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RESEARCH ARTICLE

Leukocytes Immunophenotype and Phagocytosis Activity in Pregnant and Nonpregnant Dromedary She Camels

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ABSTRACT

Pregnancy is associated with different local and systemic changes in immune cell phenotype and function. Here, we used flow cytometry and membrane immunofluorescence for the comparison between pregnant (n=18) and non-pregnant she camels (n=21) in terms of immunophenotype of blood leukocytes and phagocytosis activity of neutrophils. Camel blood leukocytes were labeled with monoclonal antibodies to camel antigens and analyzed by flow cytometry. Pregnant she camels displayed higher leukocyte counts than non-pregnant animals. Differential analysis of leukocyte populations revealed higher proportions of monocytes, lymphocytes and CD4-positive T cells but lower proportion of neutrophils in pregnant she camels in comparison to non-pregnant animals. Pregnancy was also associated with enhanced expression of the cell adhesion molecule CD11a on neutrophils but reduced CD11a expression on lymphocytes in pregnant animals. After incubation with FITC-labeled bacteria, flow cytometric ex-vivo analysis of bacterial phagocytosis by neutrophils showed higher phagocytosis capacity in neutrophils from pregnant than from non-pregnant animals. Together these results indicate significant modulating effects of pregnancy on the immune system of she camels.

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INTRODUCTION

Embryonic loss is one of the major factors responsible for high economic losses in food animals. The maintenance of pregnancy is associated with modulations in different immune mechanisms, which ensure protection against pathogens and at the same time prevent immunological destruction of the conceptus (Aluvihare *et al.*, 2004; Somerset *et al.*, 2004). The pregnancy-associated changes occur not only in the local environment of the uterus but extend also to the peripheral immune system (Ott and Gifford, 2010; Kamat *et al.*, 2016).

For different species like human (Spadaro *et al.*, 2019), cows (Leung *et al.*, 2000; Oliveira *et al.*, 2012), mares (Bazzano *et al.*, 2014; Piccione *et al.*, 2015) and sows (Zhang *et al.*, 2017), local and systemic immunomodulatory effects of pregnancy have been widely studied. According to studies in human and rodents, both lymphoid and myeloid immune cells including NK cells, αβ T cells, B cells, γδ-T lymphocytes, neutrophils, monocytes, macrophages and dendritic cells play significant role in maintaining pregnancy (Lash *et al.*, 2010; Nagamatsu and Schust, 2010; Groebner *et al.*, 2011). In dairy cows, the presence of a conceptus resulted in increased numbers of peripheral blood myeloid cells with enhanced expression of chemotactic factors, which attract these cells to the uterus (Kamat *et al.*, 2016).

Little is known about the impact of pregnancy on the immune system of dromedary she camels. The aim of the current study was, therefore, the comparative analysis of immunophenotype of blood leukocytes and phagocytosis activity of neutrophils in pregnant and nonpregnant dromedary she camels. The results of the current study would lead to a better understanding of immunology of pregnancy and the identification of the immunologic factors associated with higher pregnancy rates in she camels.

MATERIALS AND METHODS

Animals and blood sampling: In this study, 18 pregnant and 21 non-pregnant dromedary she camels (Camelus dromedarius), aged 10-14 years and maintained at the Camel Research Center, King Faisal University, Al-Ahsa, Saudi Arabia were used. The pregnant she camels were at their mid gestation (between 5th and 10th month based on sonographic examination and insemination history). Blood samples (5 ml blood from each she camel) were collected from she camels during the period between January and May 2019 by jugular venepuncture in EDTA containing vacutainer tubes (BD, Germany). All experimental procedures and management conditions used in this study were approved by the Ethics Committee at King Faisal University, Saudi Arabia (Permission number DSR 1811001).

Microscopic counting of leukocytes: Whole blood was diluted 1: 4 in PBS and was then mixed with Türk's solution (final dilution 1:20; Merck Millipore) and 10 µl of the mixture was poured onto the hemocytometer (Neubauer cell counter) for counting under the microscope. Leukocytes (in blue color were counted in four big squares of the cell counter and total leukocyte count was calculated (Camacho-Fernandez *et al*., 2018).

Monoclonal antibodies: Nine commercially available monoclonal antibodies (mAbs) were used in this study, as shown in Table 1.

