ORIGINAL RESEARCH

AcSDKP is down-regulated in anaemia induced by *Trypanosoma brucei* infection in mice

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Abstract

Background

Anaemia commonly results from destruction of erythrocytes in the peripheral blood and failure of the bone marrow haematopoietic cells to replenish the erythrocytes. The mechanisms involved in trypanosoma-induced anaemia, including the role of the bone marrow haematopoietic cells are incompletely understood. We studied the responses of a tetrapeptide, AcSDKP, and IL-10, and their association with bone marrow nucleated cells in a *Trypanosoma brucei brucei* GVR35 experimental infection model.

Methods

Mouse infection was done intraperitoneally with 1×10^3 trypanosomes/mL. Mice were either infected or left uninfected (N = 100). At days 0, 9, 16, 23, 30, 37, and 44 post-infection, mice were euthanised and blood was collected by cardiac puncture to examine for parasitaemia and packed cell volume (PCV) and then centrifuged for plasma, which was used for cytokine ELISA. The mice's femurs were also dissected and bone marrow was collected for femur cellularity.

Results

PCV dropped from 39.6% to 27% in infected animals by day 9 and remained low (relative to uninfected mice) for the duration of the experiment. AcSDKP levels decreased from day 0 (11.5×10^4 pg/mL) to day 16 (10×10^4), and increased by day 30 (12.6×10^4). There was a significant difference at day 16 (P = 0.023) between the infected and uninfected groups. By contrast, expression of IL-10 markedly increased between day 0 (18.6 pg/mL) and day 16 (145 pg/mL) and decreased by day 30 (42.8 pg/mL). There was also a significant difference in IL-10 expression between infected and uninfected mice at day 16 (P < 0.001). Bone marrow nucleated cells were significantly reduced during periods of low plasma AcSDKP and high plasma IL-10 concentrations (5.4×10^6 infected vs 6.2×10^6 on day 0 and 4.9×10^6 infected vs 10×10^6 uninfected on day 16).

Conclusions

These data unravel a possible negative feedback interaction between AcSDKP and IL-10 in trypanosome infection. More importantly, this study implicates an IL-10/AcSDKP cytokine network in the regulation of bone marrow nucleated cells and provides a new potential mechanism in the pathogenesis of trypanosoma-induced anaemia. Further mechanistic blocking experiments on AcSDKP and IL-10 are recommended to further clarify understanding of the interaction.

Introduction

Acetyl-N-Ser-Asp-Lsy-Pro (AcSDKP) is a tetrapeptide that is naturally released in organisms from its metabolic precursor thymosin β4 by prolyloligopeptidase (POP), a serine proteinase found in mammalian tissue.[1] It has been for years regarded as a physiological negative regulator of proliferation of haematopoietic stem cells and committed haematopoietic progenitors cells. It acts by blocking haematopoietic stem cells from being recruited from G_0 or early G_1 to enter into S phase. This mechanism of blocking the transition from G_0 to S phase of the haematopoietic stem cell cycle keeps haematopoietic stem cells in the quiescent phase.[2,3] Further studies have shown that AcSDKP regulates cell survival through the PI3KCA/Akt pathway.[4] It is now also regarded as an angiogenesis factor.[5] In normal physiology, AcSDKP is observed in higher titres and is thought to work best in concentrations below 10^{-7} M and above 10^{-14} M.[6] Since it is highly implicated in haematopoiesis and angiogenesis, AcSDKP has been studied in a number of conditions and disorders of the blood and the bone marrow systems.

In neoplastic diseases, including haematological malignancies and solid neoplasms, studies have revealed elevated levels of endogenous AcSDKP.[7,8] In leukaemia-inoculated mice, endogenous AcSDKP concentration has been shown to dramatically increase.[9] High levels of AcSDKP have also been demonstrated in different solid human malignancies.[10] In chronic kidney disease, accumulation of AcSDKP has been associated with the exacerbation of anaemia, though AcSDKP levels have been shown to decrease in patients with end-stage renal disease after kidney transplantation.[11] The importance of AcSDKP in anaemic conditions cannot be overemphasised.

