Lower expression of GATA3 and T-bet correlates with downregulated IL-10 in severe falciparum malaria

Anusree Mahanta and Shashi Baruah

Interleukin (IL)-10, a non-redundant anti-inflammatory cytokine is produced by different cells and its production involves activation of cell-specific transcriptional regulatory machinery in response to specific pathogen. We have previously demonstrated downregulated levels of IL-10 in severe falciparum malaria. The present study investigated transcriptional regulation of IL-10 in severe malaria. Comparative expression analysis of cell-specific signalling proteins and transcription factors for IL-10 production during the stage of active infection and with resolution of parasitaemia was performed. Interestingly, T-bet and GATA3, the Th1 and Th2 transcription factors, respectively, were downregulated in severe malaria with fold change values of 0.59 and 0.86. Increase in the levels of both the factors with resolution of parasitaemia implicated a role for parasite in depressed levels of these factors. Further support for probable parasite manipulation of GATA3 was obtained from negative correlation of GATA3 with parasitaemia. In addition, a role for interferon-α in suppressing IL-10 transcription was evident from its negative correlation with GATA3 and IL-10 levels. In summary, IL-10 transcription in Th1 and Th2 is defective and appears to have major contribution to low levels in severe malaria.

Clinical & Translational Immunology (2015) 4, e49; doi:10.1038/cti.2015.30; published online 6 November 2015
of T-helper cell transcription factors T-bet and GATA3 was downregulated in all the SM patients. Strangely, in two of the SM patients, either IFN-α (MC 40) or IFN-β (MC 41) showed enhanced expression, whereas the other genes did not show notable change in expression levels. The clinical profiles of these two patients are given in Table 1. It was interesting to note that expression of either T-bet or GATA3 or both increased on day 3 with parasite clearance in SM individuals.

k-means clustering analysis of SM group using UM as the calibrator generated three clusters based on the similar patterns of expression profile from day 0 to day 3 (Figure 1). Cluster 2 with IFN-α exhibited a rapid decrease to normal levels by day 3. In contrast, T-bet and GATA3 in cluster 3 showed a pattern of reduced expression on day 0 followed by an increase to normal levels on day 3. Clustering analysis was also performed using healthy control as the calibrator (Figure 2). TRAF3, NF-κB, IFN-α and IFN-β, which are grouped under cluster 1, exhibited high expression on day 0 followed by a steep decrease. Again, cluster 2 with T-bet, GATA3, CIITA and ETS-1 was characterised by increased expression on day 3 compared with day 0. In UM, all the three clusters exhibited decrease of expression by day 3 (Figure 2). Collectively, the results obtained hints at the possible involvement of T-cell suppression in downregulated IL-10 levels of SM group. Thus, it was important to perform further comparative analysis of NF-κB, TRAF3, IFN-α, IFN-β, CIITA, ETS-1, T-bet and GATA3 between SM and UM.

Increased expression of TRAF3, IFN-α and decreased expression of T-bet, GATA-3 in SM

The expression of TRAF3 and IFN-α in SM on day 0 was elevated with fold change levels of 2.15 ($P=0.0002$) and 3.11 ($P=0.0005$), respectively, compared with UM (Figure 3). Temporal expression analysis showed steep decrease in levels of both TRAF3 ($P=0.0002$) and IFN-α ($P=0.0002$) with parasite clearance post treatment in SM. Of note, there was decreased expression of T-bet and GATA3 on day 0.

**Table 1** Clinical data and relative mRNA expression of IL-10, IL-1β and TNF-α of two SM samples that showed different expression profile of the IL-10 transcriptional regulatory factors than the other patients

| Clinical data and cytokine expression | MC 40 | MC 41 |
|-------------------------------------|-------|-------|
| Nausea                              | Yes   | Yes   |
| Vomiting                            | Yes   | Yes   |
| Jaundice                            | Yes   | No    |
| Temperature (Fahrenheit)            | 101   | 99    |
| Hb (g dl⁻¹)                         | 7     | 7.4   |
| Red colour of urine                 | Yes   | No    |
| Pulse per min                       | 90    | 90    |
| Respiration per min                 | 18    | 16    |
| BP (mm Hg)                          | 120/70| 110/70|
| Glasgow coma scale                  | 8     | 15    |
| Blood urea (mg dl⁻¹)                | 60    | 36    |
| Serum creatinine (mg dl⁻¹)          | 2.3   | 1.1   |
| Serum sodium (mmol l⁻¹)             | 135   | 130.1 |
| Serum potassium (mmol l⁻¹)          | 3.2   | 3.22  |
| Fold change of IL-10                | 0.000179 | 1.002209 |
| Fold change of IL-1β                | 0.000163 | 1.740216 |
| Fold change of TNF-α                | 2.234853 | 3.224446 |