Separation of blood leukocytes: Separation of whole blood leukocytes was done after hypotonic lysis of erythrocytes (Hussen *et al*., 2017). Briefly, blood was suspended in distilled water for 20 sec and double concentrated PBS was added to restore tonicity. This was repeated until complete erythrolysis indicated by the formation of clear white pellet of leukocytes. Separated cells were finally suspended in membrane immuno-fluorescence (MIF) buffer (PBS containing bovine serum albumin (5 g/L) and NaN_3 (0.1 g/L)) at 5x10^6 cells/ml. The mean viability of separated cells was evaluated flow cytometrically by dye exclusion (propidium iodide; 2 µg/ml, Calbiochem, Germany) and was consistently >95%.

Immunofluorescence and flow cytometry: Separated leukocytes (5x10^6 cells / ml) in PBS containing bovine serum albumin (5 g/L) and NaN_3 (0.1 g/L) were labeled in 96 well round-bottom microtiter plates (1x10^6 / well; 20 min; 4°C) with monoclonal antibodies specific for CD4, WC1, MHCII and CD14 in three combinations, including CD4/WC1/CD14, CD14/MHCII and CD14/CD11a/CD18 (Eger *et al*., 2015; Hussen *et al*., 2018). After incubation with primary unlabeled antibodies, cells were washed twice and incubated with mouse secondary antibodies IgG1, IgM and IgG2a (BD) labelled with different fluorochromes. After washing the cells, directly labeled monoclonal antibodies were added to CD14, CD11a, CD11b and CD18. Finally, cells were washed and analyzed by flow cytometry (FACSCalibur, Becton Dickinson Biosciences). For each measurement 100,000 events were acquired and data were analyzed with FlowJo (FLOWJO LLC) (Fig. 1).

Phagocytosis assay: Heat killed staphylococcus aureus (S. aureus) bacteria (Pansorbin, Calbiochem, Merck, Nottingham, UK) were labeled with fluorescein isothiocyanate (FITC, Sigma-Aldrich, St. Louis, Missouri, USA). FITC-conjugated and heat killed S. aureus bacteria were suspended in Roswell Park Memorial Institute (RPMI) medium and adjusted to 2x10^8 bacteria/ml. Separated camel leukocytes were plated in 96 well plates (1x10^6/well) and incubated at 37°C under 5% CO_2 with labeled bacteria (50 bacteria/cell) for 40 minutes. After washing, cells were analyzed by flow cytometry (FACS Calibur, Becton Dickinson Biosciences, San Jose, California, USA). Phagocytosis-positive cells were defined as the percentage of green fluorescing cells among total cells. Phagocytosis capacity (as an indicator for the number of bacteria ingested by each cell) was defined as the mean green fluorescence intensity of gated phagocytosis-positive neutrophils.

Table 1: List of antibodies

Antigen	Antibody clone	Labelling	Source	Isotype
CD4	GC50A1	Unlabeled	WSU	Mouse IgM
WC1	BAQ128A	Unlabeled	WSU	Mouse IgG1
CD14	TÜK4	PerCP	Biorad	mIgG2a
MHCII	TH81A5	-	WSU	mIgG2a
CD11a	G43-25B	PE	BD	mIgG2a
CD18	6.7	FITC	BD	mIgG1
mIgG2a	Polyclonal	PE	Invitrogen	gIgG
mIgG1	Polyclonal	FITC	Invitrogen	gIgG
mIgM	Polyclonal	APC	Invitrogen	gIgG

Ig: Immunoglobulin; m: mouse; MHC-II: Major Histocompatibility Complex class II, g: goat, WSU: Washington State University.

