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# The Effect of Bacille Calmette-Guerin (BCG) on the Changes in Number and Functional Activities of Mononuclear Phagocytes in Malaria- infected Mice Model

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## ABSTRACT

**Introduction:** Recent studies have indicated that Bacille Calmette-Guerin (BCG) vaccination may have beneficial effect on the survival of infant living in malaria endemic area as well as of malaria-infected mice model. However, the impact of injection of BCG vaccine on the changes in number and functional activities of Mononuclear Phagocytes during malaria-infection in animal model is still poorly understood.
**Objective:** To evaluate the effect of BCG on the changes in number and functional activities of Mononuclear Phagocytes (MPs) during *Plasmodium berghei* infection.
**Methods:** Two groups of 18 Swiss mice were used. The first group was given 0.1 ml of BCG injection subcutaneously and the second was the control non-BCG group. One week after BCG injection, all mice in both groups were inoculated with $10^7$ *Plasmodium berghei* infected erythrocytes. The parasitaemia were monitored daily and the number and functional activity of splenic and peritoneal macrophages were tested.
**Results:** The parasites were detected in the blood of both groups on the first day after infection. The parasitaemia in the control group grew slowly until day 3, followed by rapid increased up to 38.96% on day 9. Parasitemia of mouse which still alive on day 12 was 59.6%. The parasitaemia of BCG-injected mice were also increased at lower rate after day 3, and the mice still survive until day 15 after infection. The number of peritoneal macrophages from BCG-injected mice increased to a higher degree compared to the non-BCG injected mice. Moreover, the phagocytic activities of peritoneal macrophages in BCG injected group were increased higher up to twice (200%) of normal levels compared to the non-BCG control group which increase only up to 1,5 times (150%) of the normal levels.
**Conclusion:** The injection of BCG on *Plasmodium berghei* infected Swiss mice resulted in the extension of survival of the mice until day 15, accompanied by higher increased in number of circulating blood, splenic and peritoneal MPs, and the phagocytic activities of peritoneal MPs up to 137% of the increased in non-BCG mice.

**Keywords:** BCG vaccine, malaria, *Plasmodium berghei,* macrophages, phagocytosis.

## INTISARI

**Pendahuluan:** Beberapa penelitian sebelumnya telah menunjukkan bahwa vaksinasi *Bacille Calmette-Guerin* (BCG) memiliki efek yang menguntungkan seperti terhadap jangkauan hidup bayi yang tinggal di daerah endemis malaria, dan juga jangkauan hidup hewan coba mencit sebagai binatang model yang diinfeksi malaria. Namun demikian, dampak pemberian dari vaksin BCG pada terhadap jumlah dan aktivitas sel fagosit pada mencit model yang diinfeksi malaria tersebut masih kurang dipahami.
**Tujuan:** Untuk mengevaluasi efek pemberian BCG terhadap perubahan jumlah dan aktivitas fungsional sel fagosit mononuklear (Mononuclear Phagocytes=MPs) selama diinfeksi dengan *Plasmodium berghei*.
**Metode:** Penelitian ini menggunakan dua kelompok kelompok mencit swiss yang masing-masing

terdiri dari 18 ekor. Kelompok pertama diberi suntikan 0,1 ml BCG secara subkutan dan kelompok kedua merupakan kelompok kontrol tanpa diberi suntikan BCG. Satu minggu setelah suntikan BCG, semua tikus di kedua kelompok diinokulasi dengan $10^7$ eritrosit terinfeksi *Plasmodium berghei*. Parasitemia dipantau setiap hari dan jumlah serta aktivitas fungsional sel makrofag dievaluasi.
**Hasil:** Parasit terdeteksi dalam darah dari kedua kelompok mencit pada hari pertama setelah infeksi. Parasitemia pada kelompok kontrol meningkat perlahan sampai hari 3, diikuti dengan peningkatan cepat hingga 38.96% pada hari 9. Parasitemia pada mencit yang masih hidup pada hari ke 12 adalah 59,6%. Parasitemia pada kelompok mencit yang diinjeksi BCG meningkat dengan kenaikan lebih rendah setelah hari 3, dan mencit masih bertahan sampai hari 15 setelah infeksi. Jumlah sel MPs dalam rongga peritoneum pada kelompok mencit yang diberi BCG meningkat lebih tinggi dibandingkan dengan pada kelompok mencit yang tidak diberi suntikan BCG. Selain itu, prosentase sel makrofag rongga peritoneum yang memfagositosis partikel latex pada kelompok mencit yang diberi suntikan BCG meningkat tinggi (hingga 2 kali dari keadaan normal) dibandingkan dengan kelompok kontrol non-BCG yang meningkatkan hanya sampai 1,5 kali.
**Simpulan:** suntikan BCG pada mencit yang diinfeksi *Plasmodium berghei* mengakibatkan perpanjangan kelangsungan hidup mencit hingga 15 hari, disertai dengan tingginya kenaikan jumlah sel Mononuclear Phagocytes dalam limpa, rongga peritoneum dan dalam sirkulasi darah, disertai dengan adanya kenaikan aktifitas fagositik sel MPs rongga peritoeum hingga 137 % dibandingkan pada kelompok mencit yang tidak divaksinasi BCG.