To the best of our knowledge, there are no studies that have examined the levels of AcSDKP in trypanosoma-induced anaemia. Although a number of studies have documented the ability of trypanosomes to produce negative regulatory cytokines *in vivo* and *in vitro,* to date there has been no study that has associated AcSDKP with trypanosomiasis or indicated any link between AcSDKP and haematopoietic stem cell numbers in trypanosoma-induced anaemia. In order to get further insight into the mechanisms behind the pathogenic events occurring in trypanosomiasis, AcSDKP was studied together with interleukin-10 (IL-10), which is a cytokine that is known to suppress the production of other cytokines. IL-10 is a type II anti-inflammatory cytokine, produced by

Th2 T-helper cells. This study investigated AcSDKP levels and IL-10 levels, in an experimental mouse model during anaemia induced by *Trypanosoma brucei* infection, in relation to the number of nucleated cells in the bone marrow.

Methods

Trypanosomes

Trypanosoma isolate *T.b. brucei* strain GVR35 was obtained from the Makerere College of Veterinary Medicine and Animal Resources and Biosecurity (COVAB) immunology laboratory. This parasite was originally isolated from the bloodstream of infected wildebeest in the Serengeti in 1966 (Serengeti/66/svrp/10).[12] *T.b. brucei* stabilates were kept frozen in liquid nitrogen and an aliquot was passaged through Wistar rats before the experiments were carried out. Stabilates were made by preserving 1 in 3 parts of blood containing the parasite with 30% glycerol–phosphate buffer saline with glucose (PSG).

Experimental mice

Female Swiss albino mice were purchased from the Uganda Virus Research Institute (UVRI) Animal House (Entebbe, Uganda) at 8 to 12 weeks old. All mice were housed at the COVAB animal facility under standard laboratory conditions according to the university's recommendations for animal care. The mice were maintained on a diet of commercial mouse pellets (Engaano Millers Ltd, Kampala, Uganda) with access to clean water *ad libitum*.

Mice were infected by intraperitoneal inoculation of 1×10^3 trypanosomes/mL. Mice were either infected or left uninfected as controls. A total of 100 mice were used, with 5 mice starting each time point. At days 0, 9, 16, 23, 30, 37, and 44 post-infection, mice were euthanised and blood was collected by cardiac puncture to examine for parasitaemia and packed cell volume (PCV). The blood was then centrifuged in EDTA tubes for 10 minutes at 1280 g to collect plasma. Centrifugation separates the blood into 3 layers: the red blood cell (RBC) layer, which sets at the bottom of the tube; followed by the buffy coat (composed of white blood cells and platelets); and the plasma layer on top. Plasma was then carefully removed using pasture pipettes and was kept frozen at -80°C for evaluation of cytokines using an enzyme-linked immunosorbent assay (ELISA) technique. Additionally, cells were flushed from the mice's dissected femurs for evaluations of femur cellularity.

Cell isolation

Bone marrow was flushed from the femurs under the sterile condition in a laminar flow hood (Aura vertical S.D.4, BIOAIR®, Italy). The hip and knee joints were cut as close to the joint as possible, exposing the marrow. Using a 5-mL syringe (BD Bioscience) with a needle, the marrow was flushed from both directions with 5 mL of ice-cold Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine (BioWhittaker®Lonza, Belgium) into 50-mL Falcon tubes (Nest®, China) until the femur colour changed from red to white. A single-cell suspension was achieved by dispersing bone marrow via suction into an empty 20-mL syringe through a 19-mm gauge needle twice. The bone marrow cell suspension was washed by centrifugation (IEC CL31R Multispeed centrifuge, Thermo Scientific) at 320 g for 10 minutes at 4°C. The cells were resuspended in R10 media made up of RPMI 1640 with L-glutamine supplemented with 10% heat-inactivated foetal calf serum (Sigma), 5 mL of 1 M HEPES (Sigma) and 5 mL of penicillin–streptomycin solution (BioWhittaker®Lonza, Belgium).

Femur cellularity

For cell counting, 20 µl of the cell suspension was mixed with 3% acetic acid with methylene blue, in 1:50 dilutions. Then 10 µl of the mixture was added into a counting slide chamber and mounted on a TC20™ automated cell counter (Bio-Rad, UK) according to the manufacturer's recommendations. The cell counter gives the number of nucleated cells per mL by counting the cells that pick up the blue dye. Each sample was counted 3 times and the means from these counts were recorded. Percent viability was also recorded for each sample. For this, 20 µl of the sample (after cell isolation) was pipetted onto a Parafilm sheet and mixed with 20 µl of trypan blue (1:1 dilution). Trypan blue dye 0.40% (Bio-Rad, UK) stains nonviable cells that have compromised membranes and was used to distinguish viable from nonviable.