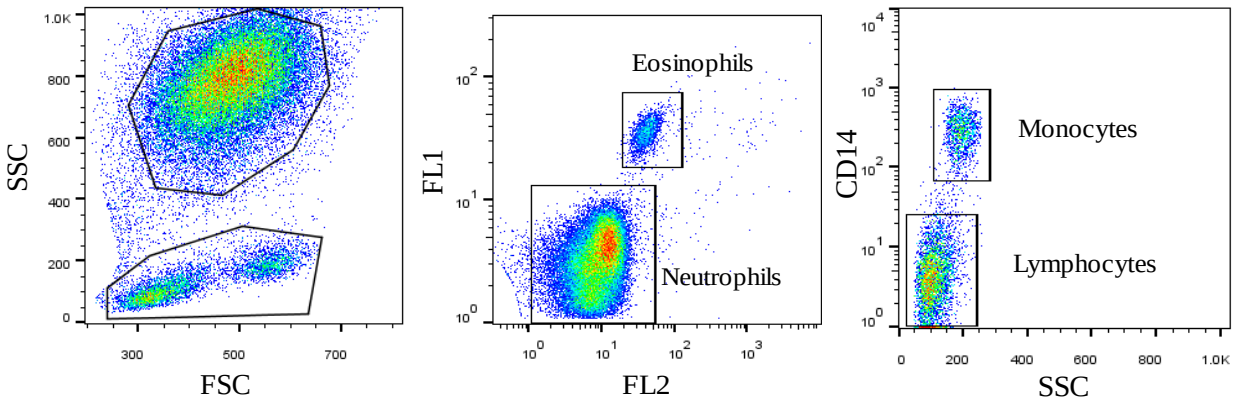

Fig. 1: Gating strategy for the identification of the main leukocyte populations in peripheral blood of she camel. In a SSC/FSC dot plot, camel granulocytes and mononuclear cells (PBMC) were gated according to their forward and side scatter characteristics. After setting a gate on granulocytes, eosinophils and neutrophils were identified according to their different auto-fluorescence intensities in the FL1 and FL2 fluorescence channels. In the PBMC gate, monocytes and lymphocytes were identified based on their different CD14 staining.

Statistical analysis: Statistical analysis was performed using the GraphPad Prism (v. 5) software. Results are expressed as means ± S.E. of the mean (SEM). Student T test was used for difference analysis between means. Differences were considered statistically significant at a p-value of less than 0.05.

RESULTS

Absolute count and relative composition of camel blood leukocytes: Flow cytometric analysis and microscopic cell counting of camel blood leukocytes revealed significant differences in absolute and relative composition of blood leukocyte between pregnant and nonpregnant she camels (Fig. 1 and 2). With a mean of 14.03±0.56 ($x10^3$) cells/µl, pregnant she camels showed higher numbers of blood leukocytes in comparison to nonpregnant she camels (10.49±0.69 $x10^3$ cell/µl). For differential analysis of the main leukocyte populations in she camel blood, leukocytes were labelled with monoclonal antibodies and analyzed by flow cytometry. Neutrophils represented relatively the main population (75.67%) of camel blood leukocytes, followed by lymphocytes (9.78% of total leukocytes), whereas monocytes (4.93%) and eosinophils (5.7%) were minor populations in camel blood. The comparison between the leukogram of the two groups revealed that the percentages of lymphocytes (11.27±0.60) and monocytes (5.59±0.39) in pregnant she camels were significantly higher than lymphocytes (8.54±0.76) and monocytes (4.54±0.33) percentages in nonpregnant she camels. On the other hand, pregnant she camels showed lower percentage of neutrophils (71.04±1.17) in comparison to non-pregnant animals (75.67±1.75). However, the percentage of eosinophils did not differ significantly between the two groups (Fig. 2).

Relative composition of camel blood lymphocytes: With a mean of 24.69±1.56% of total lymphocytes, pregnant she camels showed elevated proportion of CD4-positive T cells in comparison to 16.56±1.06 in non-pregnant animals. The two groups however showed comparable proportions of γδ T cells and B cells (Fig. 3 and 4).

Expression of cell adhesion molecules on camel blood leukocytes: Although camel neutrophils, lymphocytes and monocytes did not differ in their expression of CD18 between pregnant and non-pregnant animals, pregnant she camels showed a selective elevated CD11a expression on neutrophils but a reduced expression on lymphocytes (Fig. 5).

Impact of pregnancy on phagocytosis activity of blood neutrophils of she camels: Although the percentage of cells with phagocytosis activity was similar for neutrophils from pregnant and non-pregnant she camels, the phagocytosis capacity (the number of bacteria phagocytosed by each neutrophil) was significantly higher in pregnant she camels (976.53± 67.58) in comparison to 776.45±77.06 in non-pregnant animals (Fig. 6A & B).

