Role of TGF-β and IL-6 in dendritic cells, Treg and Th17 mediated immune response during experimental cerebral malaria

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The role of cytokines in Plasmodium infection have been extensively investigated, but pro and anti inflammatory cytokines mediated imbalance during malaria immune-pathogenesis is still unrevealed. Malaria is associated with the circulating levels of Interleukin-6 (IL-6) and transforming growth factor β (TGF-β), but association between these two cytokines in immune response remains largely obscured. Using mouse model, we proposed that IL-6 and TGF-β are involved in immune regulation of dendritic cells (DC), regulatory T cells (Treg), T-helper cells (Th17) during P. berghei ANKA (PbA) infection. Association between the cytokines and the severity of malaria was established with anti-TGF-β treatment resulting in increased parasitemia and increased immunopathology, whereas; anti-IL-6 treatment delays immunopathology during PbA infection. Further, splenocytes revealed differential alteration of myeloid DC (mDC), plasmacytoid DC (pDC), Treg, Th17 cells following TGF-β and IL-6 neutralization. Interestingly anti-TGF-β reduces CD11c+CD8α+ DC expression, whereas anti-IL-6 administration causes a profound increase during PbA infection in Swiss mice. We observed down regulation of TGF-β, IL-10, NFAT, Foxp3, STAT-3 SMAD-3 and upregulation of IL-6, IL-23, IL-17 and STAT-3 in splenocytes during PbA infection. The STAT activity probably plays differential role in induction of Th17 and Treg cells. Interestingly we found increase in STAT-3 and decrease in STAT-5 expression during PbA infection. This pattern of STAT indicates that possibly TGF-β and IL-6 play a crucial role in differentiation of DCs subsets and Treg/Th17 imbalance during experimental cerebral malaria (ECM).

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1. Introduction

The understanding of cerebral malaria is limited because of the low frequency of autopsies in most malaria endemic areas. Efficacy and/or formation of defensive mechanisms are apparently impaired by not-yet-understood parasite-induced host responses, which contribute to malaria morbidity and mortality [1]. Although there have been extensive studies on the contribution of cytokines and other inflammatory molecules during malarial erythrocytic infection, yet the mode of action of proinflammatory cytokine response that helps to activate T cells in appropriate ways to encounter pathogen needs greater attention [2]. As the immune response progresses, there is a need to contain the inflammatory response to avert immunopathology [3].

The pleiotropic cytokine IL-6 playing an important role in host response during malaria infection, were found in circulation of patients suffering from malaria caused by P. falciparum and P. vivax [4–6]. Decrease in IL-6 after administration of anti-malarial [7] is associated with decrease in hyperpyrexia [8] and parasitemia [9]. However, reports also states that IL-6 is unlikely to be involved in the pathogenesis of ECM [10–12], treatment of CM-susceptible mice with an anti-IL-6 antibody does not ameliorate cerebral symptoms, suggesting that IL-6 is not involved in cerebral malaria [12]. Earlier, we have showed the differential role of TGF-β in regulation of malaria pathogenesis, and as TGF-β mediates pro-inflammatory effects at low concentrations and anti-inflammatory effects at high concentrations, therefore it would be interesting to observe the role of TGF-β in driving a balance between clearance and control of infectious organisms on the one hand and prevention of immune-mediated pathology on the other hand [5,6]. A finely tuned balance between TGF-β and IL-6 seems to be pivotal and possibly determines the outcome of infection and may resolve the ambiguity behind the decisive role of pro and anti-inflammatory cytokines malaria pathogenesis.

The character of the immune response is critically dependent on the interplay between the innate and adaptive immune systems, dendritic cells (DCs) as strong professional APCs, play a vital role in this interaction [13,14]. Previously we have reported the role of DC subpopulations, mDC (CD11c+CD11b+) and pDC...
(CD11c"CD45R/B220") in splenocytes during PbA infection [15], although the associations between TGF-β and IL-6 in the pathogenesis of CM and their effect on the immuno regulatory function of mDC/pDC need to be studied in details. Though there are reports that plasmacytoid dendritic cells (pDC) produce IFN-γ [16,17] but, pDC functions are not only limited to IFN secretion because human and mouse pDC may secrete inflammatory cytokines such as IL-6 [18,19] and therefore neutralizing IL-6 may cause alterations in pDC function during malaria. Interestingly, pDC in tolerance induction plays a role through several mechanisms including IL-10 secretion, IDO activity, or regulatory T cell (Treg) induction [20–22]. Studies mainly reported that TGF-β modifies pDC functions in association with other immunosuppressive molecules, including IL-10 or PG-E2 [23–25]. In contrast data are emerging, that mouse and human pDC also induces IL-17 secreting Th17 cells [26,27]. Interestingly, Th17 differentiation requires at least IL-6 and TGF-β [28,29] whereas; TGF-β participates to Treg polarization [30]. Moreover, Treg functions are mainly regulated by mature DC where DC maturation leads to a blockade of the suppressive effect of Treg [31] but the role of cytokines in this regulation need to be understood clearly. In addition, it is likely that the cytokines plays and important role in Treg expansion and functions possibly regulated by the DC subtype involved in T cell stimulation.

