ 23 PP) and who met the inclusion criteria, were invited to KAMRC within 14 days of identification. All asymptomatic subjects were monitored monthly for 24 months after enrolment in the study to observe any development of active VL. This work was conducted with ethical approval (No. Dean/2017/EC/185 dated 24/10/2017) obtained from Institutional Review Committees of Banaras Hindu University, Varanasi, India. Each study patient was informed both verbally and in writing (in English and Hindi) about the nature of the study, the anticipated risks and benefits, the discomforts to which the patient will be exposed, and their right to discontinue participation at any time of their own free will. Written informed consent was obtained from all participants, and where participants were below 18 years of age, written informed consent was obtained from their legal guardian. We excluded pregnant women or lactating mothers, subjects having a vaccination within 30 days, and hepatitis B or C positive subjects. All subjects were HIV-negative and above 12 years of age.

Table 1. Demographic and clinical information on study participants

Variables	VL Group (n = 48)	ASY Group (n = 47)	EC Group (n = 59)
Age, years			
Mean ± SD	27.94 ± 14.54	36.26 ± 16.10	34.78 ± 10.29
Median	24.5	38	33
Sex, no.			
Male	31	18	32
Female	17	29	27
Illness duration, days			
Mean ± SD	26.65 ± 15.23	NA	NA
Median	28	NA	NA
Haemoglobin level, mg mL^{-1}			
Before treatment			
Mean ± SD	9.2 ± 1.60	ND	ND
Median	9.3	ND	ND
WBC, $\times 10^3$ cells mm^{-3}			
D-0			
Mean ± SD	4854.83 ± 3676.85	ND	ND
Median	4150	ND	ND
Splenic enlargement, cm			
D-0			
Mean ± SD	4.08 ± 2.88	NA	NA
Median	3	NA	NA

ND, Assay not done.

Direct agglutination test

Direct agglutination test (DAT) was performed using finger prick blood collected on Whatman filter paper. Briefly, 100 μL of blood filter paper eluate (1:400 dilutions) was serially diluted up to 1:51 200 with 50 μL DAT buffer in a V-shaped microtiter plate with one positive and one negative control. A volume of 50 μL of DAT antigen (Institute for Tropical Medicine, Belgium) was then dispensed to every well, and plates were incubated overnight at room temperature for agglutination. The DAT results were observed against a white background. Samples with a titre of 1:1600 or above were considered DAT seropositive, and asymptomatic individuals with a DAT titre ≥1:3200 were enrolled in the study.

rK39 Enzyme-Linked Immunosorbent Assay

High binding flat-bottom 96-well Nunc ELISA plates (Thermo Fisher Scientific, USA) were coated with rK39 (25 ng per well) overnight at 4°C. Plates were blocked with PBS containing 1% (w/v) bovine serum albumin (BSA) (VWR, Life Science, USA) for 2 h at room temperature. A volume of 100 μL of eluted blood from 5-mm Whatman filter paper was added and incubated for 1 h. The wells were washed four times with PBS-Tween (PBS-T) and incubated for 30 min with protein A-horseradish peroxidase (1:12 000 dilution; Bangalore Genei, India) in PBS containing 0.1% BSA and 1.0% (v/v) Tween-20. Plates were washed four times in PBS-T and incubated with 100 μL tetramethylbenzidine (TMB) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) substrate for a further 15 min. The reaction was stopped by addition of 1N H_2SO_4, and optical density (OD) measurements were undertaken at 450 nm using a microtiter plate ELISA reader (Molecular Devices, San Jose, CA, USA). Samples having ≥ 14PP were considered positive, and individuals with ≥ 20 PP were enrolled in the study.