Correlation between proinflammatory cytokines, parasitaemia and transcriptional regulatory factors

The expression pattern of IL-1β, TNF-α and IL-10 between SM and UM obtained by the SYBR Green method (Supplementary Figure 3) was similar to that obtained by TaqMan assays performed previously. In both SM and UM, IL-10 levels positively correlated with GATA3 while there was also a weak positive correlation with T-bet in SM (Figures 4a and f). An inverse correlation was found between IL-10 and IFN-α as well as between IFN-α and GATA3 in SM (Figures 4a and b). Further, a moderate positive correlation was also observed between TRAF3 and IFN-α in SM. Significantly, NF-κB showed a

(P=0.01 and $P<0.0001$) during the stage of active infection in SM followed by an increase on day 3 ($P=0.04$ for both the genes). The longitudinal expression profile of NF-κB, IFN-β, CIITA and ETS-1 was found to be comparable between the two disease groups.
weak positive association with proinflammatory cytokine TNF-α in SM suggesting the role of macrophages as inflammatory cells. Again, an exaggerated type I IFN response via TRAF3 induction could be triggered by parasite factors as suggested by the correlation pattern in Figure 4d. Interestingly, GATA3 expression seemed to decrease with increasing parasitaemia further supporting the role of decreased Th2 response in active infection (Figure 4e). However, T-bet did not exhibit any significant correlation with parasite density.

Altered expression of SOCS3 in SM

Comparative analysis revealed reduced expression levels of SOCS3 (Figure 5) in SM on day 0 ($P=0.0007$) compared with UM followed by an increase on day 3 ($P=0.02$). As expected, the temporal expression pattern was similar to that of IL-10 determined in our previous study. Notably, SOCS3 expression is reported to be directly regulated by IL-10.

**DISCUSSION**

IL-10 has a non-redundant role in maintaining the delicate balance between inflammation and immunoregulation during an immune response against pathogens. The finding of association of decreased IL-10 with severe malaria in our previous study prompted us to investigate the macrophage, Th1 and Th2 cell-specific transcriptional regulation of this cytokine between SM and UM. Initial clustering analysis revealed differential clustering of the regulatory factors between SM and UM on the basis of change in expression profile with resolution of parasitaemia.

The most interesting observation was a marked downregulation in SM of Th1 and Th2 transcription factors, T-bet and GATA3, respectively. Importantly, results also showed a modest increase in the levels of the two factors with resolution of parasitaemia. At this point, it is interesting to ask if there could be a role for parasite factors in depressed expression of T-bet and GATA3. We analysed in context of parasitaemia and observed a negative correlation with GATA3. Considering that GATA3 is a critical transcription factor for IL-10 by Th2 cells, it may be speculated that this pathway could be involved in the observed suppressed levels of IL-10. Another significant observation in SM was that proinflammatory cytokine IFN-α showed a moderate positive association with parasitaemia while correlating negatively with GATA3 and IL-10. A recent finding of special interest in this context is the ability of IFN-α to selectively repress GATA3 expression in fully committed Th2 cells by histone modifications. Another recent study has shown the Pf genome to contain over 6000 AT-rich immunostimulatory motifs, which potently induce type I IFN transcription via an unidentified receptor that signals through STING-mediated pathway. This view of the possible involvement of
plasmodial DNA-induced type I IFN in malaria pathophysiology prompted us to hypothesize whether differential load of AT-rich DNA between SM and UM determines the extent of GATA3 suppression via IFN-γ induction. Notably, high induction of TRAF3 observed in SM supports hyperactivation of IFN-γ pathway. Although T-bet was not correlated with parasitaemia like GATA3, a similar role of increased IFN-γ in manipulating Th1 response towards downregulated IL-10 levels may be suggested. This is based on the reported involvement of type I IFNs in dampening the development of Th1 responses during malaria infection. It is important to mention that IL-10 produced by IL-10+/IFN-γ+ co-producing Th1 cells has been demonstrated to regulate severe malaria pathogenesis in both human and experimental malaria. Also, the switch from effector cytokine-producing IFN-γ+IL-10− Th1 cells to regulatory cytokine-producing IFN-γ+IL-10+ Th1 cells is dependent on a high environmental IL-2 production as well as T-bet. Our data suggest that this switch is not operational in SM because there is not only low T-bet but also low IL-2 as reported in our previous study. Further, T-regulatory (T-reg) cell activity is expected to be downregulated because dynamic expression of T-bet and of GATA3 is known to be associated with activated T-reg cells. Consistent with this, expression of CTLA4 and Foxp3 (determined previously), the markers of T-reg cell function, was downregulated in SM.