The balance between pro-inflammatory and regulatory immune mechanisms is crucial in determining the outcome of many parasitic infections [32,33]: pro-inflammatory responses, lead to severe disease is also essential to clear infection and need to be very tightly regulated to maintain immune homeostasis. In several murine models of malaria, upregulation of Treg may influence disease outcome but its highly controversial [34–39]. Depending on the mouse–parasite combination studied, Treg were found to prevent severe symptoms [38,40], or to contribute to disease severity [34,36] or to have no effect on disease outcome at all [33,35,37]. In some cases, even in very similar models discordant results were reported [34,36,37,39]. In the current study we neutralized the major cytokines and characterized the frequency, phenotype and function of Treg cells in non-congenic polymorphic Swiss Albino mice to understand the duality in Tregs immune response during cerebral malaria. Recently, the Th17 subset of CD4+ T-cells has gained attention among malaria researchers because of its role in autoimmune diseases and chronic inflammation and in responses to extra cellular pathogens such as bacteria and fungi [41]. Earlier we have shown that Th17 cells are activated during acute PbA and P. yoelii infection [42], whereas no association of Th17 cells and cerebral malaria in PbA infected IL-17−deficient mice was observed [43]. Further, erythrocytic-stage P. chabaudi infection confirms IL-17A and IL-17F- producing CD4+ T-cells mainly in the liver; whereas, IL-17A- deficient mice showed no significant alterations [44]. Therefore, defined role of Treg and TH17 cells induction during Plasmodium infections in association with cytokines needs further attention in malaria immunopathology.

Interestingly, TGF-β and IL-6 trigger the coordinated activation of SMAD3 and STAT-3 to induce the transcription factor RORγt necessary for Th17 differentiation [45]. Mechanistically, the activation status of STAT-3 and STAT-5 following TGF-β or IL-6 plus TGF-β was evaluated in more details using neutralization antibodies. These studies indicate that possibly TGF-β is a central cytokine in the differentiation of both Treg and Th17 cells and IL-6 is the key cytokine regulating Treg/Th17 balance. Significantly, our findings will possibly throw some light on how IL-6 and TGF-β contribute to pathogenesis during ECM and affects the DCs, Treg/Th17 cells response during ECM. In this study, we raised the question that possibly TGF-β and IL-6 deficient milieu during PbA infection may influences the capacity of pDC or mDC to polarize naive CD4+ T cells to Treg or Th17 cells. In the present study, we performed a comparative analysis of DCs, Treg and Th17 immune response after IL-6 and TGF-β neutralization during PbA infection.

2. Materials and methods

2.1. Chemicals and reagents

Giemsa’s azur eosin methylene blue solution (#109204), hematoxylin and eosin stains for microscopy were purchased from Merck Chemicals (Mumbai, India). Phosphate buffer saline (PBS), sodium bicarbonate, sodium azide and RNase A were procured from Himedia, Mumbai, India. Fetal Bovine Serum from Axxora and Invitrogen, Darmstadt, Germany. RPMI 1640 medium with 2.05 mM l-glutamine, HEPES buffer, Antimycotic solutions, Gentamycin solution were purchased from Hyclone, USA. Fetal calf serum was procured from Hyclone. FITC-anti-CD11c (#117305), PE-anti-B220 (#103207), PE-anti-CD86 (#105007), PE-Cy5.5-anti-CD40 (124624), FITC-anti-CD80 (#104705), FITC-anti-CD69 (#104505), FITC-anti-CD62L (#104405), FITC-anti-MHCII (#107605), PE-IL-10 (#505007), CD3 FITC-anti-CD4, PE-anti-CD8, PE-anti-CD25 (#102007), Alexa Fluor-anti-FoxP3 (#320013), PE anti-IL-17 (#506903), LEAFPE Purified anti-mouse IL-6 (#504506), LEAFPE purified anti mouse LAP/TGF-β1 (#141304), purified anti-CD16/32 (#101301), permeabilization buffer (#421002), fixation buffer (#420801) and isotye controls were purchased from Bio legend (New Delhi, India); APC Anti-mouse RORγt (#17-6988-80) and respective APC Rat IgG2ak isotype Control (#17-4321) were procured from e bioscience, Inc. (San Diego, CA, USA). Antibodies against Stat-3 (#4904), Stat-5 (#9383) and β-actin (#4967), AP-linked anti-rabbit (#7054), AP linked anti-mouse (#7056) secondary antibodies were purchased from the Cell signaling Technology (Cell Signaling Technology, Inc, Denver, MA, USA). All solutions were prepared with commercial reagents of at least pre-analysis quality and with sterilized 18 MX milllQ water. When necessary, the specific origins of reagents are listed in the text.

