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Experimental Study / Deneysel Çalışma

Splenectomy and *Salmonella Typhi* Exposure Lead to Quantitative Alterations of Bone Marrow B220$^+$ Cells and CD4$^+$ T Cells on BALB/c Mice

Splenektomi ve Salmonella Typhi Maruziyeti BALB/c Farelerde Kemik İliği B220$^+$ Hücreleri ve CD4$^+$ T Hücrelerinin Kantitatif Değişimine Neden Olur

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Objectives: This study aims to determine the quantitative alterations of relative density of bone marrow B220$^+$ cells and CD4$^+$ T cells in BALB/c mice which underwent splenectomy and exposed to *Salmonella typhi (S. typhi).*

Materials and methods: Splenectomy was performed under ketamine anesthesia (65 mg/kg BW). Exposure of *S. typhi* was performed by injection of acute doses (10^9 cells/mL) at two weeks after splenectomy and repeated at two weeks after the initial dose. The relative density of bone marrow B220$^+$ cells and CD4$^+$ T cells were quantified by BD FACSCalibur™ flow cytometer. Statistical analysis was performed using two-way ANOVA and Tukey HSD test. A p value of <0.05 was considered significant.

Results: All treatments had significant effects on observed variables. Splenectomy significantly depleted the relative density of B220$^+$ cells to 5.51% compare with controls (17.34%). *Salmonella typhi* exposure alone did not significantly alter the relative density of B220$^+$ cells (17.92%). Splenectomy and *S. typhi* exposure decreased B220$^+$ cells to 5.90%. CD4$^+$ T cells had various responses to all treatments. Compared to the controls (0.28%), splenectomy significantly increased the relative density of CD4$^+$ T cells up to 0.43%. *Salmonella typhi* exposure alone and splenectomy in combined with *S. typhi* exposure significantly increased the relative density of CD4$^+$ T cells up to 0.55% and 0.50%, respectively.

Conclusion: These results indicated that splenectomy was the leading factor affecting the relative density of B220$^+$ cells, rather than *S. typhi* exposure, whereas *Salmonella typhi* exposure was the main factor which increased the relative density of CD4$^+$ T cells. This phenomenon also reflects the pathological condition due to *S. typhi* exposure.

Key words: B220$^+$ cells, CD4$^+$ T cells, *Salmonella typhi*, splenectomy.

Amaç: Bu çalışmada splenektomi yapılan ve *Salmonella typhi'ye (S. typhi)* maruz bırakılan BALB/c farelerde kemik iliği B220$^+$ hücreleri ve CD4$^+$ T hücrelerinin rölatif yoğunluğundaki kantitatif değişiklikler incelendi.

Gereç ve yöntemler: Ketamin anestezisi altında splenektomi yapıldı (65 mg/kg BW). Splenektomiden iki hafta sonra akut doz enjeksiyonu ile *S. typhi* maruziyeti yapıldı (10^9 hücre/mL) ve doz, ilk dozdan iki hafta sonra tekrarlandı. Kemik iliği B220$^+$ hücreleri ve CD4$^+$ T hücrelerinin rölatif yoğunluğu, BD FACSCalibur™ akım sitometri ile belirlendi. İki yönlü ANOVA ve Tukey HSD testi ile istatistiksel analiz yapıldı. P<0.05 değerleri anlamlı kabul edildi.

Bulgular: Tedavilerinin tümü, gözlenen değişkenler üzerinde anlamlı etkilere sahipti. Splenektomi,B220+ hücrelerin oranınını splenektomi yapılmayan farelerdeki %17.34 oranından %5.51'e anlamlı olarak düşürdü. *Salmonella typhi* maruziyeti tek başına B220$^+$ hücrelerinin rölatif yoğunluğunu anlamlı düzeyde değiştirmedi (%17.92). Splenektomi yapılması ve *S.typhi* ile karşılaşma B220+ hücreleri %5.9'a düşürdü. CD4$^+$ T hücreleri tüm tedavilere karşı farklı yanıtlar verdi. Kontrollere kıyasla (%0.28), splenektomi CD4$^+$ T hücrelerinin rölatif yoğunluğunu anlamlı düzeyde %0.43'e kadar artırdı. *Salmonella typhi* maruziyeti tek başına ve splenektomi ile birlikte, CD4$^+$ T hücrelerinin rölatif yoğunluğunu sırası ile %0.55 ve %0.50'ye kadar anlamlı düzeyde artırdı.