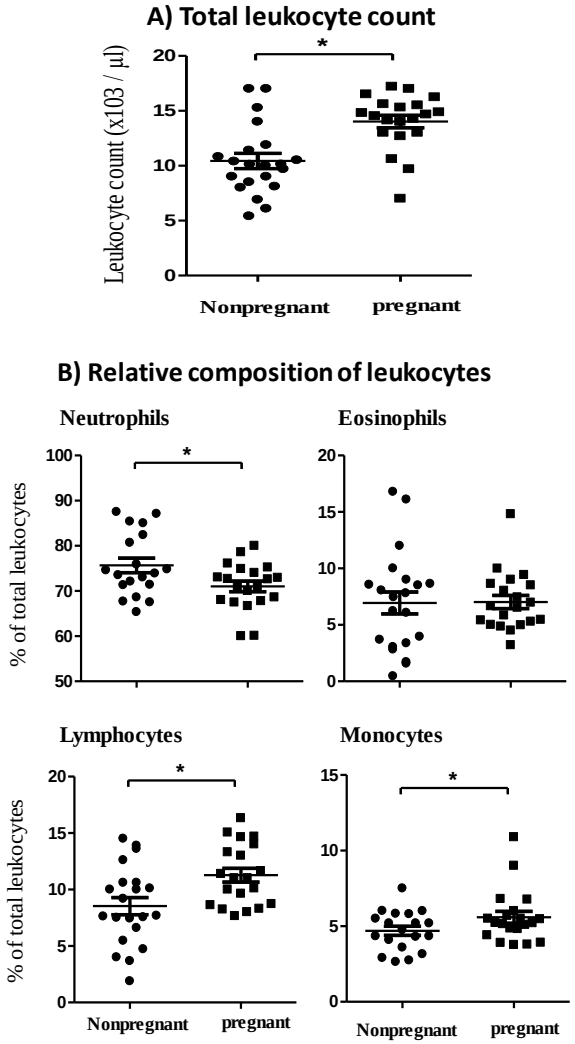

Fig. 2: Total and differential cell count of camel blood leukocytes. A) Total leukocyte count in pregnant and non-pregnant animals is presented as scattered dot plots (*=P<0.05). B) Percentages of neutrophils, eosinophils, lymphocytes and monocytes are presented as scattered dot plots for pregnant and non-pregnant animals (*=P<0.05).

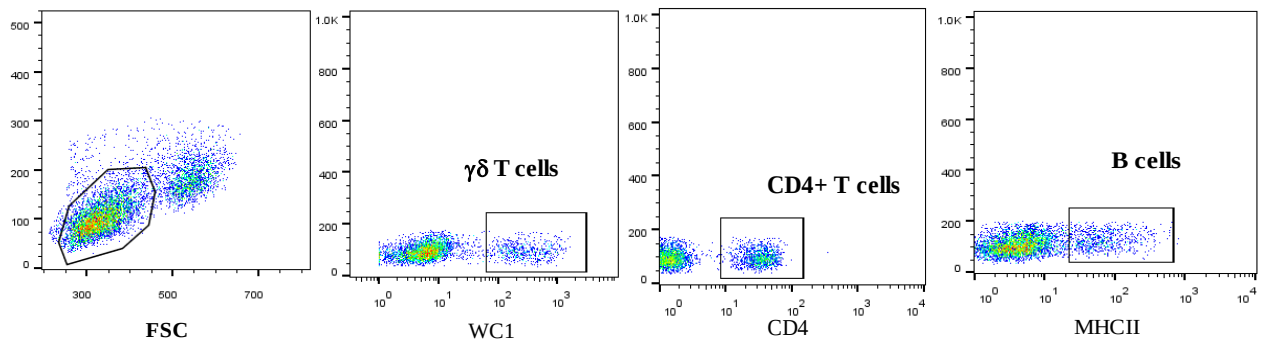

Fig. 3: Gating strategy for the identification of lymphocyte populations. In a SSC/FSC dot plot, a gate was set on lymphocytes according to their forward and side scatter characteristics. WC1-positive lymphocytes were shown in a SSC/WC1 plot. CD4-positive lymphocytes were gated in a SSC/CD4 plot. B cells were shown in a MHCII / SSC plot as MHCII+ lymphocytes.