2.2. In vivo malaria infection

Male Swiss albino mice (~25 g each; aged 6–8 weeks) were maintained in sterilized cages and absorbent media. All animals were provided rodent chow (National Institute of Nutrition) and filtered water ad libitum. Animal experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration No: 885/ac/05/CPCSEA) and as approved by the Institutional Animal Ethics Committee (IAEC), University of Calcutta, and conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Parasite strain P. berghei ANKA obtained from National Institute Malaria Research Centre, New Delhi. Parasitized mouse red blood cells (pRBC) from a liquid N2 preserved stabilize were injected (1 x 10^6 pRBC) in 100 μl PBS by intraperitoneal injection. Control mice received an equal number of uninfected mouse red blood cells in 100 μl PBS by intraperitoneal injection. Control mice received an equal number of uninfected erythrocytes. Parasitemia, survivability and ECM symptoms of mice (n = 60) were also observed daily as described previously [46,47].

To examine the effect of neutralization of TGF-β on the course of malaria infection, mice were administered with 10 μg of TGF-β antibody, by intraperitoneal injection 1 d before malaria infection and on days 2, 5, and 7 after infection. Control mice received 10 μg of polyclonal mouse IgG1. Our preliminary results and previous study on SB-431542 (TGF-β inhibitor) suggests the above dose...
regimen, as continuous dose of TGF-β results mice death on 3–4 dpi.

To examine the effect IL-6 neutralization in malaria infected mice, mice were infected with PbA and given (20 μg/day) IL-6 in 100 μl of PBS by intraperitoneal injection on the day of infection and then till the last day of mice survival. Control mice received 20 μg of polyclonal mouse IgG1. To examine the effect of both the cytokines, another group was used. Isotype Rat IgG1, κ was administered accordingly the regime of cytokine dose separately.

2.3. Flow cytometry

Whole spleens were aseptically removed from euthanized mice of all experimental groups and splenocytes were obtained by mechanical disruption through a cell strainer. Erythrocytes were lysed by incubation with ACK lysis buffer for five minutes followed by washing with cold DMEM. In brief, for analysis of spleen DC subpopulation and phenotype, single-cell suspensions of splenocytes were first blocked with anti-CD16/CD32. Cells were washed, stained with APC-CD11c, FITC-CD11c, FITC-CD11b, FITC-B220, FITC-CD80, PE-CD86, FITC-CD40, PE-CD8, FITC-MHC-II, and determined by gating on viable forward versus side scatter gates. The difference of DC activation between all experimental group of mice and control mice were determined by the numbers and percentage of CD11c+DCs expressing MHC II, CD40, CD86 or CD80 molecules. The difference of DC subsets was determined by numbers and percentage of CD11c+DCs expressing MHC II, CD40, CD86 or CD80 molecules.

2.4. Flow cytometric analysis of Treg and TH17 splenocytes during PbA infection

Spleen cells collected from mice at 8 DPI after PbA infection were previously stimulated with PMA and ionomycin for 2 h at 37 °C and then Golgi stop (BD Bioscience) was added to each reaction (1:500 (v/v)). After continued co-culture for 4 h at 37 °C, cells were washed (in CSB). For surface staining FITC-anti-CD4 and PE-anti-CD25 (Biolegend) were added and incubated for 30 min at 4 °C. These cells were then incubated with anti-CD16/32 (Fc-block) and stained with surface markers, followed by fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin. The cells were then fixed washed twice with cell staining buffer and intracytoplasmic staining was performed using APC-conjugated anti-Foxp3 for Treg cells and PE-IL-17, APC-RODyt for TH17 cells respectively along with respective isotype controls. The cells were then analyzed and viable cells were gated by forward and side scattering.

2.5. Immunohistochemistry

Sections (5 μm) were cut from paraffin embedded spleen tissues and mounted on positively charged super frost slides (Export Mengel CF). Tissues were deparaffinized, rehydrated through graded alcohols, and then blocked for endogenous peroxidase in 3% hydrogen peroxide in methanol. All tissues were pre-blocked in Tris buffered saline containing 0.3% Triton, and 0.5% blocking agent (BSA, SRL, India, and batch No. 832095) and incubated with STAT-3 and STAT-5 primary antibody (1:30) overnight at 4 °C for positive control. Antiseric was used for STAT-3 and STAT-5 were diluted 1:30 in Tris buffered saline containing 0.3% Triton, and 0.5% blocking agent. Immunoreactive complexes were detected using DAB system (Bangalore Genel DAB system, Cat #SFE5). Slides were counterstained briefly in haematoxylin (MERCK), mounted in DPX (MERCK).