Sonuç: Bu bulgular, *S. typhi* maruziyetine kıyasla, splenektominin B220$^+$ hücrelerinin rölatif yoğunluğunu etkileyen başlıca etmen olup, *S. typhi* maruziyeti CD4$^+$ T hücrelerinin rölatif yoğunluğunu artıran ana etmendir. Bu fenomen, *S. typhi* maruziyeti nedeni ile ortaya çıkan patolojik durumu yansıtmaktadır.

Anahtar sözcükler: B220$^+$ hücreleri, CD4$^+$ T hücreleri, *Salmonella typhi*, splenektomi.

The spleen is a secondary lymphoid organ that is important for many reasons. It is the place where antigen-exposed lymphocyte destruction and opsonin production occur. In addition, it also acts as a blood reservoir and is the main location for the reticuloendothelial system, humoral responses (both of T cell-induced activation and antibody production by plasma cells,[1] and macrophage microbicidal activity.[2] In cases involving leuchemy, splenomegaly, lymphoma, systemic lupus, and sickle cell anemia, the spleen functions abnormally, requiring a splenectomy or spleen removal.[3] In patients who have undergone a splenectomy, the absence of the spleen can lead to chaotic predisposition towards several infections, which are generally known as overwhelming post-splenectomy infections (OPSIs). These include bacterial pathogens such as *Streptococcus, Staphylococcus*, gram-negative pathogens, and viral infections like *Haemophilus influenza (H. influenza)*. They are also more susceptible to parasitic infections like Plasmodium spp.[4]

Salmonella typhi (*S. typhi*) is an intracellular gram-negative pathogen that is both facultative and cosmopolite in nature. It causes localized and systemic infection as well as an enteric fever known as typhoid, an endemic disease that is still a common health problem in developing countries. For instance, typhoid fever in Indonesia has an incidence rate of 350,810 cases per one million people and a mortality rate of 2%.[5] Eradication of intracellular pathogens such as Salmonella requires a cellular-mediated immunity response.[2] Lipopolysaccharide (LPS) of gram-negative bacteria is a potent inducer of innate and adaptive immune responses mediated by $CD4^+$ helper T cells. This response is the result of the phagosomal localization of Salmonella or its antigens in which the phagocytes acquire, process, and present pathogen-derived peptides in the context of major histocompatibility complex (MHC) class II molecules. These peptides then engage and activate $CD4^+$ T cells via the T-cell receptor (TCR). Another induced subset of immune cells is the $B220^+$ cells, which necessitate the humoral responses. These cells mediate the clearance of extracellular Salmonella from infected tissue by producing antibodies triggered by bacterial surface antigens such as LPS and flagellin.[6,7] A lack of these T cells along with antibody depletion or a lack of B cell functions leads to an increased susceptibility to Salmonella infection.[8-10]

Macrophage activation by the LPS-induced $CD4^+$ T cell subset causes the secretion of Th1 and $B220^+$ cells, which subsequently leads to the secretion of the T-helper cell type 2 (Th2) cytokine. This, in turn, kickstarts the cellular and humoral responses. Mukherjee et al.[11] discovered that this Th1 and Th2 cytokine secretion takes place at different levels while investigating a case of Salmonella infection in which she determined which immune pathway was activated. Although the cellular immune response is part of the first aid process for Salmonella infections, protection by the humoral response is needed for creating comprehensive protection. Prolonged inflammation due to the cellular-mediated immunity response also has side effects. Although the inflammation acts as a protective mechanism, it also causes tissue disruptions and leads to a disease state.[11,12] After a splenectomy, a patient is able to survive without a spleen because the liver takes over part of the work that was done by this organ. *Salmonella typhi* infection leads to a systemic infection that penetrates into the lymphoid tissues, causing the development of acute inflammation along with focal necroses, bleeding, and the perforation of several organs, including the spleen, liver, and gallbladder as well as the bone marrow.[5] The presence of acute and chronic inflammation in these organs could exacerbate the condition of patients who underwent the splenectomy; therefore, a control mechanism is needed to ensure balance in the immunity systems in order to know whether the cellular or humoral pathway has been activated. This is important since the humoral response is preferable in such conditions.