Human PBMC isolation and RNA extraction

Blood was collected from asymptomatic individuals, VL patients and ECs into 15-mL Falcon tubes (BD Biosciences)

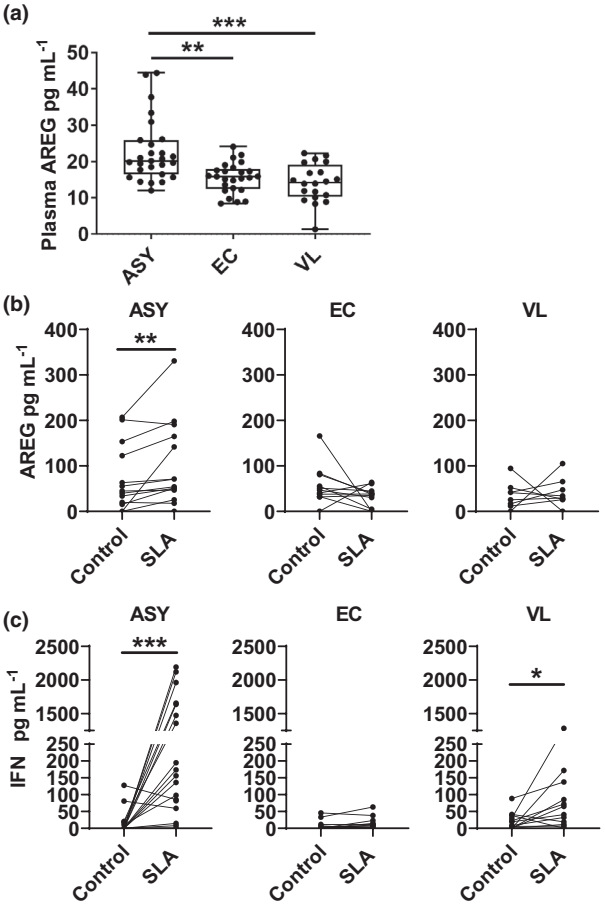

Figure 5. Amphiregulin (AREG) is a marker of asymptomatic *L. donovani* infection. AREG was measured in plasma from (ASY; $n = 27$) individuals, endemic controls (EC; $n = 25$) and visceral leishmaniasis (VL; $n = 20$) patients **(a)**, as well as from supernatants from whole blood assays stimulated with soluble Leishmania antigen (SLA) or media alone (control), as indicated, from ASY ($n = 15$), EC ($n = 12$) and VL ($n = 12$) **(b)**. IFNγ was measured from the same WBA cell culture supernatants **(c)**. The box shows the extent of the lower and upper quartiles plus the median, while the whiskers indicate the minimum and maximum data points **(a)**. Paired samples are shown in **b** and **c**; $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$. Significance was assessed by the Kruskal–Wallis test **(a)** or the Wilcoxon matched-pairs signed rank test **(b**, **c)**.

containing 150 U heparin. Blood was layered over Lymphoprep (StemCell Technologies, Vancouver, Canada) to isolate PBMCs. CD4+ T cells were isolated from PBMCs by magnetic-activated cell sorting (MACS) using MS Columns and anti-human CD4 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and stored in RLT buffer (Qiagen, cat #79216) at −80°C till further use. Cells were homogenised using the QIAshredder and RNA isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA digestion was performed using either the RNAse-free DNase set or DNase Max kit (QIAGEN). RNA was quantified using the Qubit RNA HS Assay kit on a Qubit 4 Fluorometer (Thermo Fisher, Waltham, MA, USA). The quality of RNA was determined using the RNA 6000 Nano kit, run on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions where an RNA Integrity Number (RIN) value above 8 was optimal.

RNA-sequencing

Libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit, High Throughput (Illumina, San Diego, CA, USA) with Superscript III Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA) and Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Libraries were quantified using the Qubit DNA HS Assay kit (Thermo Fisher), and quality was assessed using the DNA 100 kit (Agilent Technologies), run on a 2100 Bioanalyzer. 75-bp, paired-end RNA-sequencing was performed on the NextSeq 550 using the NextSeq 500/550 High Output Kit v2 (150 cycles) (Illumina). Each flow cell contained 12 libraries.