Bone marrow cell cultures

From the bone marrow cell suspension after cell isolation, a cell suspension of concentration 10^6 cells/mL was removed from the tubes and was incubated (CO_2 incubator, LEEC Research) at 37°C and 5% CO_2 for 48 hours in duplicate wells. After incubation, supernatant from the wells was collected and analysed for cytokines.

Measurement of cytokine levels

Murine cytokine and capture detection antibody against murine IL-10 and AcSDKP were used for sandwich ELISA to detect these cytokines in the plasma and cell culture supernatant fluids following the manufacturers' recommendations. For AcSDKP (TSZ Scientific, Waltham), specific microtiter plates, which were commercially pre-coated with solid-phase antibody, were used. For IL-10, specific (BD OptEIA) mouse IL-10 ELISA kits were used. Each sample of plasma and supernatant was tested for each cytokine in duplicate. The detection limit for IL-10 was 30 pg/mL; the detection limit for AcSDKP was 15 pg/mL. Absorbance was read at a wavelength of 450 nm using a microwell reader (Dynex technologies, MRX).
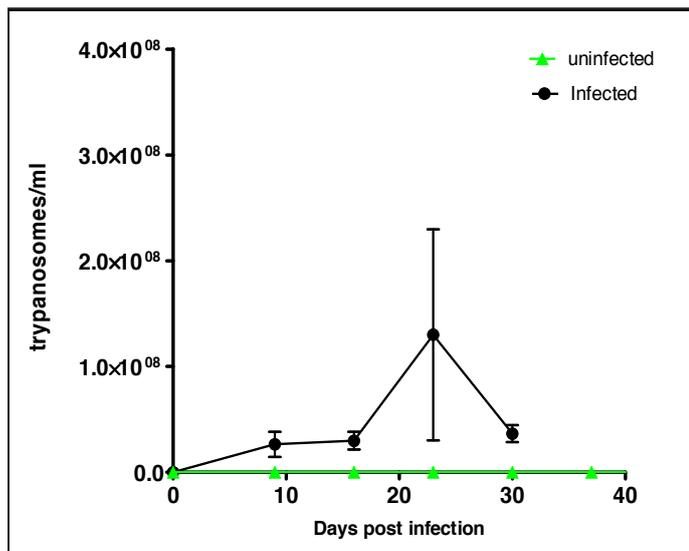
Statistical analysis

Levels of cytokines were quantified and tested. Changes in PCV levels and nucleated bone marrow cell numbers were evaluated. For each variable, results were expressed as the mean response of the 5 infected mice tested individually (± standard error of the mean, SEM) compared with the same variables assessed in the 5 uninfected control mice. Evidence for associations between cytokines and haematological derangements was examined using Student's t-test. The significance level was set at 0.05. Results are representative of 2 similar independent experiments. All data were double-entered into a secure electronic database using GraphPad Prism 5.