2.6. RNA isolation, reverse transcription, and RT-PCR

After treatment, total RNA extraction was performed using TriZol reagent (Bangalore Genei, India) followed by DNase treatment, phenol–chloroform extraction, and ethanol precipitation. RNA purity was checked by spectrophotometry, next, 1 μg of RNA was transcribed using the reverse transcription system (MMLV High Performance Reverse Transcriptage, Epicentre Biotechnologies) (Random hexmer – Promega # C1181). GAPDH was used as constitutive expression. The following primers were used for the PCR reactions: TGF-β, IL-6, IL-10, Foxp3, NFAT, STAT-3, STAT-5, IL-17, IL-23, SMAD-3 and GAPDH. The following primers were used for the PCR reactions: TGF-β; Forward primer: ACTGC-TATGCTGCTGCTTACT and Reverse primer: TGGCCCTGTGACAC CTTGCTTC, IL-6; Forward primer: CACGACGTGCGCATC GGGGG and Reverse primer: CTGCGCCTAGTCTCCAGG TCT, IL-10 Forward primer: ACTGCTATGCTGCTTACT and Reverse primer: TGGCCTTGTAGACACCTTTGCCT. Slides: Forward primer: GCAGCACAGTTGACCTCAC and Reverse primer: AGTGTCTGCT ATAGTGAGAGTGA, NFAT; Forward primer: ACAACATGAGAC CACCATCGACT and Reverse primer: GCCATGACTTGTGAACAC GAAA, STAT-3; Forward primer: CTGTTCTACTCT ACGCCGACAT and Reverse primer: GATCCCATGCA ACGTGGA CGG, STAT-5; Forward primer: GCTGACTCATCGCTTIC and Reverse primer: GACCGTGGCTTCTATCAGTGA, IL-17; Forward primer GCTCCA GAAGGCCC TAT and Reverse primer: CTTCCTGCAATGTTACA, IL-23; Forward primer: AGCGCCGATGTAACTCTACTAAGGA and Reverse primer: GTCTTATGAGG AGGTGTGAAAGTGC, SMAD-3; Forward primer: TAAGTAAGCCGCTTAC AGCACCT and Reverse primer: CACCCCCACACCTTTGTAGCTT and GAPDH; Forward primer: CCATGGAGAAG GCTGGGG and Reverse primer: CAAACCTGTCATGAGTACG.

2.7. Statistical analysis

Values between groups on same or different dpi were analyzed using two-way ANOVA, followed by Bonferroni multiple comparison post-test. All values are shown as mean ± SD, except where otherwise indicated. To assay the frequency distribution of all the data sets we have performed either normality or homogeneity distribution tests using Origin Pro 8. For non-parametric data, a Kruskal-Wallis test with Dunn’s multiple comparison post-test was used. Data were analyzed and when appropriate, significance of the differences between mean values was determined using Student’s t-test. Survival curve analyses were performed using the Graph Pad Prism software version 3.03. Significant differences between survivals were assessed by Log rank test. p < 0.05 were considered significant (p < 0.05, p < 0.01 and **p < 0.001).

3. Results

3.1. Characteristics of PbA infection in Swiss albino mice

After PbA infection, characteristics on infection in all experimental groups were shown in Fig. 1. Mice belonging to the same age group and having the similar body weight were used for all experiments. Parasitemia increased significantly till 8 dpi, in case of PbA infected, PbA infected + anti-TGF-β, and PbA infected + anti-TGF-β + anti-IL-6 experimental group. In contrast, parasitemia didn’t rose significantly in case of PbA infected + anti-IL-6 group compared to other experimental groups (Fig. 1A). Interestingly, after administration of anti-TGF-β a significant rise in parasitemia within 2dpi was observed compared to other experimental groups along with early death. It is evident from observation that most of the mice dies during 4–8 dpi, except in anti-IL-6 administered group, (p < 0.05) compared to other experimental groups and
When compared to PbA anti-IL-6 and anti-TGF-β infection. (A) Resulting parasitemias were expressed as a percent parasitemia vival percentage of mice were observed (Fig. 1B). We also observed matched controls. Statistically significant differences in the survival percentage of mice were observed (Fig. 1B). We also observed the experimental cerebral malaria symptoms in all experimental groups; interestingly the mean neurological score in all experimental groups was higher than the PbA+ anti-IL-6 group (Fig. 1C). The clinical ECM scores were expressed as mean neurological score on 4, 6 and 8 dpi from respective experimental groups of mice (n = 5/group). Data is representative of five comparable experiments and represented as mean ± SD values. *p < 0.05, Student’s t-test) between groups on respective days.

3.2. Comparison of splenic DCs expression and maturation during PbA infection

To elucidate the immunological mechanisms that could contribute to more rapid death of PbA-infected mice supplemented with anti-TGF-β we investigated changes in CD11c+ DCs and matched controls. Statistically significant differences in the survival percentage of mice were observed (Fig. 1B). We also observed the experimental cerebral malaria symptoms in all experimental groups; interestingly the mean neurological score in all experimental groups was high than the PbA+ anti-IL-6 group (Fig. 1C). The clinical ECM scores were higher in the anti-TGF-β groups than the control group (Fig. 1C). Taken together, these data suggest that anti-TGF-β treated mice died early from immune mediated pathology whereas, anti-IL-6 delays the death due to ECM.