**Kata kunci**: vaksin BCG, malaria, *Plasmodium berghei*, makrofag, fagositosis

## INTRODUCTION

Bacille Calmette-Guerine (BCG) immunization to children under five years old is a common national policy in developing countries where TBC cases are still relatively high including in Indonesia. These policy has long been implemented with the objective to prevent children from and to control the spread of tuberculosis in the community. The diseases usually affects people with moderate to low social and economic status. It has been reported that one to three million people are killed because of this diseases[1]. In the last decade an increased in number of TB cases has been noticed in most of developing countries, which are influenced by some factors such as the development of new and more simple and rapid diagnostic tools, the increased of community knowledge and educational status related to the increased in awareness to their health status and the most prominent one is due to the emergence of TB drug resistance. Therefore, the effort in combating tuberculosis have been re-activated in most of developing countries through the widenned of TB vaccination coverage, more accurate diagnosis, community health education, and promote the quality of treatment.

Despite tuberculosis, malaria is also represent another major public health problem in Indonesia and other tropical countries. Most of Indonesian population are resident of areas where malaria are endemic or high risk for malaria infection. Malaria also affect people with low social and economic status and causes much burdent and death especially in children and pregnant women[2]. Efforts in controlling malaria have been conducted through many different ways include the implementation of effective surveilance system, early diagnosis, improve case management and prompt treatment, vector control and prevention. However, some epidemic attack have been reported in some areas, indicating that malaria control programme

still need to be evaluated and improved. Some effort on malaria prevention that have been conducted are by some vector control such as insecticide residual spraying (IRS), insecticide impregnated bed net or long lasting impregnated net (LLIN), however, malaria prevention by mean of vaccination seem still to be in a long waiting from reallity. In developing tropical countries like Indonesia BCG vaccination are given to all children where they also lives in malaria endemic areas, and the impact of BCG vaccination on the burden due to malaria infection have never been studied[3].

Roth *et al*[4] have reported their epidemiological studies in children living in malaria endemic area in Guinea Bisau, where tuberculosis is still wide spead among population and vaccination programme in childrens relatively have a good caverage in the country[4]. These studies concluded that children who have BCG scar have a longer survival as compared to children who did not have BCG scar. Studies in rodent malaria have also been conducted using mice infect with *P. berghei*, and it was shown that injection of $10^7$ of *Mycobacterium bovis* BCG strain into mice prevent *P. berghei* sporozoite infection[5]. Similar studies using rodent malaria model of *Plasmodium yaelii* have also been conducted and the result showed that injection of BCG resulted in the extention of survival of A/J mice infected with lethal strain of *P.yoelii*[6].

Preliminary studies have been carried-out in our laboratory to investigate the effect of BCG injection on the changes in the numbers of progenitor cells in the bone marrow of Swiss mice, and the result indicated that injection of BCG into Swiss mice infected with *P. berghei* resulted in the higher increased in number of mononuclear phagocyte progenitor in the bone marrow and higher increased in number of circulating blood monocytes as well as blood lymphocytes during the infection[7].

The objectives of this study are to evaluate the effect of BCG on the changes in the number and functional activities of mature mononuclear phagocytes in the peritoneal cavity of mice infected with *P. berghei*.

