Immunophenotyping of blood mononuclear cells from *P. berghei* (NK-65) infected and immunized BALB/c mice

Vineet Kumar¹ · Aruna Rakha² · Ruchika Saroa¹ · Upma Bagai¹

Received: 2 September 2015 / Accepted: 17 May 2016 / Published online: 3 June 2016
© Indian Society for Parasitology 2016

Abstract The present study demonstrates the alterations in the frequencies of helper, cytotoxic and suppressor T cells in blood of *P. berghei* infected and TPA immunized rodent host towards the induction of protective immune response. Statistically significant ($p < 0.005$) number of CD4⁺ T cells was recorded on D9 [Post Challenge (PC)] meanwhile CD4⁺ Treg cells were found to be declined in immunized mice as compared to infected. This increase in frequencies of T helper cells points towards the stimulation of strong Th2 immune response in immunized mice. Statistically significant ($p < 0.005$) decline was observed in the frequencies of CD8⁺ T cells on D9 (PC) in immunized mice. In infected controls cytotoxic T cells were also found to be increased after 24 h post infection. Due to strong Th2 immune response, evident from lower frequencies of CD4⁺ Treg cells in immunized mice, complete protection was obtained. T reg population was found to be higher in infected controls and all control mice died.

Keywords *Plasmodium berghei* · T helper · T cytotoxic · T suppressor cells

Introduction

Immunity to asexual stages during *Plasmodium* infection involves both antibody-dependent and T cells dependent mechanisms which are essential in the induction and

maintenance of immunity (Troye-Blomberg et al. 1994). Infection with *Plasmodium* causes T cell activation, T cell lymphopenia and T cell dysfunction in host (Kemp et al. 2002). T-helper and T-cytotoxic cells play critical roles in eliminating malarial parasites. It has been reported that in *P. c. chabaudi* AS infected host protective immunity to blood stage malaria requires both CD4⁺ T cells and antibodies. Sequential activation of Th1 and Th2 cells is also critical for protection as it contributes to protective immunity during infection and the balance between these two subsets determines the outcome of the disease (Wipasa et al. 2002).

Cellular immunity during liver stage is mostly mediated by CD8⁺ T cells, CD4⁺ T cells, NK cells and T cells. Helper T cells assist B cells to secrete a high level of protective antibodies, which help in the induction of cytotoxic T cells and directly inhibit the development of liver stage of *Plasmodium* (Taylor-Robinson 1995). CD8⁺ T lymphocytes are very important in the immune response against *Plasmodium* infection and have been established as key effectors in protective immunity against pre-erythrocytic-stages of infection. Effector function of these cytotoxic cells is associated with the production of inflammatory cytokines such as IFN- γ or TNF- α , which mediate elimination of the parasite within the hepatocytes by the NO pathway. The thrombospondin-related anonymous protein (TRAP; also known as sporozoite surface protein 2) of *P. yoelii* has been shown to induce CD8⁺ T cells (Ho et al. 1995).

Treg cells play a significant role in the modulation of inappropriate immune responses that lead to the induction of pathogenic CD8⁺ T cell clones during cerebral malaria. These cells prevent the development of parasite specific Th1 memory cells (Nie et al. 2007). Tregs prevent the development of parasite-specific Th1 memory cells during a parasitic infection demonstrating a regulatory role for this cell population in the control of pathogenic responses

✉ Upma Bagai
upmabagai@yahoo.co.in

¹ Parasitology Laboratory, Department of Zoology,
Panjab University, Chandigarh 160014, India

² Department of Translational and Regenerative Medicine,
PGIMER, Chandigarh 160012, India

leading to fatal disease. They are the subset of immunosuppressive lymphocytes that are known to inhibit both the IL-2 production and cell proliferation of conventional CD4⁺ T lymphocytes (Thornton and Shevach 1998).

Therefore, the present study was undertaken to investigate the role of various T cell populations (CD3⁺, CD4⁺, CD8⁺ and regulatory T cells) in blood of lethal *P. berghei* (NK-65) infected and total parasite antigen (TPA) immunized rodent host towards the induction of protective immune response.