Results

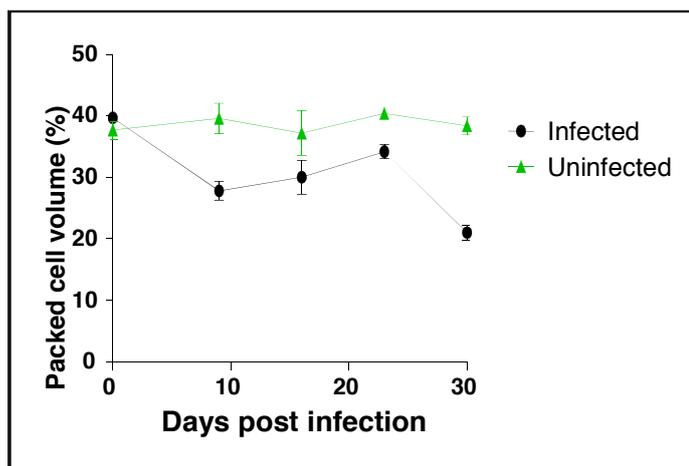### Trypanosoma infection and anaemia

Parasitaemia responses in infected and control animals are reported in Figure 1. In uninfected mice, no death was recorded. In infected mice, parasitaemia appeared by day 9 (2.64×10^7 trypanosomes/mL [$\pm 1.1 \times 10^7$]) and peaked by day 23 (1.3×10^8 trypanosomes/mL [$\pm 9.9 \times 10^7$]) and was reduced by day 30 post-infection to 3.65×10^7 trypanosomes/mL ($\pm 8.3 \times 10^6$). However, the infected mice failed to control parasitaemia after the first wave and therefore experienced a high rate (90%) of mortality during this period. A wave is an observed surge of parasitaemia, which occurs in cycles and is moderated by host immune responses.

A reduction in the cell volume (Figure 2) was observed among infected mice. In uninfected mice, no such reduction was seen and PCV ranged from 37% to 42%. In infected mice, PCV levels decreased early by day 9 post-infection (PCV dropped from 39.6% [± 0.3] pre-infection to 27.8% [±

1.5]). Anaemia was seen to be severe at day 30 post-infection (21% [± 1.1]) (P < 0.001). There was a significant difference between infected and uninfected mice (P = 0.02) over the entire period.

Bone marrow nucleated cells numbers during *T.b. brucei* infection

Femoral cellularity among the mice is shown in Figure 3. The levels of bone marrow nucleated cells in uninfected mice ranged from 6.2×10^6 cells/mL ($\pm 9.4 \times 19^5$) to 10.9×10^6 cells/mL ($\pm 8.2 \times 10^5$). There was a gradual increase from day 0 (6.2×10^6 cells/mL [$\pm 9.4 \times 10^5$]) to day 16 (10.9×10^6 cells/mL [$\pm 8.2 \times 10^5$]) but thereafter it remained almost constant until the end of the experiment. The mean number of bone marrow nucleated cells of infected mice decreased to 3.26×10^6 cells/mL ($\pm 4.7 \times 10^5$) by day 9 (P < 0.01). By day 30 the bone marrow nucleated cells had increased to 7.75×10^6 cells/mL ($\pm 1.3 \times 10^6$). There was a significant difference between infected mice (3.26×10^6 cells/mL [$\pm 4.7 \times 10^5$]) and uninfected mice (8.7×10^6 cells/mL [$\pm 10 \times 10^6$]) on day 9 (P = 0.0042) and day 16 (4.99×10^6 cells/mL [$\pm 5.1 \times 10^5$] and 10.9×10^6 cells/mL [$\pm 8.2 \times 10^5$], respectively [P = 0.0051]). However, by day 30 the number of nucleated cells in infected mice increased to levels comparable to the numbers in uninfected mice (7.75×10^6 cells/mL [$\pm 1.3 \times 10^6$] and 9.27×10^6 cells/mL [$\pm 1.5 \times 10^6$], respectively).

IL-10 and AcSDKP levels in mice during *T.b. brucei* infection

Plasma levels of IL-10 are shown in the Figure 4. In uninfected mice the plasma IL-10 levels ranged from being undetectable to 22 pg/mL. Plasma levels of IL-10 in infected mice increased from 14.67 pg/mL (± 8.7) at baseline to 145 pg/mL (± 28.7) by day 16 and decreased to 42.8 pg/mL (± 8) by day 30. The lowest levels of IL-10 were on day 30 (42.8 pg/mL), which was significantly different to the levels observed in uninfected mice (6.67 pg/mL [± 1]) (P < 0.001).