A loss of spleen function in the immune system also means the loss of large amounts of macrophage deposits, B cells, T cells, molecules, and opsonin, which performs various roles in the immunity process. Homeostasis is constantly occurring under these circumstances in an effort to compensate for the loss of the spleen in order to maintain immunity against antigens through lymphopoiesis. Components of the immune cells develop from pluripotent stem cells in the bone marrow through the lymphopoiesis process.[4,13]

In addition, B cells and $CD4^+$ T cells are critical for the clearance of Salmonella infection;[10,14] however, little is known about the response of these cells during OPSIs. Therefore, it is necessary to study the population of B cells and $CD4^+$ T cells in the bone marrow since that is not only the primary location in which lympophoiesis is carried out to compensate for the spleen removal, but it is where most *S. typhi* infections occur. This resembles the OPSI phenomenon that occurs in patients who undergo a splenectomy.

MATERIALS AND METHODS

Completely random factorial experiments were conducted with three replications in four groups: the control group, the splenectomy group, the *S. typhi* exposure group, and the splenectomy group followed

by *S. typhi* exposure. All experiments were performed according to institutional guidelines concerning the care and use of experimental animals.

Splenectomy

In this experiment, we used female, six-week old BALB/c mice and performed the splenectomy based on the method used by Teixeira et al.[15] The mice (Mus musculus BALB/c) were premedicated with a subcutaneous injection of atropine sulphate (0.04 mg/kg body weight).[16,17] After 10 minutes, they were injected intramuscularly with xylazine (12 mg/kg body weight) and then anesthetized with ketamine (65 mg/kg body weight) intraperitoneally. After the splenectomy, the mice were subsequently maintained in a pathogen-free chamber for two weeks until they were stable and ready for the next treatments. Peripheral blood counts also measured before the splenectomy and at one week and one month after the surgery.

Salmonella typhi preparation and exposure

The *S. typhi* specimens were from the microbiology laboratory of the Medical Faculty at the University of Brawijaya in Malang, Indonesia. These bacteria were rejuvenated and confirmed by salmonella-shigella (SS) and bismuth sulphite agar (BSA) plate cultures followed by gram-stain and catalase reactions (data not shown). The *S. typhi* were prepared for exposure according to the process used by Weinstein et al.[18] and employed at the infective log phase (A600=3.35-0.50; agitated in Luria-Bertani (LB) broth at 37 °C for 24 hours). The number of cells was determined by direct counting using a memocytometer. The *S. typhi* (10^9 cells/ml) were suspended in 100 µl of sterile phosphate buffered saline (PBS) and intraperitoneally injected for exposure treatment. This dosage was sufficient for acute infections and close to a lethal dose of *S. typhi*.[19] A booster was given a couple weeks after the first injections, and the mice were then assessed by noting all variables a week after the booster. The presence of *S. typhi* in the mice was also confirmed through blood cultures on SS and BSA agar plates followed by a catalase reaction test and a biochemical assay.

Bone marrow isolation and flow cytometry assay. Bone marrow cells were isolated from the femur by PBS flushing and filtered through a BD Falcon™ nylon cell strainer (BD Biosciences, San Jose, CA, USA) (100 µm). They were then collected into microtubes. The filtrate was centrifuged at 3200 rpm 4 °C for two minutes. The supernatant was discarded, washed once, and then centrifuged again to obtain a pellet of bone marrow cells, which was co-incubated with monoclonal, FITC-conjugated anti-mouse CD4 and PE-Cy7-conjugated Rat anti-mouse CD45R/B220 antibodies (BD Biosciences, San Jose, CA, USA) for 15 minutes. Next, the pellet was resuspended in 500 µl PBS and assessed via a BD FACSCalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The data was then processed using the BD CellQuest Pro™ software (BD Biosciences, San Jose, CA, USA) and statistically analyzed via two-way analysis of variance (ANOVA) (α=0.05). This was followed by a post-hoc Tukey's honestly significant difference (HSD) test to determine major changes and differences among the $B220^+$ cells and $CD4^+$ T cells relative to the density mean values.