3.3. Comparison of DC subpopulations in splenocytes during PbA infection

Spleen from all experimental groups were isolated on 8 dpi along with matched controls and were submitted to CD11c+CD40+ and CD11c+CD80+ during PbA infection on 8dpi whereas pronounced expression of CD11c+CD40+ and CD11c+CD80+ was observed after anti-TGF-β administration with respect to PbA and matched controls. But after administration of anti-IL-6 alone we observed decrease in these DC maturation markers compared to PbA and PbA + anti-TGF-β infected group. On the other hand decrease in number of maturation profile of CD11c+CD40+ and CD11c+CD80+ was observed when anti-IL-6 and anti-TGF-β was administered with respect to PbA infection (Fig. 3A–D). Interestingly we observed significant increase in CD11c+CD86+ numbers on 8dpi during PbA infection with respect to matched controls, whereas decrease in number was observed when anti-TGF-β was administered alone or together with IL-6 group with respect to PbA infection. Further we observed significant decrease in CD11c+CD86+ numbers in anti-IL-6 group compared to infected untreated group. As shown in Fig. 4, the percentage of MHCII+ DCs on 8dpi during PbA infection we observed increase in number of CD11c+CD40+ and CD11c+CD80+ during PbA infection on 8dpi, whereas pronounced expression of CD11c+CD40+ and CD11c+CD80+ was observed after anti-TGF-β administration with respect to PbA and matched controls. But after administration of anti-IL-6 alone we observed decrease in these DC maturation markers compared to PbA and PbA + anti-TGF-β infected group. On the other hand decrease in number of maturation profile of CD11c+CD40+ and CD11c+CD80+ was observed when anti-IL-6 and anti-TGF-β was administered with respect to PbA infection (Fig. 3A–D). Interestingly we observed significant increase in CD11c+CD86+ numbers on 8dpi during PbA infection with respect to matched controls, whereas decrease in number was observed when anti-TGF-β was administered alone or together with IL-6 group with respect to PbA infection. Further we observed significant decrease in CD11c+CD86+ numbers in anti-IL-6 group compared to infected untreated group (Fig. 3E, F). We also observed increase in numbers of CD11c+MHCIId+ DCs on 8 dpi during PbA + anti-TGF-β treated group compared to PbA infection, whereas significant decrease was observed in PbA + anti-IL-6 and PbA + anti-IL6 + anti-TGF-β groups compared to PbA infected group (Fig. 3G, H). It is likely that splenic DCs from all experimental groups appears functionally different in terms of the induction of cytokine during the course of infection, suggesting possible role of TGF-β and IL-6 in DC maturation during PbA infection (Fig. 3A–H).

Fig. 1. Effect of anti-TGF-β and anti-IL-6 neutralization in Swiss Albino mice during PbA infection. (A) Resulting parasitemias were expressed as a percent parasitemia (mean ± SD), measured daily on Giemsa stained blood smears. Parasitemia was evaluated statistically by Two-way ANOVA with Bonferroni post-test. It shows that anti-IL-6 and anti-TGF-β administration causes significant difference in parasitemia when compared to PbA infection (p < 0.05), whereas no significant difference between PbA and PbA+anti-IL-6+ anti-TGF-β group (p > 0.05). (B) Survival was assessed daily during infection period, significant differences between survival were assessed by Log rank test, with a different profile between IL-6 (p < 0.001) and TGF-β (p < 0.01) treated group w.r.t. PbA infected controls. (C) Cerebral symptoms were expressed as mean neurological score on 4, 6 and 8 dpi from respectively experimental groups of mice (n = 5/group). Data is representative of five comparable experiments and represented as mean ± SD values. *p < 0.05, Student’s t-test) between groups on respective days.
compared to infected untreated group. Interestingly, on 8 dpi a significant increase in percentage of CD11c+B220+ DC population was observed after anti-IL-6 administration compared to infected untreated group. Interestingly when we compared the CD11c+-B220+ DC numbers after anti-IL-6 treatment a pronounced increase was observed compared to anti-TGF-β group, whereas, no significant change was observed when compared to infected untreated group (Fig. 4A–C).