## MATERIALS AND METHODS

Two groups of 15 female, 8 weeks old Swiss mice (from the Department of Parasitology, Faculty of Medicine UGM) were used in the experiment, the first group were injected with 0,1 ml of BCG (Bio Farma, Bandung, Indonesia) containing 0.9375 $\mu^2$g of BCG into subcutaneous tissue at the proximal site of the right hind leg. The other group was the control group without BCG injection. One week after BCG injection all mice in the two group were infected with $10^7$ *P. berghei* infected erythrocytes intraperitoneally. The rodent malaria model of *P. berghei* used was the ANKA strain originated and maintain at the Department of Parasitology Faculty of Medicine, Universitas Gadjah Mada. The parasitaemia of all mice were monitored daily by microscopic examination of thin blood smears prepared from the tail blood of each mice. On days 0 (before *P. berghei* infection), day 3, day 6, day 9 and day 12 after infection, 3 mice from each groups were sacrifized using deep narcose, macrophages were isolated from the peritoneal cavity of each mice, washed 3 times using RPMI solution and the numbers of peritoneal cell were counted.The peritoneal cells were then cultured in the wells of 24 well plates containing coverslips at the density of $10^5$ cells/well. The plate were incubated in 5% $CO_2$, at 37$^o$C for 24 hours and on the following day the plate were wahed using pre-warmed RPMI to remove non-adherent cells and the remaing adherent peritoneal macrophages were further cultured and ready for functional activity assay.

### Phagocytosis Assay

On the following days the cultured cells

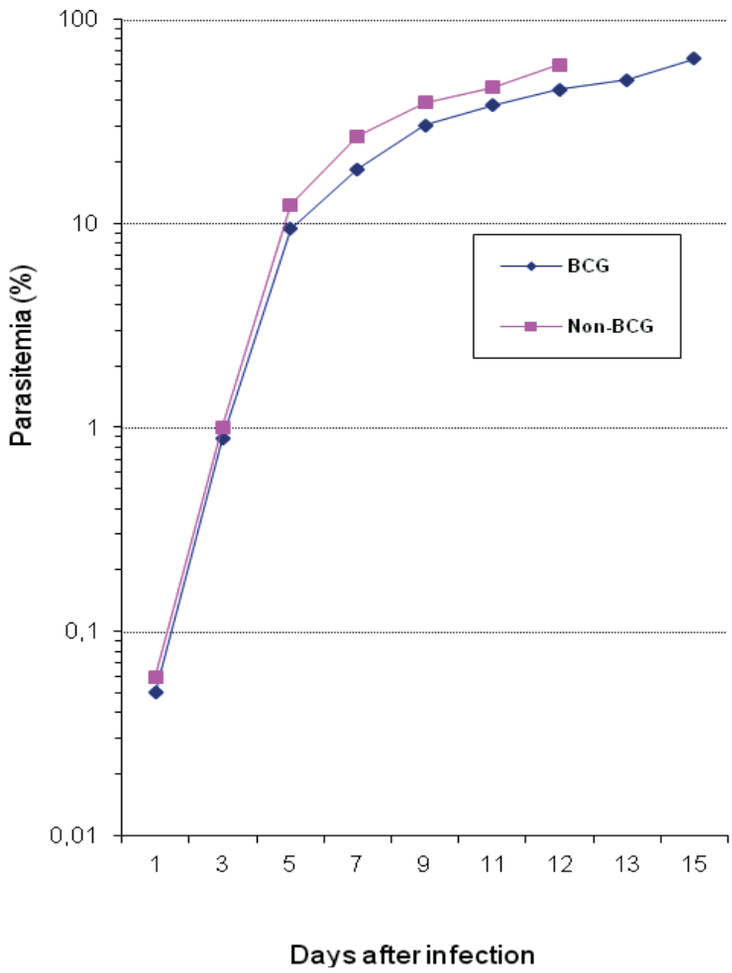


Figure 1. Parasitemia of Swiss mice previously injected with BCG or without BCG during *P. berghei* infection

were washed 3 times using pre-warmed RPMI medium to washed-out non-adherent cells and the remaining adherent cells were mostly monocyte-macrophages lineage. After washing, 300 µl of RPMI containing $10^6$ latex beads (2-3 µm diameter) were overlayed on each well of cultured cells and the plate were further incubated at 37°C in 5% $CO_2$ incubator for 60 minutes. After incubation time was completed all unphagocytosed latex particles were washed-out by vigorous pipetting and repeated three times with PBS. The cells on the coverslips were then fixed with absolute methanol for 1 minutes and after drying the cells were stainned with 5% Giemsa for 20 minutes. The cells were then washed using distilated water and the coverslips were taken-out from wells and air dried. Coverslips were then fixed on glass slide and examined under the microscope. The number of cells that engulfing latex particles and the average numbers of latex beads phagocytosed per-cells were counted.