RESULTS

The flow cytometry analysis of the bone marrow showed no significant increase in the relative number of $CD4^+$ T cells (0.43%; $p>0.05$) during the splenectomy treatment compared with the controls (0.38%). This corresponds with the study by Willführ and Westermann[20] which stated that the number of T cells was not affected by splenectomies, and there was no compensation for the absence of peripheral T cells. We also found a significant increase in the relative number of $CD4^+$ T cells due to the exposure to *S. typhi* (0.55%) and splenectomies that were followed by exposure to *S. typhi* (0.50%). This also corresponds with Wahyuni[21] who revealed a higher relative number of $CD4^+$ T cells in peripheral lymphoid organs of mice treated with *S. typhi*, although the difference was insignificant.

It is not known how an antigen that infects the tissue of lymphoid organs affects the way the immune response is generated because the same antigen can cause different immune responses in different organs.[22] Involvement of cellular and humoral immune responses in cases where infection occurs may feature a systemic response, which is mainly mediated by the liver, spleen, intestines, and gut-associated lymphoid tissues (GALT). *Salmonella typhi* can enter into mucosal tissues in that area, and after growing in the submucosal region of the liver and spleen, this bacteria progressively reaches the blood vessels and circulates throughout the body, even entering the bone marrow.[23]

When there are huge numbers of $CD4^+$ T cells in the bone marrow, this is anomalous and indicates infection. These cells also play a role in the elimination of intracellular pathogens such as *S. typhi*,[24] with flagellin (H antigen) of Salmonella being a specific target.[10] The doses used in our study (10^9 cells/ml) had a modulated immune response. In a study by Srinivasan et al.,[19] doses of 10^7-10^9 were able to increase the level of $CD4^+$ T cell clonal expansion and the expression of CD11a. In addition, Sood et al.[25] reported that the higher number of $CD4^+$ T cells in the bone marrow is

linear, as evidenced by the increased ratio of CD4+ T cells/CD8+ T seen in Peyer's patches and the spleens of mice treated with exposure to the *S. typhi* outer membrane protein (OMP). Furthermore, the authors noted a massive expansion of CD4+ T cells relative to the number found in the bone marrow after *S. typhi* exposure, indicating that this bacteria is capable of causing systemic infection that can reach the bone marrow. *Salmonella typhi* causes disease by establishing colonies and then multiplying within the host body.[26]

Peritrichous bacteria have flagella that actively engage with the surrounding cells, but they also have pili that serve as intermediaries for sex and adhesion.[27] These bacteria are capable of causing systemic infection in the first two weeks after the original infection.[28] In addition, they are very dangerous because they can enter various tissues and organs through the bloodstream. Future development of this type of bacteria can occur in the gallbladder, bone marrow, and spleen and can be a source of infection.[26]

The existence of CD4+ T cells in the bone marrow also allows for the secretion of cytokines in the microenvironment of the bone marrow. This stimulates the immune cells that are needed to eliminate the penetrating antigen. The cytokines can also induce the proliferation and differentiation of immune cells, which are hemopoietic precursors in the bone marrow.[29] This same process occurred in our study with a significant increase in the B220+ cells relative to the number ($p>0.05$) of mice exposed to *S. typhi* versus the control group. These B220+ cells express a surface molecule of B220 (CD45R). In mice, all cells that are committed to the B cell lineage express CD45R molecular surface, both from a progenitor cell, the cell c-kit + pro-B in the bone marrow, and the liver fetus until there are mature B cells in the peripheral circulation (sIgM+ sIgD+) and activated B cells.[30] Therefore, B220 (CD45R) is used as a molecular marker of B cells in mice.

In this study, the relative number of bone marrow B220+ cells in the control group (17.34%) and *S. typhi* exposure group (17.92%) did not differ significantly, but patients who undergo a splenectomy could see a significant reduction of up to 5.51% in the relative amount of B220+ cells and this increases to 5.90% if the splenectomy is followed by exposure to *S. typhi* (Figure 1).