Materials and methods

Experimental animal

For the present study, inbred mice from BALB/c strain of either sex (20–25 g) were used as experimental model. Mice were procured from IMTECH (Institute of Microbial Technology), Chandigarh and were given pellet feed and water. For handling and care of mice, guidelines approved by the Institutional Animal Ethics Committee (45/99/CPCSEA) of the Panjab University, Chandigarh were followed.

Maintenance of parasite and experimental design

Lethal *P. berghei* (NK-65) was maintained in BALB/c host by intra-peritoneal (i.p) inoculation of 1×10^6 parasitized RBCs from infected to naive mice. Giemsa-stained thin blood smears were studied to check the course of parasitemia (Santiyanont 1985).

For the present study following groups of mice were used:

GP-I: Placebo control BALB/c mice/ 1×10^6 *P. berghei* infected BALB/c mice (n = 30).

GP-II: Immunized BALB/c mice (n = 35).

GP-III: Normal BALB/c mice (n = 15).

Isolation of total parasite antigen (TPA)

Blood was obtained by jugular vein incision from infected mice and subjected to saponin lysis to obtain cell free parasite. Then it was centrifuged at 800 g for 15 min after sonication on ice for 30 s to obtain the total parasite antigen (TPA) (Banyal et al. 1979). The protein concentration in TPA was estimated using BSA standard (Lowry et al. 1951).

Immunization of animals

On Day 0 (D0), mice of GP-II group were injected subcutaneously (s.c.) with 100 µg of TPA emulsified in

Freund's complete adjuvant (FCA). Two booster doses were administered with similar amount of protein in Freund's incomplete adjuvant (FIA) on D14 and D28. Mice of group-1 (GP-I) were administered with PBS (0.01 M, pH 7.2) along with Freund's adjuvant (FCA/FIA) on similar days (Bagai and Pawar 2013). On D35, both groups were challenged with 1×10^6 *P. berghei* infected erythrocytes.

Preparation of blood samples for Flow cytometry analysis (FCM)

Blood of experimental animals was subjected to density gradient centrifugation by using Histopaque 1077 (Czuprynski and Brown 1998). Isolated lymphocyte cell count was done by Neubauer's haemocytometer.

The upper white layer of mononuclear WBCs was aspirated after centrifugation of blood overlaid on Histopaque at 300 g for 30 min in REMI (8°C). 2 ml of FACS (Fluorescent activating cell sorter) buffer was added to it after draining out plasma. It was centrifuged at 300 g for 5 min, supernatant was discarded and pellet of mononuclear cells (MNCs) was suspended in FACS buffer.

Quantification of T helper and T cytotoxic cells

Anti-mouse CD3⁺ (FITC conjugated, clone 17A2 mAb), CD4⁺ (PE conjugated, clone H129.19 mAb), CD8⁺ (APC conjugated, clone 53-6.7 mAb) were used for quantification of T helper and T cytotoxic cells. These antibodies were purchased from BD Biosciences, Singapore. Isolated mononuclear cells were quantified by FACS Calibur BD in Central Sophisticated Instrumentation Faculty (CSIF) at PGIMER, Chandigarh after collecting 10,000 events. The results were analyzed using BD FACS DIVA software (Bierer et al. 1989).

Quantification of T suppressor cells

Isolated MNCs from various groups were processed for T suppressor cell analysis. Antibodies viz; anti mouse CD4⁺ (FITC conjugated), CD25⁺ (APC conjugated), FoxP3⁺ (PE conjugated) were purchased from BD Biosciences, Singapore. Samples were analyzed using BD FACS DIVA software on flow cytometer (FACS Calibur BD) after collecting 10,000 events (Hori et al. 2003).

Statistical analysis of data

Data collected from various experiments was subjected to statistical analysis. Mean $\pm$ SD of five mice was used to obtain data. Student's *t* test was applied to determine the

statistical significance and inter group difference of various parameters. Data of the immunized and control groups were compared and the p value <0.05 has been termed as statistically significant value.