## RESULTS AND DISCUSSION

**Evaluation of parasitaemia of the mice.** Parasitaemia of the experimental mice were monitored daily by preparing thin blood smears of both BCG and non-BCG injected mice. The percentage of infected red blood cells were calculated from the number of infected red blood

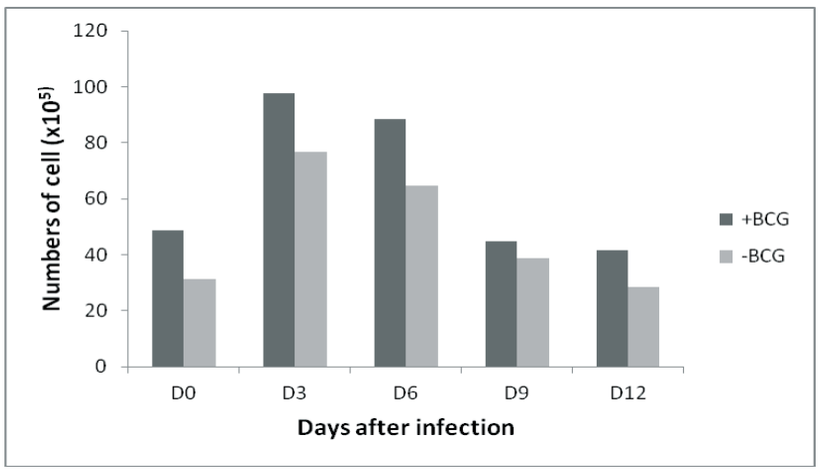


Figure 2.Changes in numbers of peritoneal macrophages of mice previously injected with BCG or not, during *P.berghei* infection.

cell found within at least 1000 erythrocytes during microscopic examination of mouse thin blood smears. During the study the clinical condition of all experimental mice in all groups were monitored dailly, and any changes on behavior of physical condition such as rigorous body, low body temperature, pale and weakness of the mouse, all were recorded. During the study two mice from non-BCG injected mice were died on day 10 after infection and one mouse that still servive on day 12 was clinically very ill, with weak appearance, pale, rigorous, body temperature low and the parasitemia was 59.6% ; whiles the mice in BCG-injected group which still survive on day 15 were not expressing significant clinical manifestation. The average parasitaemia of those mice was about 38%. Simillar results was also been reported where injection BCG into mice also resulted in the prolonge of the survival of mice infected with P. *yoelii* strain 17 XL which in mice usually produced fatal infection[6]. The result of the parasite count is presented in figure 1.

As shown in Figure 1, the parasitemia of mice previously injected with BCG increased in a slightly lower degree as compared to the control group that are without injection of BCG. Although this difference was statistically not significant, however, injection of BCG in the experimental group seem resulted in slight prolonge of the mouse survivals as indicated by the earlier dead of 2 mice in the control non-BCG group on day 10 after infection, as compared to the experimental group where all three mice still survive on day 12 after infection without significant appearance of clinical manifestation. The mechanism how BCG injection may prolong the survival of the lethally infected mice still need to be studied in more detail. Increased survival that corelated with BCG was also observed in an epidemiological study in children living in malaria endemic area in Guinea Bisau[4]. Injection of BCG probably resulted in the stimulation or activation of reticuloendothelial cells component including macrophages which in turn may resulted in the increased of some fuctional effector activities of these cell to combate the parasite and therefore increasing it survival the mice.

**Changes in number of peritoneal macrophages.** Changes in numbers of peritoneal macrophages of mice during *P. berghei* infection

is evaluated on day 0 (just before infection) day 3, 6, 9 and 12 after infection in both BCG injected and non-BCG injected mice. As much as 6-8 ml of cold RPMI was injected into peritoneal cavity of each mice after exising the skin covering the peritoneum. Gentle abdominal masage was carried out to let the cells enter the medium and then aspiration was done using the same spuit injection to take out the medium and cells as much as possible. Suspension of peritoneal cell was washed 3 times by centrifugation in RPMI medium, and the number of peritoneal cells yield from each mice were counted in haemocytometer chamber. After last washing the cells were resuspended in culture medium and incubated at 37$^{o}$C, 35% $CO_2$ overnight. The changes in numbers of peritoneal macrophages during the course of *P. berghei* infection in BCG and non-BCG injected mice are shown in Figure 2. As shown in Figure 2, the number of peritoneal macrophages increased during early stages of infection i.e. on day 3 and day 6 after infection both in BCG injected and non-BCG injected mice. The increased of the number of peritoneal macrophages was significantly higher in BCG injected mice compared to the control non-BCG mice ($P<0.05$). During later stages of infection, the number of peritoneal macropohages decreased back to normal level from day 9 to 12 after infection.