DISCUSSION

Our results demonstrated that a splenectomy is the only factor that affects the relative amount of B220+ cells in the bone marrow. This result contradicts our initial hypothesis and the conclusions of several related studies which showed that patients who had a splenectomy had an higher number of these cells as compensation for the loss of B lymphocytes when the spleen was removed.[31-33]

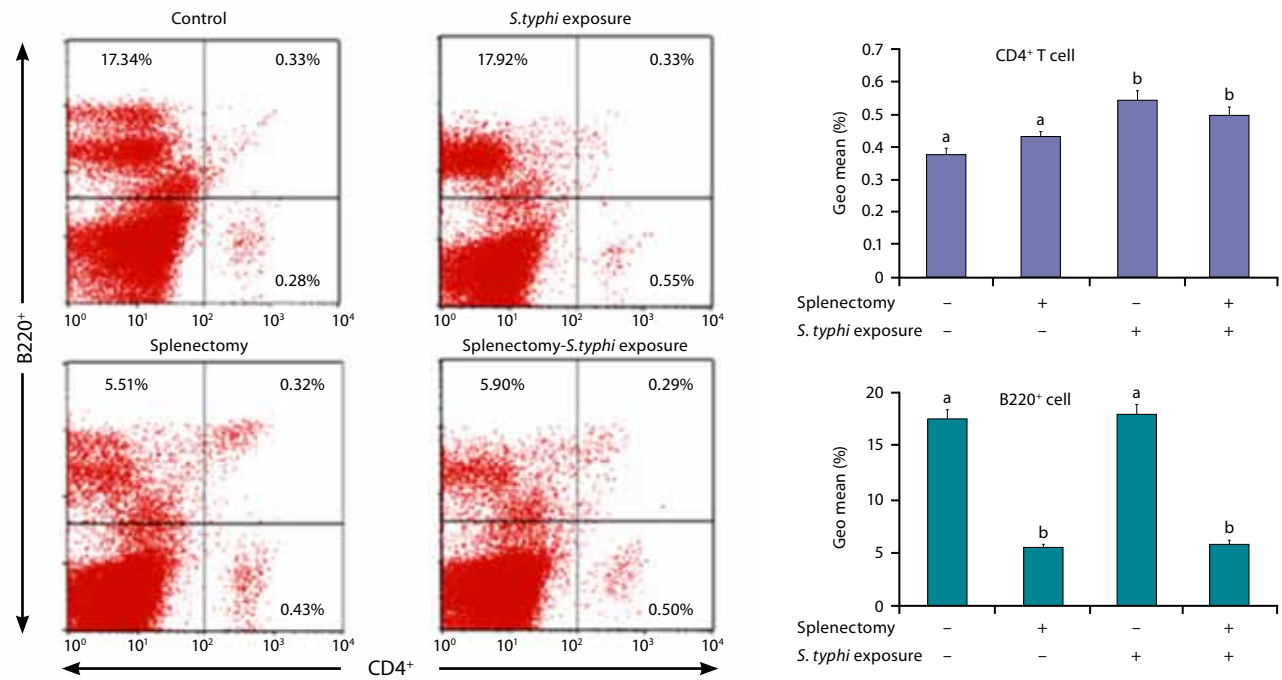

Figure 1. The relative numbers of bone marrow B220+ cells and CD4+ T cells. The corresponding lowercase letters above the deviation line showed no significant difference ($p>0.05$) based on Tukey's High Significant Differences test at a 95% significance level.

We also found that relative number of B cells in the central lymphoid organs (bone marrow) drops dramatically after a splenectomy. The explanation for this phenomenon involves several possible mechanisms. First, this decline indicates that other organs were not able to effectively compensate for the large numbers of B cells that were lost after the splenectomy. A second possibility is that the higher migration rate and lower proliferation rate were responsible.[34] Massive migration of immature B cells into the peripheral circulation has also been described by Ellis et al.[32] who reported that the high number of peripheral lymphocytes in their patients indicated a large amount of mature B lymphocytes. This was caused by the increased migration of newly formed B cells from the bone marrow which served as a homeostatic mechanism for the recovery of B cells in the peripheral circulation due to castration. Normal migration usually ranges from 10-15% to fill the peripheral B cells, but in certain cases, such as when castration occurs, this percentage can involve as many as 45% of the total number of circulating B cells and increase the number of lymphocytes both in the peripheral lymphoid organs and central lymphoid organs.