Results

Course of infection in immunized mice

Parasite appeared on day 3 post challenge (PC) in GP-II though maximum infection (10.28 ± 1.01 %) was observed on D12 (PC). No parasite was observed in blood smear after D18 and all the animals of immunized group survived the challenge. Parasite appeared on D2 [post infection (PI)] in GP-I mice and typical *P. berghei* infection pattern was observed. Maximum infection of 43.56 ± 2.21 % was recorded on D8 (PI). All the animals of control group died by D9 (Fig. 1).

Immunophenotyping of various T cells

Mice of various groups were sacrificed on D0, D1, D3, D5, D6, D7, D9 and D20.

CD3⁺ cells

The percentage of CD3⁺ T cells was found to be significantly higher ($p < 0.005$) in immunized group as compared to controls. In TPA immunized blood, number of CD3⁺ cells was observed to be significantly increased after 24 h of infection and remained higher till D9 (PC). In control group, cell number increased significantly after 24 h of infection, followed by a decline on D7 (PI) after which all control mice died by D9 (PI) (Figs. 2, 3).

Fig. 1 Course of parasitaemia in GP-I and GP-II groups of mice after infection with 1×10^6 *P. berghei* infected cells. The parasitaemia was determined by examining Giemsa- stained thin blood smears of individual mice daily after injection. Parasitaemia (mean + SD) of the immunized and control mice is plotted versus days post-infection

Analysis of T helper and T cytotoxic cells

T helper cells in blood of TPA immunized mice were found to be significantly ($p < 0.005$) elevated as compared to controls. In immunized mice, CD4⁺ T cell number was also found to be increased till D9 (PC) as compared to controls. After parasite clearance on D20, cells were significantly declined. Due to the weak Th2 immune response, all the mice of control group died, whereas, immunized mice exhibited higher frequencies of CD4⁺ T cells. It points to strong Th2 immune response leading to complete survival in this group. Increase in the CD4⁺ T cells in infected controls was also observed till D7 (PI) (Figs. 2, 3).

In TPA immunized mice, statistically significant ($p < 0.005$) higher percentage of CD8⁺ T cells was recorded as compared to controls. In immunized group, percentage of CD8⁺ T cells was found to be significantly high till D3 (PC), whereas, in infected controls, CD8⁺ T cells were found to be high till D7 (PI) and declined on D9 (PI). After parasite clearance (D20) the percentage of CD8⁺ T cells was found to be declined in GP-II (Figs. 2, 3).

T suppressor cell analysis

In GP-II mice, the frequencies of CD4⁺ Treg cells were found to be significantly ($p < 0.005$) declined as compared to controls. In immunized group percentage of CD4⁺ Treg cells were found to be reduced on D6 (PC), whereas, in controls it increased on D1 (PI) and remained so till all mice of control group died. In GP-II, higher numbers of CD4⁺ Treg cells were observed on D6 (PC) which declined on D9 (PC). It points towards the stimulation of humoral immune response as evident from increased CD4⁺ T cells which decreased after D20 (Fig. 4).

Discussion

During blood-stage *P. chabaudi* infection in mice, a switch from a Th1 to a Th2 response takes place, which is necessary for host survival and cure of the infection. The cellular composition of peripheral blood is drastically altered around peak parasitaemia, with a sharp decrease in both frequencies and absolute numbers of CD3⁺ T cells (Helmby et al. 1998). Frequencies of CD3⁺ T cells were found to be significantly ($p < 0.005$) decreased with rise in infection in controls as compared to immunized in present study too.

CD4⁺ T cells have been reported to mediate protective immunity to control blood stage parasites especially in parasite clearance (Wipasa et al. 2002). During self clearing of *P. chabaudi* infection, a 50 % decrease was

Fig. 2 Flow cytometry analysis showing the percentage of CD3⁺, CD4⁺ and CD8⁺ cells in blood of normal and *P. berghei* (NK-65) infected BALB/c mice on various days of experiments. D0 (A, B, C)-Normal, D1-D9 (D-R) *P. berghei* infected

observed in number of CD4⁺ T cells on D9 to D18 (PI). Dick et al. (1996) revealed that the malaria antigens induced expansion of CD4⁺ cells from malaria non-

exposed individuals. Higher CD4⁺/CD8⁺ T-cell ratios in the malaria-exposed individuals result from stimulation with live intact parasite antigens.