These increased in the number of macrophage in the peritoneal cavity during *P. berghei* infection are in accordance with the well-known hall-mark of malaria infection which is consisted of marked splenomegaly and macrophage hyperplasia within the spleen dan other organ. Splenomegaly is the enlargement of spleen that clinically sometimes can be observed and in human physically can be examine by palpating the abdominal wall under the left castae arcus. Splenomegaly is also a common feature in the population of high malaria endemic areas. In holo-endemic malaria areas the enlargement of spleen may very extensive and may reached down to umbilicus or even reached to right ischia iliaca. Spleen represent an important immune organ during malaria infection. This organ function as a filter to trapped and killed the malaria infected red blood cells which passing this organ. During the life, the blood cells circulated throughout all the organs and tissues including the spleen. When erythrocytes reach the splenic arteriolae end, the cells passing through spleenic pulp via inter cellular space in which many lymphoid cells such as B lymphocytes, T lymphocytes and more importantly activated macrophages are abundance in numbers and in a ready stage to phagocytosed infected cells and to secreted toxic products such as reactive oxygen intermediates or reactive nitrogen intermediates that can killed the nearby infected cells as well as free parastes.

**Changes in functional activity of macrophages.** Functional activities of peritoneal macrophages that was examined in the study was phagocytosis activity using latex paticles which is represent non-specific phagocytosis activity of the cells. Extracted peritoneal macrophages from both BCG and non-BCG injected mice were cultured on coverslips in 24 well culture plate. Following 24 hour incubation, the cell were overlayed with 10 times cell numbers of latex beads and further incubated at 37$^{o}$C, 35% $CO_2$ for 60 minutes. The cells were then washed by vigorously pipetting with PBS to remove all the unphagocytosed particles. After fixation with absolute methanol the cells were Giemsa stainned for 15 minutes. Cells were then washed with tap water, the coverslips were taken out from the wells and dried at room temperature and put on glass slide with parafin gel and finally examined under the microscope. The cells phagocytossing latex particles will be clearly

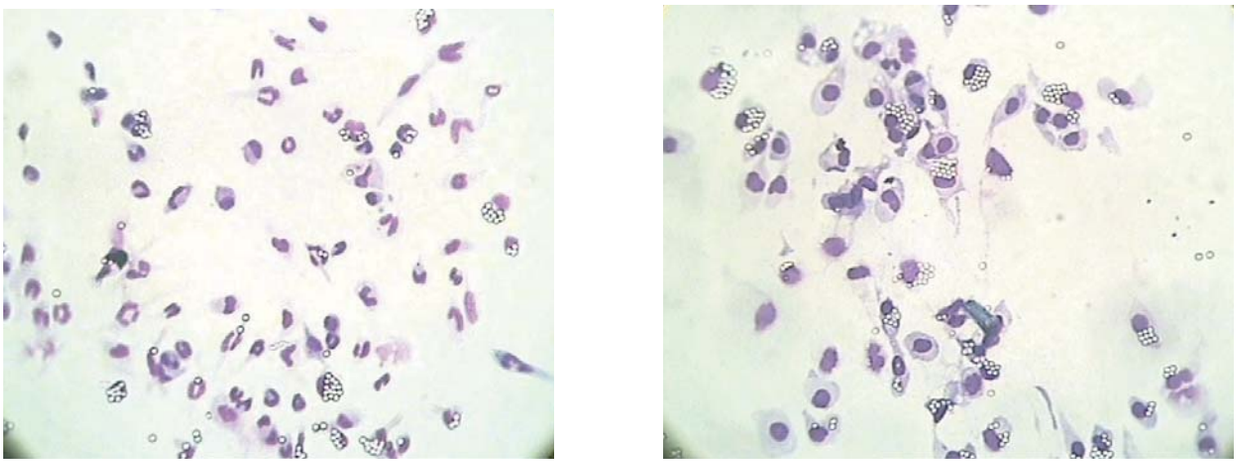

Figure 3. Peritoneal macrophages from BCG injected (left) and non-BCG injected (right) mice phagocytosing latex particles.