The blood stage of a Pba infection induces a significant increase in the percentages and number of CD11c+CD11b+ DC cells in 8 dpi spleen compared to uninfected mice. On the other hand, anti-TGF-β administration augments the CD11c+CD11b+ DC percentage and numbers compared to Pba infection. Further there was no significant change in splenic CD11c+CD11b+ DCs in Pba + anti-IL6 + anti-TGF-β group compared to Pba infected group. Interestingly a significant decrease in percentage and numbers of CD11c+CD11b+ DCs was observed in Pba + anti-IL-6 group compared to infected untreated group (Fig. 4D–F). We then compared the ratio of mDC to pDC in all experimental groups. As shown in Fig. 4G, the ratio of mDC to pDC significantly increased in the spleen of Pba + anti-TGF-β group compared to infected untreated group, while there was significant decrease in ratio after anti-IL-6 administration compared to infected untreated group. Taken together these results suggest that the cytokine imbalance causes significant alterations in mDC/pDC during Pba infection (Fig. 4G).

### 3.4. Alterations in the frequencies of CD11c+CD8α+ DC cells in the spleen during Pba infection

Further we observed the effect of IL-6 and TGF-β on another subset of DC CD11c+CD8α+ during Pba infection which is required for Treg induction. We observed a significant decrease in percentage, whereas significant increase in number of splenic CD11c+CD8α+ at 8dpi in Pba infected group compared to matched controls. In contrary when anti-IL-6 was administered we observed significant upregulation in percentage change and numbers of CD11c+CD8α+ compared to Pba infected group. In anti-TGF-β + Pba group significant decrement in percentage and number of CD11c+CD8α+ DC was observed compared to infected untreated group. Interestingly, we observed no difference in percentage and numbers of CD11c+CD8α+ DCs when both anti-IL-6 and anti-TGF-β were administered compared to infected untreated group (Fig. 5A–C).
3.5. Effect of IL-6 and TGF-β on Th17-Tregs expression in spleen during PbA infection

To analyze the role of anti-IL-6 and anti-TGF-β in induction of Th17 and Tregs cells we performed flowcytometry of CD4+IL-17+RORγt+ (Th17) and CD4+CD25+Foxp3+ (Tregs) on 8dpi splenocytes from all infected groups along with matched controls. As described earlier percentage and number of Th17 increased on 8 dpi during PbA infection with respect to control. Whereas in anti-TGF-β group there was a significant increase in the percentage and number of Th17 cells at 8 dpi with respect to infected untreated control. In contrary IL-6 administration causes significant decrease in percentage and number of Th-17 cells compared to PbA infected group. Further we observed a tardy increase in percentage and number of Th17 cells when anti-IL-6 and anti-TGF-β were administered together compared to infected untreated group (Fig. 6A, B).

Regulatory T cells (Tregs) are T-cell subgroups that have different immunoregulatory effects than Th1 and Th2. As we showed previously, percentage of Treg cells decreased on 8 dpi, whereas number of Treg cell increased during PbA infection with respect to matched control. Interestingly in anti-IL-6 group we observed a significant increase in percentage and number of Treg cells compared to PbA infection (Fig. 6C, D). In contrary, PbA + anti-TGF-β group showed a significant decrease in percentage and number of Treg cells compared to PbA infection. But when both anti-IL-6 and anti-TGF-β were administered no significant change in percentage and number of Treg cells were observed with respect to PbA infection (Fig. 6C, D).

3.6. Effect of IL-6 and TGF-β on key regulators involved in Treg/Th17 induction during PbA infection

Next we observed the expression pattern of key regulators involved in the Treg/Th17 induction and the effect of anti-IL-6 and anti-TGF-β neutralization during PbA infection. The levels of IL-6, IL-23, STAT-3, SMAD-3 and IL-17 mRNAs were found elevated...
in 8 dpi spleen after PbA infection with respect to matched control. In anti-TGF-β group mRNAs levels of all the factors elevated, whereas opposite was observed in SMAD-3 compared to PbA infection. On the other hand no significant change was observed in IL-6 and IL-17 mRNA levels when both IL-6 and TGF-β were administered together, except SMAD-3 mRNA level elevated, whereas IL-23 and STAT-3 mRNA level decreased compared to infected untreated group. Further anti-IL-6 administration showed decrease in mRNA levels of IL-6, IL-17, IL-23 and STAT-3, except SMAD-3 compared to infected untreated group. (Fig. 7A). In addition we observed levels of TGF-β, IL-10, NFAT and Foxp3 mRNAs in 8 dpi spleens of all experimental groups along with matched controls. Interestingly, we observed decrease in mRNA level of all the factors during PbA infection with respect to control. whereas,
anti-IL-6 administration causes significant increase in mRNA levels of all the factors compared to infected untreated group. In contrary pronounced decrease in expression of all the factors in PbA + anti-TGF-β group compared to PbA infected group was observed. Further when both anti-IL-6 and anti-TGF-β were neutralized we observed increase in TGF-β, IL-10, STAT-5, NFAT and Foxp3 mRNAs compared to infected untreated group (Fig. 7B).