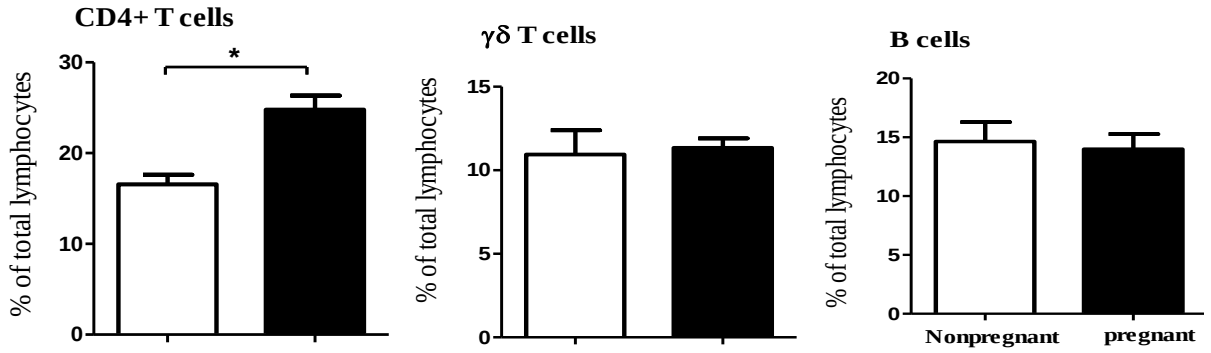

Fig. 4: Relative composition of blood lymphocytes in peripheral blood of pregnant and non-pregnant Dromedary camels. The percentages of gd T cells, CD4-positive T helper cells and B cells under total lymphocytes are presented as mean ± SEM (*=P<0.05).

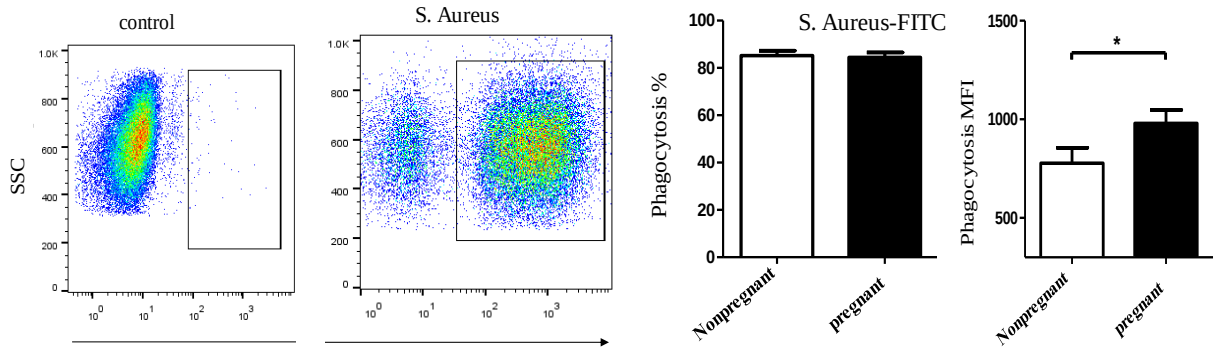

Fig. 6: Flow cytometric analysis of phagocytosis of bacteria by camel neutrophils. A) Gating strategy for phagocytosis analysis (representative results from negative and positive control are shown). B) The percentage of phagocytosis-positive neutrophils and phagocytosis capacity (number of bacteria ingested by each cell) of camel neutrophils are presented as means ± SEM (*=P<0.05).