Alternatively, macrophages could also be the source of IL-10 production downstream of MyD88 and TRIF signalling. Data revealed increased levels of p38 and NF-κB in some patients as well as of IFN-α and IL-27 in some others. In fact, upregulated expression of macrophage induction factors as well as modest levels of IL-10 was observed in one of the patients (MC3) indicating these cells to be involved in some amount of IL-10 production. Exceptions to this were two patients (MC 40 and 41) who had no detectable levels of both macrophage and T-helper cell regulatory factors. Instead, they demonstrated an exaggerated inflammatory response both in terms of type I IFNs and other inflammatory cytokines TNF-α and IL-1β as well as very low levels of IL-10. Notably, MC 40 and 41 samples had complications of acute renal failure coupled with low Glasgow coma score and severe anaemia, respectively, emphasizing the role of inflammation in severe malaria pathogenesis.

It is evident that IL-10 mediates anti-inflammatory response via SOCS3 and its production, in turn, is induced by IL-10. Our data revealed a similar temporal expression profile of SOCS3 as that of IL-10 with depressed levels on day 0 followed by a steady increase on day 3. Hence, it follows that deficient production of SOCS3 resulted in inefficient regulation of over-exaggerated inflammation in severe malaria.

Pathogens during coevolution with their hosts have developed many immune evasion strategies in order to achieve persistence. Critical factors like NF-κB and MAPKs have also been targeted by pathogens for immune subversion. Significantly, many viruses have been reported to manipulate cytokine response either by initiating a cytokine storm or by expressing IL-10 homologues. Our data suggest two-pronged immune evasion strategy by falciparum pathogen. On one hand, the pathogen mediates activation of proinflammatory pathways involving IFN-α and NF-κB. On the other hand, a depressed IL-10 response together with suppression of IL-2, T-bet and GATA3 tends to suggest compromise of regulatory
functions of T cells in falciparum malaria. Studies using purified cell populations will elucidate the role of different cell types in differential regulation of IL-10 in severe malaria. This propensity for a strong inflammatory response by upregulating proinflammatory with concomitant downregulation of anti-inflammatory response is likely to facilitate sequestration as exposure to proinflammatory cytokines is known to enhance endothelial activation.15

In conclusion, our data suggest differential transcriptional regulation of IL-10 expression between SM and UM. Although Th1 and Th2 arm appeared to be operating optimally in UM, a suppressed IL-10 response in SM seemed to be mediated by manipulating T-bet and GATA3 expression with validation at the protein levels could be exploited to target exaggerated inflammation.

MATERIALS AND METHODS

Study site, study design and subjects

The detailed description of the study site and the study population used in this study have been previously described.1,12 Briefly, malaria patients stratified into SM and UM based on the World Health Organisation guidelines were followed up in a longitudinal study by multiple sampling at the stages of active infection before treatment (day 0), parasite clearance (day 3) and resolution of clinical symptoms (day 7). After obtaining written informed consent from the participants, venous blood was collected in RNAlater (Ambion, Austin, TX, USA) from the patients for all the 3 days. Only one sample for healthy control was collected. In addition, clinical data and parasitaemia were recorded. In the present study, 10 samples each of healthy control and of SM and UM groups with follow-up till day 3 were included. The study was approved by the Tezpur University Ethical Committee (DoRD/TUEC/10-14/453 dated 23/09/10).

Analysis of gene expression by real-time quantitative reverse transcription-PCR

cDNA was generated from samples as previously described.6 Expression analysis was performed using SYBR Green chemistry on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). In addition to the regulatory factors, expressions were also checked for IL-10, TNF-α and IL-1β. Primers for some of the genes were designed using Primer Express software (Applied Biosystems) (Table 2) while for the other genes commercially available RT² qPCR Primer Assays (Qiagen, Hilder, Germany) were used. IDT OligoAnalyzer tool (Integrated DNA Technologies, Coralville, IA, USA) was used to screen designed primers for secondary structure formation. Real-time PCR was carried out in 10 μl reaction volume consisting of 1 x SYBR Green PCR master mix, 0.4 μM of each primer, 100 ng cDNA and water to adjust the volume using standard conditions. Gene expression analysis using TaqMan-based assay (Applied Biosystems) was performed only for CTLA4 gene. Expression levels were normalised using GAPDH as the endogenous control while controls were used as the calibrator. The relative fold changes were calculated based on the 2^-ΔΔCT method. Melt curve analysis was performed to confirm the presence of specific amplification products.