3.7. Immunohistochemical expression of STAT-3 and STAT-5 during PbA infection

Further the immunohistochemical staining of STAT-3 and STAT-5 were documented in the nucleus in the spleen of 8 dpi mice from all experimental groups along with respective control mice. STAT-3 expression were predominantly distributed in the spleen tissue in PbA infected mice, followed by lower expression of STAT-5 during PbA infection compared to matched control. In PbA + anti-TGF-β group we observed pronounced increase in STAT-3 expression compared to PbA infection, whereas STAT-5 expression decreased. Interestingly in PbA + anti-IL-6 group significant decrease in STAT-3 expression was observed, whereas STAT-5 expression was high compared to PbA infection. Further in PbA + anti-TGF-β + anti-IL-6 group no significant change in STAT3 and STAT-5 expression was observed compared to PbA infection (Fig. 8A, B).

4. Discussion

Plasmodium blood-stage infection induces strong cytokine responses that facilitate parasite clearance, but also may trigger pathology in the host [49]. TGF-β is a T cell inhibitory cytokine that contributes to the regulation of innate responses in malaria infections of mice [50] and humans [51]. TGF-β present during the first 2 days of blood-stage infection in mice completely inhibits pro-inflammatory cytokine [52] whereas; IL-6 presence plays a detrimental role in malaria infection. The present study was designed to investigate the effects of the pro-inflammatory IL-6 and anti-inflammatory TGF-β cytokines during PbA infection. Survival and weight change exhibited by experimental groups of mice showed a distinct relationship with parasitemia level. In our previous study we have showed that downregulation of TGF-β and upregulation of IL-6 in spleen plays important role during the course of PbA infection [42]. We have earlier showed that as the infection progresses and parasitemia reaches its peak resulting in ECM nearly around 8dpi, TGF-β level decreases and IL-6 level increases [42]. Therefore in current study, we studied the effect of anti-IL6 and anti-TGF-β separately or together on DCs subsets, Treg and Th17 cells mediated immune response during PbA infection.

DCs are antigen-presenting cells that take part in both innate and adaptive immune responses [53]. Although different effects have been described depending on the parasite strain used or time
Fig. 6. Proportions of TH17 and Treg cells and effect of IL-6/TGF-β neutralization during PbA infection. Flowcytometric analysis demonstrated Th17 and Treg cell populations in matched controls and all experimental infected groups. Mice were infected with $1 \times 10^6$ infected RBCs. (A, B) At day 8 dpi along with respective control spleen cells were stained with FITC-CD4 and stained intracellularly for PE-IL-17 and APC RORγt. Quadrant markers were set based on staining with PE Rat IgG1 (IL-17) and APC Rat IgG2a (RORγt) isotype controls on FITC CD4 gated on splenocytes for analysis of Th17 cells. Representative bar diagram showing the percentage change and count of Th17 expression on splenocytes gated on CD4 T cells from all experimental groups. (C, D) At day 8 dpi along with respective control spleen cells were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD25, and stained intracellularly for APC Foxp3. Quadrant markers were set based on staining with PE Rat IgG1 (CD25) and Alexa Fluor® 647 Mouse IgG1k (Foxp3) isotype controls on FITC CD4 gated on splenocytes for analysis of Treg cells. Representative bar diagram showing the percentage change and count of Foxp3+ CD25+ expression on splenocytes gated on CD4 T cells from all experimental groups. Data is representative of five (n = 5) comparable experiments and represented as mean and ± SD values. The results were evaluated by ANOVA. Significant differences between infected and control mice are marked (*p < 0.05), whilst differences between infected groups on respective days are marked (by letters). The same letter indicates no significant differences and different letters indicate significant differences.
after infection, the cytokine responses of DC were markedly affected by *Plasmodium* [48,54]. High expression of MHC II is crucial for DCs to present antigens to CD4+ Th cells [55]. We found that blood-stage *PbA* infection induces splenic CD11c+DCs positive for MHC II, CD80, CD86 and CD40 at 8dpi. However, it is noticeable that the magnitude of increased expressions of MHCII on DCs from *PbA* infected mice was less than that from TGF-β depleted mice, which was consistent with the involvement of TGF-β in immune response. In light of these findings, it seems likely that TGF-β inhibits the development of immune response possibly by impairing DC

Fig. 7. (A, B) Temporal expression of IL-6, IL-23, STAT-3, SMAD-3, IL-17, IL-10, TGF-β, STAT-5, NFAT and Foxp3 mRNAs in spleens during *PbA* infection. Total RNA was extracted from all experimental group on 8 dpi and respective controls. The RNA samples were obtained from five mice per time point. RT-PCR was performed, and the PCR products were electrophoresed on 1.5–2.5% agarose gels containing 0.5 μg of ethidium bromide per ml and observed with a UV transilluminator.

Fig. 8. Representative immunohistochemistry of (A) STAT-3 and (B) STAT-5 in spleen harvested and fixed from control and 8 dpi mice from all experimental groups, red arrows represent expression of STAT-3 in spleen tissue sections. Whereas, green arrows represent STAT-5 expression in spleen tissue sections. Magnification = 40×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
phenotypic maturation. In addition to the maturation, IL-6 depletion shows contradictory results, showing decreased inflammation which possibly favours delay in parasite activity.