Our study also calculated the number of peripheral blood components after the splenectomy (data not shown). The quantification of peripheral blood lymphocytes one week after the splenectomy (75 cells/mm^3) showed a statistically significant decrease ($p<0.05$) compared with the pre-splenectomy levels (91 cells/mm^3). The possibility of migration to the periphery was also indicated by a decrease in the number of B cells. However, according the B cell measurements taken at one-month intervals after the procedure, the number of lymphocytes gradually returned to normal pre-surgical levels. The flow cytometry analysis of the bone marrow in this study was conducted at the same time as the quantitative analysis of the blood components one month after the splenectomy. This suggests the possibility that the decrease in the bone marrow B cells was caused by the high migration rate in order to maintain the number of peripheral B cells. However, the normally expected high rate of B cell lymphopoiesis in the bone marrow did not occur due to the splenectomy. To achieve homeostasis, a high migration rate should be followed by an increase in the rate of lymphopoiesis, as indicated by the higher number of lymphocytes in both the peripheral organs and central organs, for example the bone marrow and thymus,[32,35,36] but there was no homeostatic anomaly in our study. Foster and Trejdosiewicz[37] explained why this might happen by showing that a splenectomy does not affect the proliferation of B cells in the bone marrow and lymph nodes. This result is also supported by the persistence of T cells and memory B cells that already exist in the lymphoid organs and an increase in Kupffer cell activity, indicating that the rate of proliferation was not affected.[38] Milićević et al.[34] found that the migration and proliferation of B cells was influenced by surface molecules such as lymphocyte function-associated antigen 1 (LFA-1), intercellular adhesion molecule 1 (ICAM-1), L-selectin, 4-integrins, the IL-2-R chain, CD44, and MHC class II, which affects the initial attachment of B cells in the endothelium and their infiltration into the tissue. Removing the spleen caused a decrease in the expression of LFA-1 and ICAM-1 in B cells, which would support the acceleration of B cell migration to the peripheral organs and increase in blood circulation. These results prove that the spleen is needed for the expression of LFA-1 and ICAM-1 in B cells. When the B cell proliferation in the bone marrow was calculated, stable numbers were reported after the splenectomy, indicating that the high number of peripheral B cells due to the splenectomy was mainly caused by migration rather than proliferation. Part of the marginal zone of the spleen plays an important role in the regulation of the expression of LFA-1 and ICAM-1 because this is only done in the spleen[39] and cannot be replicated elsewhere after the splenectomy. In addition, the marginal zone is the only area that develops slowly, and we found that B cells in this location do not express much CD2, which causes B cell lymphocytosis after procedures such as a splenectomy. A decrease in the expression of LFA-1 and ICAM-1 also causes B cell's affinity in peripheral tissue is reduced and speed up the circulation into the lymph circulation.[40]

In addition, some studies have shown that the number of T cells is not affected by a splenectomy. The existence of empty space not compensated for by the T cells resulted in an increased demand for peripheral B cells in the Willführ and Westermann study;[20] hence, migration from the bone marrow increased. The existence of these events causes the re-infiltration of B cells into tissues at an increased rate and leads to the migration of B cells from the bone marrow into the blood and peripheral organs. Acceleration of this migration may be one factor that can cause a patient to be vulnerable to infection after a splenectomy since the B cells do not remain on the peripheral organs as long, thus mediating cellular interaction that initiates and regulates the immune response against an antigen.[41] Another mechanism that also exacerbates the decrease in the number of B cells is the extensive selection of peripheral immature B cells that migrate from the bone marrow, of which only 30% pass this selection process.[42,43]

One limitation of this study was that there was no differential count of B cells and T cells in the total peripheral lymphocytes, so the possibility of B cell migration to compensate for the number of peripheral B cells due to the splenectomy could not be proved exactly. In addition, the possibility of extensive selection towards B cells in the bone marrow could not be proved by the presented methods. Further studies are needed to determine the exact number of emigrant cells from the bone marrow in order to differentiate between the amount of mature peripheral B cells ($B220^{+Lo}$ $CD24^{+hi}$) and emigrant B cells ($B220^{+hi}$ $CD24^{+Lo}$).

Conclusion

In this study, the splenectomized and unsplenectomized mice which were exposed to *S. typhi* had massive increases in the relative number of bone marrow $CD4^+$ T cells. However, the relative number of $B220^+$ decreased significantly when a splenectomy was performed. In addition, *S. typhi* exposure also did not significantly affect the relative number of $B220^+$ cells. This proves that quantitative alterations of $CD4^+$ T cells are primarily affected by *S. typhi* exposure and that the relative number of $B220^+$ cells are primarily affected by splenectomies.

Declaration of conflicting interests

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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