Statistical analysis

k-means clustering was performed to group the genes according to homogenous pattern of longitudinal gene expression. Clustering analysis for SM group was performed using healthy controls and UM as calibrator separately. The genes that clustered differently between SM and UM were further individually analysed for comparison of expression between the groups and within the same group using the unpaired and paired Student's t-test, respectively. Heat map analysis was also performed to check whether clusters of similar genes correspond to clusters of similar samples. Correlations between parasitaemia, cytokines and regulatory factors were evaluated by Pearson's correlation coefficient test. XLSTAT 2015 version (Addinsoft, New York, NY, USA) and GraphPad Prism version 5.0 (La Jolla, CA, USA) were used to carry out the statistical analyses.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Department of Biotechnology Institutional Biotech Hub (Grant no: BT/22/NB/2011) and the Department of Biotechnology (Grant no: BT/CP/11/NE/TBP/2010).

1 Cunningham AJ, Riley EM, Walther M. Stuck in a rut? Reconsidering the role of parasite sequestration in severe malaria syndromes. Trends Parasitol 2013; 29: 585–592.
2 Saravia M, O’Garra A. The regulation of IL-10 production by immune cells. Nat Rev Immunol 2010; 10: 170–181.
3 Iyer SS, Cheng G. Role of Interleukin 10 Transcriptional regulation in inflammation and autoimmune disease. Crit Rev Immunol 2012; 32: 23–63.
4 Kubo M, Matsumura Y. Transcriptional regulation of the anti-inflammatory cytokine IL-10 in acquired immune cells. Front Immunol 2012; 3: 275.
5 Redford PS, Murray PJ, O’Garra A. The role of IL-10 in immune regulation during M. tuberculosis infection. Mucosal Immunol 2011; 4: 261–270.
6 Mahanta A, Kar SK, Kakati S, Baruah S. Heightened inflammation in severe malaria is associated with decreased IL-10 expression levels and neutrophils. Innate Immun 2014; 21: 546–552.
7 Shomaker J, Saravia M, O’Garra A. IL-10 does not remodel immune responses in children 5 years of age or older. J Immunol 2006; 177: 3470–3479.
8 Huber JP, Gonzales-van Horn SR, Roybal KT, Gill MA, Farrar JD. IFN-γ suppresses GATA3 transcription from a distal exon and promotes H3K27 trimethylation of the CNS-1 enhancer in human TH2 cells. J Immunol 2019; 142: 5687–5694.
9 Sharma S, De Oliveira RB, Kalantari P, Parroche P, Gautaguy N, Jiang Z et al. Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome. Immunol 2011; 35: 194–207.
10 Gazzinelli RT, Kalantari P, Fitzgerald KA, Golenbock DT. Innate sensing of malaria parasites. Nat Rev Immunol 2014; 14: 727–757.
11 Walther M, Jeffries D, Finney OC, Njie M, Ebonyi A, Deininger S et al. Distinct roles for FOXP3+ and FOXP3+ CD4+ T cells in regulating cellular immunity to uncomplicated and severe Plasmodium falciparum malaria. PLoS Pathog 2009; 5: e1000364.
12 Freitas do Rosário AP, Lamb T, Spence P, Stephens R, Lang A, Roers A et al. IL-27 promotes IL-10 production by effector TH1 CD4+ T cells: a critical mechanism for protection from severe immunopathology during malaria infection. J Immunol 2012; 188: 1178–1190.
13 Cope A, Le Frere G, Cardone J, Kemper C, The Th1 life cycle: molecular control of IFN-γ to IL-10 switching. Trends Immunol 2011; 32: 278–286.
14 Xu FY, Sharma S, Edwards J, Feigenbaum L, Zhu J. Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. Nat Immunol 2015; 16: 197–206.
15 Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 2010; 10: 552–555.
16 Neves BM, Lopes MC, Cruz MT. Pathogen strategies to evade innate immune response: the interplay between PTEN and IL-10. Innate Immun 2015; 15: 53–68.
17 Cunningham AJ, Riley EM, Walther M. Stuck in a rut? Reconsidering the role of parasite sequestration in severe malaria syndromes. Trends Parasitol 2013; 29: 585–592.
18 Huber JP, Gonzales-van Horn SR, Roybal KT, Gill MA, Farrar JD. IFN-γ suppresses GATA3 transcription from a distal exon and promotes H3K27 trimethylation of the CNS-1 enhancer in human TH2 cells. J Immunol 2019; 142: 5687–5694.
19 Tisoncik JR, Korth MJ, Simmons CP, Farrar J, Martin TR, Katze MG. Into the Eye of the Cytokine Storm. Microbiol Mol Biol Rev 2012; 76: 16–32.
20 Alcami A, Koszinowski UH. Viral mechanisms of immune evasion. Immunol Today 2000; 21: 447–455.
21 Mahanta A, Kakati S, Baruah S. The association of IL-8-251T/A polymorphism with complicated malaria in Karbi Anglong district of Assam. Cytokine 2014; 65: 210–216.

This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-sa/4.0/