DCs are capable of initiating an antigen-specific immune response and presenting antigen to helper T cells, and shaping the subsequent immune response [48]. Further, the splenic DC compartment is a heterogeneous population with subsets of cells differing across a spectrum of functions and morphologies [56], in which mDC and pDC can exhibit distinct levels of proliferation under different infections conditions [15]. Our data showed that within 8 days of the onset of experimental blood-stage malaria infection, mDC (CD11c+CD11b+) multiplication was observed. In addition, anti-TGF-β resulted in predominant proliferation, whereas anti-IL-6 caused suppression. This result is also consistent with findings that the ratio of mDC to pDC was significantly higher, but the increase in Tregs was comparatively slower than the prominent increase in Th17 cells during PbA infection. Further the opposite pattern was observed with pDC (CD11c+CD45R/ B220+) during PbA infection and with TGF-β and IL-6 neutralization. Although pDC have a pro-inflammatory role, particularly against viral infection [57,58], these cells have been shown to possess immuno-regulatory activity in mice [59] and humans [17].

As production of cytokines by DCs play a precarious role in promoting the differentiation of Th0 cells into phenotypes such as Th1, Th2, Th17, or regulatory T (Treg) cells [60,61]. We thought that the distinct DC subpopulation proliferation possibly determines the Treg/Th17 polarisation under the presence and absence of TGF-β and IL-6 in PbA infected Swiss mice, which needs to be further confirmed with in vitro studies. DC can also be subdivided based on the expression CD8α [62,63]. CD8α+ DCs were initially identified in the mouse spleen with a propensity to drive iTreg induction [64]. CD8α+ DCs can drive iTreg differentiation both in vitro and in vivo in presence of low dose of the antigen without addition of any exogenous TGF-β. However, iTreg induction mediated by CD8α+ DCs is dependent on TGF-β, as addition of TGF-β neutralizing antibody suppresses iTreg differentiation [65]. Furthermore, we also observed downregulation of CD11c+CD8α after anti-TGF-β treatment. However, in absence of exogenous TGF-β, CD8α splenic DCs are better at stimulating nTregs rather than driving iTreg differentiation which could be an important aspect for future research [65].

Importantly, excessively proliferative Th17 play an immunosuppressive role in establishment of early effective Th1 immune response, leading to the persistent rise of parasitaemia and mortality as a final consequence in susceptible Swiss mice. The suppression of inflammatory response in the malaria infection has been addressed in our study through anti-IL-6 administration which possibly presents an increased survival associated with higher Treg cell and lower Th1 cell responsiveness against non lethal P. yoelii infection [42]. Consistent with these findings, in this study, we found that the Th17 cell increased profoundly with respect to Treg cells in Swiss mice. These findings support the notion that TGF-β protected whereas, IL-6 accelerated the immunopathology during ECM.

Previous studies have reported that IL-1β and IL-6, but not TGF-β, are responsible for Th17 differentiation in humans [66], but others have suggested that TGF-β dosage plays an essential role in the differentiation of naïve human CD4+ T cells toward the Th17 lineage [67]. Therefore the interplay between these cytokines seems to be an important factor in disease outcome. There are also reports that Treg expansion inhibits Th17 development, an effect that is dependent on interleukin-17 and IL-23 [43,68,69], on the other hand, Treg cell controls inflammation through multiple mechanisms including production of the cytokine IL-10 and TGF-β [69]. Furthermore, in the case of Treg differentiation, TGF-β-induced transcription factor STAT-3, SMAD3 possibly cooperates with NFAT to induce the expression of Foxp3 [70]. In contrast, TGF-β and IL-6 trigger the coordinated activation of SMAD3 and STAT-3 to induce the transcription factor ROyr necessary for Th17 differentiation [45] which also supports our hypothesis. STAT-3 is a signaling protein that is activated by IL-6 and IL-21 which, binds to the IL-17F ioci and IL-17A [71]. Interestingly there are reports that STAT3/-/- mice display a severe reduction in ROyr expression and Th17 development, leading to increased Treg cells [72]. Taken together, STAT-3 directly regulates the differentiation of Th17 cells and targeting STAT-3 is one way to reset the Th17/ Treg cell imbalance observed in malaria. These findings suggest that regulation of the STAT and SMAD signaling pathways might contribute to the action of TGF-β mediated immune response during malaria.

Significantly, our findings will possibly throw some light on how TGF-β and IL-6 modulates the immune responses during PbA infection and their possible effect on DCs, Tregs and Th17 cells respectively. Therefore, this is the probably the first report showing that TGF-β and IL-6 imbalance possibly contribute to differential immune response establishment during the PbA infection.

Conflict of interests

The authors declare no commercial or financial conflict of interest.

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References