Differential gene expression and pathway analysis

Cutadapt[58] (version 1.11) was used for trimming adapter sequences from fastq files. STAR[59] (version 2.5.2a) was used for sequence alignment to the GRCh37 assembly with the gene, transcript, and exon features of Ensembl (release 89) gene model. RNA-SeQC[60] (version 1.1.8) was used to compute quality control metrics, and RSEM[61] (version 1.2.30) was used to quantify reads. Normalisation and differential gene expression analysis were performed using the edgeR package,[62] and pathways analysis was performed using ingenuity pathway analysis (IPA; winter 2018 release; QIAGEN).

RT-qPCR

Peripheral blood mononuclear cells were isolated from blood layered over Lymphoprep, and CD4+ T cells were enriched by MACS from PBMCs using the anti-human CD4 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. RNA was then extracted and reverse transcribed to cDNA (both PBMC and CD4+ T cells). RT-qPCR for amphiregulin was performed on an ABI Prism® 7500 real-time PCR system (Applied Biosystems, Waltham, MA, USA) using the TaqMan Gene Expression Assay (Assay ID: Hs00950669_m1; Applied Biosystems). Relative quantification was performed using the comparative CT method relative to 18S ribosomal RNA (rRNA) (Assay ID: Hs99999901_s1; Applied Biosystems).

Preparation of soluble *Leishmania* antigen

Soluble Leishmania antigen (SLA) was prepared as previously described.[63] Briefly, *L. donovani* amastigotes from clinical isolates (Kala-azar Medical Research Center, Muzaffarpur, Bihar, India) were grown in Medium 199, Hanks' Balanced Salts (M199; Thermo Fisher) until transformed into promastigotes, then cultured. $2–3 \times 10^9$ stationary-phase promastigotes were harvested from culture and centrifuged at 3900 *g* for 20 min to obtain a parasite pellet, which was

washed three times with cold 1 x PBS and resuspended in solution (10 mM Trizma hydrochloride solution (TRIS-HCl; Sigma-Aldrich), 1 mM pH 8.0 ethylenediaminetetraacetic acid (EDTA; Amresco, ELITechGroup, Puteaux, France), 1.6 mM phenylmethanesulphonyl fluoride (PMSF; HiMedia, Mumbai, India) and 50 mg mL^{-1} N-acetyl-L-leucyl-Lleucyl- L-argininal (leupeptin; Amresco)) at a concentration of 2–3 $\times$ 10^9 parasites m^{-1}. The parasite suspension was sonicated 4–5 times for 15 s at 10 Hz and centrifuged at 2000 *g* for 30 min at 4°C. The lipid layer was removed from the surface of the supernatant, and the remaining solution was ultracentrifuged at 100 000 *g* for 4 h at 4°C. The supernatant was removed, dialysed against the PBS overnight and stored at –80°C until used. Protein concentration was measured using a Micro BCA Protein Assay Kit (Thermo Fisher), as per the manufacturer's instructions.

Ex vivo whole-blood assay

Heparinised blood was collected from asymptomatic individuals, active VL patients and endemic controls. To remove background plasma cytokine readings, the plasma was removed and the whole blood was washed once with 1 $\times$ PBS. The autologous plasma was then replaced with an equal volume of HI-FBS. The blood was divided equally in 4 polypropylene culture tubes (BD Biosciences; cat #352063) containing 1 mL in each tube. Whole-blood cells were cultured in the absence or in presence of SLA. A non-stimulated group (PBS was used as placebo) was included as a negative control and the levels of IFNγ /AREG detected were subtracted from corresponding antigen-stimulated samples. Whole-blood cultures will be kept at 37°C and 5% (v/v) CO_2 for 24 h, and then, supernatants were collected. AREG levels were measured using a human amphiregulin ELISA kit (Thermo Scientific; Cat# EHAREG), and IFNγ levels were measured using an ELISA kit (BioLegend, San Diego, CA, USA; Cat# 430104) in supernatant according to the manufacturer's instructions.