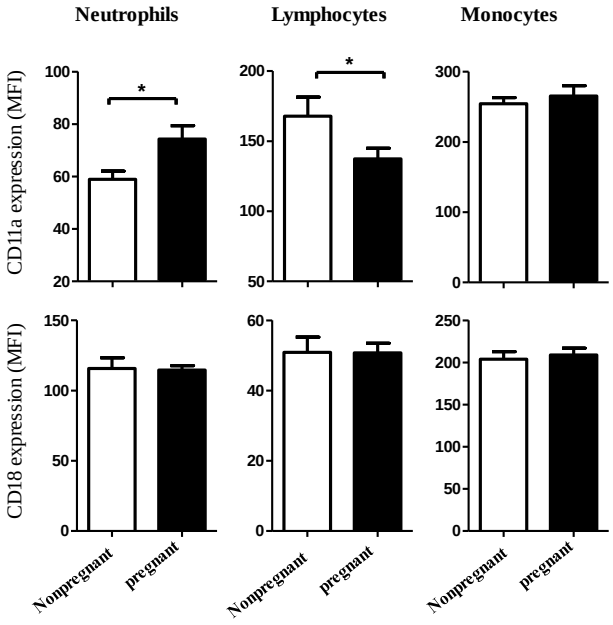

Fig. 5: Adhesion molecules expression on leukocyte populations in peripheral blood of Dromedary camels. The differential expression of the adhesion molecules CD11a and CD18 was estimated as the mean fluorescence intensity (MFI) of each molecule on blood neutrophils, lymphocytes and monocytes. Data for pregnant and non-pregnant she camels are presented graphically as mean ± SEM (*=P<0.05).

DISCUSSION

Similar to findings in pregnant mares (Aoki *et al.*, 2016) and women (Tan and Tan, 2013), pregnant she camels showed significantly more leukocytes in blood than non-pregnant females. This pregnancy-related leukocytosis has been linked to higher cortisol concentrations in serum of pregnant animals (Bazzano *et al.*, 2014).

Polymorphonuclear neutrophilic granulocytes play a key role in bacterial elimination mainly through their phagocytic and bactericidal functions (Hussen *et al.*, 2016). In the current study, the reduced proportion of neutrophils in pregnant than non-pregnant she camels is contrary to findings in the human system, where higher percentages of neutrophils were found in the blood of pregnant women (Zhang *et al.*, 2017). This neutrophil decrease may be due to enhanced extravasation of these cells and their migration to the uterine tissue. This is also supported by the enhanced expression of the cell adhesion molecule CD11a on blood neutrophils from pregnant she camels in the current study. Ex-vivo analysis of bacterial phagocytosis by neutrophils revealed higher phagocytosis capacity of cells from pregnant she camels. This enhanced antimicrobial activity of neutrophils may compensate their reduced percentage observed in blood of pregnant animals. Similar enhancing effect of pregnancy on neutrophils phagocytosis capacity was reported in the pregnant mare (Barriga *et al.*, 1994). In dairy cows however, pregnancy is associated with impaired antimicrobial functions of neutrophils (Saad *et al.*, 1989; Sheldon *et al.*, 2009). These differences may rely on species-specific regulation of phagocytosis during pregnancy.

The increased CD4-positive T cells in blood of pregnant she camels is in line with reports from studies on the bovine immune system during pregnancy, where more CD4+CD25+T cells were found in blood of pregnant cows (Oliveira and Hansen, 2008; Oliveira *et al.*, 2012). Similar increase has also been shown for human and mice during pregnancy (Aluvihare *et al.*, 2004; Somerset *et al.*,

2004). Whether the increased camel CD4-positive T cells have a regulatory phenotype, like their counterparts in women and cows (Oliveira and Hansen, 2008; Oliveira *et al.*, 2012) is still to be investigated.

In our study, the higher proportion of monocytes in pregnant she camels also agrees with finding in pregnant cows, where the presence of conceptus was correlated with increased numbers of monocytes in peripheral blood (Kamat *et al.*, 2016). This increase may be explained by enhanced need for macrophage accumulation in the pregnant endometrium, as has been reported in pregnant cows (Oliveira and Hansen, 2008).

In summary, this is the first study on the impact of pregnancy on phenotype and function of blood leukocytes in she camels. We found that pregnant she camels showed higher number of total leukocytes, higher proportions of lymphocytes, monocytes and helper T cells but reduced proportions of neutrophils in comparison to non-pregnant animals. Furthermore, neutrophils from pregnant she camels displayed higher phagocytosis capacity than cells from non-pregnant animals.

Conclusions: Together these results indicate significant modulating effects of pregnancy on the immune system of she camels. Further studies are needed for the characterization of molecular mechanisms responsible for these changes.

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Authors contribution: JH, HJ conceived and designed the study. JH, TS and NA collected the samples. JH, TS, FA and AIA prepared the samples for flow cytometry. JH, NA, AIA and FA analyzed the labelled cells by flow cytometry. JH, TS and HJ analyzed the data by Flow Jo. All authors read and approved the final manuscript.

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