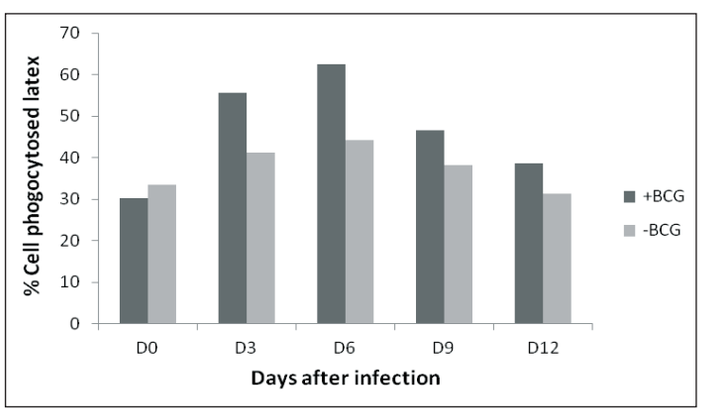


Figure 4. Changes in the percentage of peritoneal macrophages of mice previously injected with BCG or not phagocytosing latex particles during *P. berghei* infection

seen having a number of latex particles within its cytoplasm. As can be seen in Figure 3, the differences in phagocytosis activity of peritoneal macrophages from BCG and non BCG injected mice are distinct.

As seen in Figure 3, the majority of peritoneal macrophages from BCG injected mice are phagocytosing latex particle as compared to the peritoneal macrophages from control non BCG group, furthermore, if we count the average number of latex particles in each cells it is also clear the the macrophages from BCG injected mice phagocytosed higher number of latex particles as compared to the cells from control non-BCG mice. These condition probably also indicated that macrophages in BCG injected mice are more activated following the BCG injection and when these cells were exposed to latex particle the performed phagocytosis activity and engulfing more latex particle, on the other hand the cells from non BCG injected mice are probably still in resting stages and therefore the

only phagocytosing less latex particles.

To quantify the increase of the phagocytosis activity of the cell during the course of *P.berghei* infection can also be done by calculating the percentage of cell both from BCG and non BCG injected mice which phagocytosing latex particles, and the result are shown in Figure 4.

As shown in Figure 4. the percentage of peritoneal macrophages phagocytosisng latex particles increased higher in BCG injected mice since day 3 post infection, reaching a peak on day 6 then goes down back to normal value on day 12 post infection. In BGC injected mice the percentage of peritoneal macrophages phagocytosing latex particles increased on day 6 until more than twice (200%) of the normal level. In the non-BCG control mice the percentage of cell phagocytosing latex particles also increased on day 6 after infection but only about 1,5 times (150%) of the normal levels, then goes down again to normal levels on day 9 post infection. This figure probably also indicated that during early stages of malaria infection parasite antigen begin to stimulate immune responses that lead to the increased of phagocytosis activity of macrophages in the spleen and other compartment including peritoneal macrophages. These increased of responses are the effort of the host immune system to combat and to eliminate the invading parasites by secreting cytokine such as interferon gamma by T lympocytes which will stimulate and activated resting macrophages anywhre in the body to became more activited and ready to caryout its effector function. Increased secretion of reactive oxygen intermediate by macrophages during early stages of malaria infection have also been reported in *P. vinkei petteri* infection in mice[8]. These increased of functional activities were also been demonstrated in mice stimulated by immunization using crude *P.vinckei petteri* blood stage antigens one week prior to homologus parasite infection[8]. Spleen which consisted of many different white blood cells are usually enlarged during rodent malaria infection. Masive mononuclear phagocyte progenitor proliferation are also occur in the bone marrow in order to fullfilled the increased demand of mature cells to combate and eliminate the invading malaria parasite in the blood circulation as well as in other organs. Spleenic macrophages of malaria infected mice usually also full of dark brown malaria pigment, indicating the increased of phagocytosis activity of those cell populations.

## CONCLUSION

All of these finding indicated that macrophages plays important role in the defence against malaria infection, and finally from all the above data can be concluded that : (1) Injection of BCG into Swiss mice infected with *P. berghei* resulted in the increase of parasitaemia at lower stages as compared to the control non-BCG injected mice. (2) During *P. berghei* infection the number of peritoneal macrophages increased at higher levels on day 3 and 6 after infection as compared to control non-BCG injected mice. (3) During *P. berghei* infection the percentage of peritoneal macrophages phagocytosing latex particles increased higher on day 3 and 6 after infection as compared to control non-BCG injected mice.

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