Fig. 3 Flow cytometry analysis showing the percentage of $CD3^+$, $CD4^+$ and $CD8^+$ cells in blood of TPA immunized BALB/c mice on various days of experiments. D0 (E)-Pre-challenge, D1-D9 (F- I)-Post challenge, D20 (J)-After parasite clearance

Fig. 4 CD4⁺ T-reg analysis of blood of normal, *P. berghei* (NK-65) Infected (GP-I) and TPA immunized (GP-II) BALB/c mice on various days. Normal D0 (A); Infected D1, D3, D5, D7 (C, D, E); Pre challenge D0 (H); Post challenge (I, J); After parasite clearance D20 (K)

Increased frequencies of CD4⁺ and CD8⁺ T cells and their respective cytokines in spleen of TPA immunized mice have been reported earlier in our laboratory too (Kumar et al. 2015). The number of CD4⁺ T cells was also observed to be significantly ($p < 0.005$) increased till D7 (PI) and declined on D9 (PI) leading to death of all the mice in infected control group. Whereas, in TPA immunized mice, CD4⁺ T cells significantly ($p < 0.005$) increased on D1 (PC) and remained so till the parasite was cleared from blood and all mice survived the challenge. The consequences of increased number of CD4⁺ cells in *P. vivax* infected people render a proinflammatory environment including induction of nitric oxide synthase, antimicrobial peptides and other cytokines leading to a favorable immune response against the parasite (Imai et al. 2010).

Lau et al. (2011) reported 30 % splenic CD8⁺ T-cells in blood stage *P. berghei* ANKA infection. These cells are responsible for primary parasite specific CTL response. In previous studies also lower percentage of CD8⁺ peripheral blood lymphocytes was reported indicating that immune suppression is more pronounced in *P. falciparum* than *P. vivax* infection. During *P. falciparum* infection CD8⁺ cells have been reported to decrease in both percentage and absolute number (Lau et al. 2011).

CD8⁺ T cells provide protection in mice from asexual stages of *Plasmodium* via secretion of IFN- γ and its cytotoxic activity. IFN- γ from CD8⁺ T cells activates the macrophages leading to the phagocytosis of parasitized erythrocytes (Imai et al. 2010). In present study also, TPA immunized mice showed significant increase in the frequencies of CD8⁺ T cells in blood as compared to infected controls indicating the activation of cytotoxic T cells and preventing infection by secreting their respective cytokines. Earlier in our study, the frequencies of CD8⁺ T cells and IFN- γ were also found to be high in TPA immunized serum of mice, providing complete protection against parasite (Kumar et al. 2015).

During *Plasmodium* infection, Treg cells have been shown to inhibit effector T cell responses resulting in inadequate immune response to the pathogen. A tight balance between CD4⁺Foxp3⁺ regulatory T cells and effector CD4⁺ Th1 cells is necessary to effectively control and eliminate *Plasmodium* infection (Ho et al. 1995). In correlation with above findings, our study showed that infected controls had higher number of CD4⁺ Treg cells till D9 (PI), whereas, in TPA immunized group, it was significantly ($p < 0.005$) decreased on D6 (PC). After parasite clearance (D20), significant increase was found in frequencies of CD4⁺ Treg cells.

In conclusion, our data showed that humoral as well as cell mediated immune responses were stimulated in lethal *P. berghei* (NK-65) infected mice. TPA immunized group

of mice showed generation of effective humoral as well as cell mediated immune response which was confirmed by the continuous increase in the frequencies of helper T cells and cytotoxic T cells leading to complete protection. However, Treg cells were declined in immunized group, whereas, these increased in control group.

Acknowledgments It is a pleasure to acknowledge the technical work of CSIF for immunophenotyping by flow cytometer (FACS Calibar B.D.) at PGIMER, Chandigarh. Authors are also thankful to University Grant Commission, New Delhi for providing financial assistance under UGC-Major Research Project and UGC-CAS programme of Zoology department Panjab University, Chandigarh.

Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

References

- Bagai U, Pawar A (2013) A blood stage fraction of *Plasmodium berghei* induces protective and long lasting immune response in BALB/c mice. *Parasitol Int* 62:329–336
- Banyal HS, Pandey VC, Dutta GP (1979) Subcellular fractionation and localization of marker enzymes of erythrocytic stages of *Plasmodium knowlesi*. *Ind J Parasitol* 3:9–14
- Bierer BE, Sleckman BP, Ratnosky SE, Burakoff SJ (1989) The biologic roles of CD2, CD4 and CD8 in T cell activation. *Annu Rev Immunol* 7:579–599
- Czuprynski CJ, Brown JF (1998) In vitro analysis isolation and preparation of lymphocytes from infected animals. In: Kaufmann SHE, Kabelitz D (eds) *In immunology of infection*. Acad Press, New York, pp 189–194
- Dick S, Waterfall M, Currie J, Maddy A, Riley E (1996) Naive human alpha beta T cells respond to membrane-associated components of malaria-infected erythrocytes by proliferation and production of interferon-gamma. *Immunology* 88:412–420
- Helmby H, Kullberg M, Troye-Blomberg M (1998) Expansion of IL-3-responsive IL-4-producing non-B non-T cells correlates with anemia and IL-3 production in mice infected with blood-stage *Plasmodium chabaudi* malaria. *Eur J Immunol* 28:2559–2570
- Ho M, Sexton MM, Tongtawe P, Looareesuwan S, Suntharasamai P, Webster HK (1995) Interleukin-10 inhibits tumor necrosis factor production but not antigen-specific lymphoproliferation in acute *Plasmodium falciparum* malaria. *J Infect Dis* 172:838–844
- Hori S, Nomura T, Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057–1061
- Imai T, Shen J, Chou B, Duan X, Tu L, Tetsutani K, Moriya C, Ishida H, Hamano S, Shimokawa C, Hiseada H, Himeno K (2010) Involvement of CD8⁺ T cells in protective immunity against murine blood-stage infection with *Plasmodium yoelii* 17XL strain. *Eur J Immunol* 40:1053–1061
- Kemp K, Akanmori BD, Adabayeri V, Goka BQ, Kurtzhals JA, Behr C, Hviid L (2002) Cytokine production and apoptosis among T cells from patients under treatment for *Plasmodium falciparum* malaria. *Clin Exp Immunol* 127:151–157
- Kumar V, Rakha A, Saroa R, Bagai U (2015) CD4⁺T cells expansion in *P. berghei* (NK-65) infected and immunized BALB/C mice. *J clin Exp Pathol* 5:3
- Lau LS, Fernandez Ruiz D, Davey GM, de Koning-Ward TF, Papenfuss AT, Carbone FR, Brooks AG, Crabb BS, Heath WR

- (2011) Blood-stage *Plasmodium berghei* infection generates a potent, specific CD8+ T-cell response despite residence largely in cells lacking MHC I processing machinery. *J Infect Dis* 204:1989–1996
- Lowry OH, Rasebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with phenol reagent. *J Biol Chem* 193:265–275
- Nie CQ, Bernard NJ, Schofield L, Hansen DS (2007) CD4 + CD25 + regulatory T cells suppress CD4+ T-cell function and inhibit the development of *Plasmodium berghei*-specific TH1 responses involved in cerebral malaria pathogenesis. *Infect Immun* 75:2275–2282
- Santiyanont R (1985) Parasite identification, counting and staining. In: Panyim S, Wilairat P, Yuthavong Y (eds) Application of genetic engineering techniques in tropical diseases, pathogens with special reference to plasmodia. A laboratory manual of selected techniques. UNDP/WorldBank/WHO special program for research and techniques in tropical diseases, Bangkok, pp 413–448
- Taylor-Robinson AW (1995) Regulation of immunity to malaria: valuable lessons learned from murine models. *Parasitol Today* 11:334–342
- Thornton AM, Shevach EM (1998) CD4 + CD25 + immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287–296
- Troye-Blomberg M, Berzins K, Perlmann P (1994) T-cell control of immunity to the asexual blood stages of the malaria parasite. *Crit Rev Immunol* 14:131–155
- Wipasa J, Elliott S, Xu H, Good MF (2002) Immunity to asexual blood stage malaria and vaccine approaches. *Immunol Cell Biol* 80:401–414