Levels of IL-10 in supernatant were analysed after 48 hours of *in vitro* culture without physical priming with trypanosome (Figure 5). Cells in uninfected group could not produce IL-10 in the supernatant. In infected animals IL-10 was expressed by day 16 (1644 pg/mL [± 722]) and reduced by day 30 (76 pg/mL [± 46]) (P < 0.001).
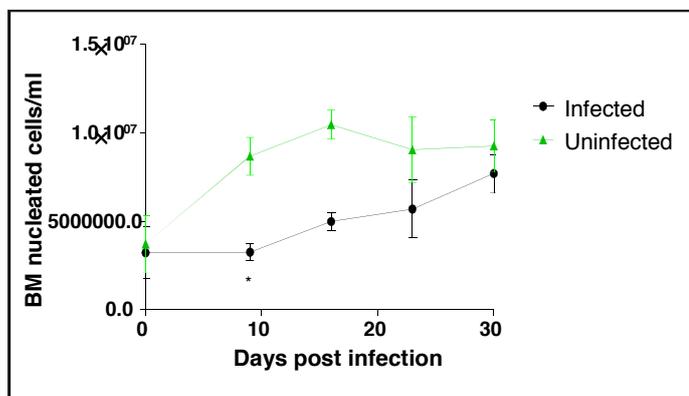

Figure 1: Mean parasitaemia in mice at different time points infected with 103 parasites/mL of *T. brucei* GVR 35 (black), or uninfected (green)

Data (±SEM; n=5) are representative of 2 independent experiments.

Figure 2: Packed cell volume values in mice (n = 5) at different time points infected with *T.b .brucei* GVR 35 (black) or uninfected mice (green)

On day 30 post-infection, the decline in the average PCV of the infected mice was significantly greater (p < 0.001) than in the uninfected mice.

Figure 3: Total bone marrow cellularity of mice at different time points

Mean cellularity was significantly lower in infected mice than in controls Data (±SEM; n=5) are representative of 2 independent experiments. Significant differences between infected animals and uninfected (day 9) *P=0.0042

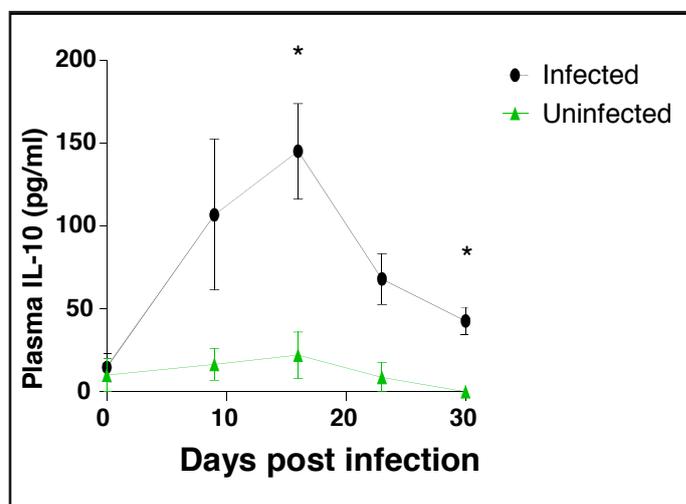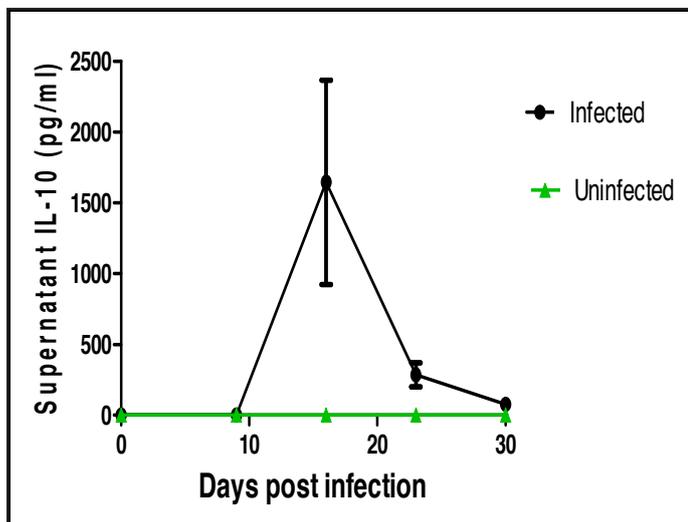**Figure 4: Plasma IL-10 levels in mice. IL-10 was quantified in *T.b. brucei* GVR 35 infected (black), and uninfected mice (green)**