Flow cytometry

FACS staining was performed for the phenotypic characterisation of amphiregulin present on CD4$^+$ T cells from different subject groups; asymptomatic individuals (n = 15), VL patients (n = 11) and endemic controls (n = 17). CD4$^+$ T cells and their different subsets were measured by expression of different chemokines/surface markers. Fixable Zombie Aqua™ fixable viability kit (BioLegend; cat #423102) was used to exclude dead cells from the analysis. Briefly, for each sample, 1 $\times$ 10^6 PBMC were dispensed in BD-polystyrene round-bottom FACS staining tubes (Corning, Mexico; cat# 352054). Thereafter, cells were incubated in the dark at room temperature for 30 min in 100 μL 1 $\times$ PBS with Zombie Aqua™ dye (Detected on BV-510) for dead cell staining. A volume of 2 mL FACS staining buffer (5% (v/v) FCS in 1 X PBS) was added to each tube and washed twice. Surface staining was performed in FACS staining buffer with antibodies against CD3ε APC-Cy7 (BD Biosciences; cat #557832), CD4 AF®-700 (BD Biosciences cat #557922), CD45RA FITC (BD Biosciences cat #555488), CD25 APC (BD Biosciences cat #340939), CD127 PerCP-Cy5.5 (BD Biosciences cat #8560551), CD197 PE-Cy7 (BD Biosciences cat #557648), CD194 BV-605 (BD Biosciences cat #359418), CD196 BV-650 (BD Biosciences cat #563922), CD185 BV-421 (BD Biosciences cat #562747), CD183 PE-CF594 (BD Biosciences cat #562451), CD38 BV-711 (BD Biosciences cat #563965) and human amphiregulin PE (Invitrogen, cat# 12-5370-42) for 30 min in the dark. Finally, stained cells were washed twice with 2-mL FACS staining buffer and resuspended in 300 μL of FACS staining buffer. Freshly stained cells were acquired by Flow cytometer (BD LSRFortessa flow cytometer, BD Biosciences) using FACS Diva software. FACS data were analysed by FlowJo version 10 software (Tree Star (BD)).

Statistical analysis

Statistical analysis was performed exclusively in GraphPad Prism 7.02 (GraphPad Software, La Jolla, CA, USA). Analysis of human qPCR and cellular assays was performed using a one-way ANOVA, with either a Kruskal–Wallis or a Tukey's multiple comparisons test, as appropriate, to assess more than 2 groups within an experiment. A Wilcoxon signed-rank test was used to compare matched-pairs. SPICE analysis was performed using SPICE version 5.3 (M. Roeder, Vaccine Research Centre, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, USA; http://exon.niaid.nih.gov).[64] $P < 0.05$ was considered significant. Graphs depict Box and Whisker plots showing minimum and maximum values or connecting lines when data were paired.

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AUTHOR CONTRIBUTIONS

Siddharth Sankar Singh: Data curation; Formal analysis; Investigation; Writing – review & editing. **Shashi Bhushan Chauhan:** Data curation; Formal analysis; Investigation; Methodology. **Susanna SS Ng:** Data curation; Formal analysis; Investigation; Methodology; Software; Writing – review & editing. **Dillon Corvino:** Formal analysis; Investigation; Methodology; Software; Visualization; Writing – review & editing. **Fabian de Labastida Rivera:** Formal analysis; Investigation; Methodology; Project administration. **Jessica A Engel:** Formal analysis; Investigation; Methodology. **Nic Waddell:** Supervision; Data curation; Formal analysis. **Pamela Mukhopadhay:** Data

curation; Investigation; Methodology; Software. **Rebecca L Johnston:** Data curation; Formal analysis. **Lambros T Koufariotis:** Data curation; Software. **Susanne Nylen:** Funding acquisition; Investigation; Project administration; Writing – review & editing. **Om Prakesh Singh:** Investigation; Project administration. **Christian Engwerda:** Conceptualization; Investigation; Funding Acquisition; supervision; Writing– review & editing. **Rajiv Kumar:** Conceptualization; Investigation; Funding Acquisition; supervision; Writing– review & editing. **Shyam Sundar:** Conceptualization; Funding acquisition; Supervision.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.