Data (± SEM; n=5) are representative of 2 independent experiments. Significant difference between infected and uninfected on day 16 and 30 are indicated as *P=0007 or *P<0.00001.
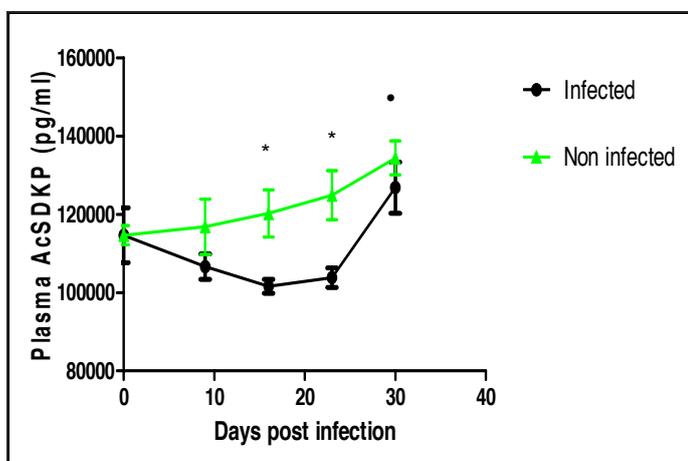
Figure 5: Supernatant IL-10 levels in mice. IL-10 was quantified in *T.b. brucei* GVR35 infected (black), and uninfected mice (green)

Data (± SEM; n=5) are representative of 2 independent experiments.

Figure 6: Plasma AcSDKP levels in mice. At different times points, plasma levels were quantified in *T.b.brucei* GVR35 infected (black) and uninfected mice (green)

Data (± SEM; n=5) are representative of 2 independent experiments. Significant differences between infected and controls are indicated as *P=0.0230 (day 16) or *P=0.0096 (day 23). Differences that are not significant are indicated as ˙P=0.4408..

The normal plasma AcSDKP in uninfected group ranged from 11.4×10^4 pg/mL ($\pm 2 \times 10^3$) to 13.4×10^4 ($\pm 4 \times 10^3$) as shown in Figure 6. Concentrations of AcSDKP in plasma from infected mice were reduced from pre-infection levels of 11.4×10^4 pg/mL ($\pm 7 \times 10^3$) to 10.6×10^4 pg/mL ($\pm 3 \times 10^3$) on day 9 (P < 0.001). The AcSDKP concentration reduced further than the baseline level on day 16 to 10.1×10^4 pg/mL ($\pm 1.7 \times 10^3$). However, an increase of AcSDKP on day 30 to 12.6×10^4 pg/mL ($\pm 6.5 \times 10^3$) was observed (P < 0.01). There was significant difference between AcSDKP levels in infected mice as compared to uninfected mice (10.1×10^4 pg/mL [$\pm 1.7 \times 10^3$] vs 12×10^4 pg/mL [$\pm 6 \times 10^3$] [P = 0.023]) on day 16 and on day 23 (10.4×10^4 pg/mL [$\pm 2.5 \times 10^3$] vs 12.5×10^4 pg/mL [$\pm 6 \times 10^3$] [P = 0.001]). However, there was no significant difference between the infected and uninfected mice on AcSDKP levels by day 30 (12.6×10^4 pg/mL [$\pm 6.5 \times 10^3$] vs 13.4×10^4 pg/mL [± 4 x10³] [P = 0.44]).

Supernatant AcSDKP concentration in *in vitro* bone marrow cultures was not detectable.

Discussion

Infection-induced anaemia is an important morbidity factor in African trypanosomiasis and has been linked to

destruction of the red blood cells due to cellular injury.[13] Packed cell volume (PCV) is a simple red blood cell count measurement that expresses the percentage of the blood's volume taken up by the red blood cells. Anaemia is said to start early in trypanosomiasis[14] and resolves in late stage of the disease.[15] Recovery from anaemia is characterized by low or absence of parasitaemia in the blood.[14] The present data indicates that PCV experiences sharp fall during early stage of infection corresponding to the appearance of parasitaemia in the circulation and persists throughout the experiment. The anaemia did not resolve despite reduced levels of parasitaemia and become severe anaemia (21%) after the first wave. This corresponded with high mortality rate of infected animals.

Appearance of parasitaemia in the circulation also corresponded to decrease in bone marrow cellularity. It has been reported elsewhere that in trypanosoma infection, the bone marrow is severely stressed by the parasite infection affecting the production of mature red blood cells.[16] Therefore it can be speculated that early reduction in the bone marrow cellularity observed in this study is an indication of a direct trypanosome effect on the bone marrow cells. This speculation is justified by the fact that the bone marrow cellularity returned to levels almost similar to those of uninfected mice after the first wave in infected mice. The data also implies that though parasitaemia may affect bone marrow cellularity at the early stage, it had little or no effect during the chronic phase of the disease.

While in infected mice PCV maintained fluctuating low levels throughout the course of infection, bone marrow cellularity was gradually increasing. The bone marrow compartment seems to recover from the initial wave and this recovery of bone marrow late in infection may indicate that some of the haematopoietic cells may escape the effects of the trypanosomes.[16]

A decrease in bone marrow nucleated cell numbers corresponded with increased IL-10 production in infected mice IL-10 is an intermediate acting factor that works in complex interactions with other cytokines in regulating haematopoiesis. The high levels of IL-10 seen in association with low levels of bone marrow nucleated cell numbers may suggest that IL-10 is part of a cytokine cascade that affects the proliferation of the bone marrow progenitors.

AcSDKP expression was observed in plasma of both the infected and uninfected animals. The plasma levels observed in uninfected mice was consistent with literature elsewhere where it shows that AcSDKP is maintained in stable levels in normal plasma.[17,18] However, the course of AcSDKP in trypanosomiasis studies has not been reported before and here we observed that in *T. brucei* infected animals, AcSDKP was down-regulated after infection and returned to levels comparable to those observed at pre infection during the terminal stage. Though the reduced levels were observed with appearance of parasitaemia, the lowest levels were not associated with high parasitaemia but suggest that the down regulation was due to the infection. Production of AcSDKP was not observed in bone marrow cell culture supernatants. Though this may mean that *T. brucei* failed to induce production of AcSDKP *in vitro*, it has been reported that AcSDKP is degradable if not preserved in medium such as captopril or lisinopril that prevents its breakdown.[19] It is a possibility that the low undetected supernatant AcSDKP levels in the supernatant may be as a result of degradation

of the cytokine by Angiotensin Converting Enzyme (ACE) which degrades it using its N-terminal active site. Alternatively, the 48-hour period that we cultured the bone marrow cells probably was not long enough for the cells to produce an AcSDKP output that could be detectable.

Comparisons between bone marrow nucleated cells and expression of AcSDKP showed that AcSDKP was down-regulated during the time period where there was reduction of bone marrow nucleated cells. Later as the infection progressed towards the terminal stage, both AcSDKP and bone marrow nucleated cells were seen to be increasing to levels comparable to those observed in uninfected mice (Figure 3 and Figure 6). Cells in the bone marrow especially the haematopoietic stem cells proliferation are thought to be inhibited by AcSDKP as it antagonises the production of stimulatory factors by stromal cells. Stimulatory factors are important as they regulate a variety of cell growth processes. Once there is disturbance in the production of these stimulatory factors there is an imbalanced proportion of high proliferative potential colony-forming unit (HPP-CFU-1) in cell cycle.[20] This imbalance therefore may contribute to reduced numbers of nucleated cells that could be produced in the bone marrow. On the other hand the increasing bone marrow nucleated cell numbers during the terminal stage can be attributed directly to the levels of AcSDKP itself. This can be argued by the fact that AcSDKP was not at its optimum concentration therefore could not exert its inhibitory role on the proliferation of stem cells. The concentration above 10^{-7} M and below 10^{-14} M of AcSDKP are known to have no effect on both mice and human bone marrow cells.[6]

Comparisons between IL-10 and AcSDKP cytokine production showed that the period when plasma levels of IL-10 were elevated it corresponded to the period where AcSDKP levels were reduced. This is not surprising as IL-10 is known to suppress production of other cytokines that are produced by macrophages. For example, IL-10 suppresses the production of M-CSF, G-CSF and MIP-1αby macrophages[21–23] which are important in the differentiation, proliferation and maturation of cells. AcSDKP has also been shown to be produced by macrophages *in vitro*[24] bringing the possibility that there could be an indirect inhibition of stimulatory molecules on this macrophages by IL-10 to produce AcSDKP. Due to suppressive nature of IL-10 on immune processes, it is plausible then that it may also suppress AcSDKP production, but this needs to be further investigated.

Interestingly on day 30 while low levels of plasma IL-10 were observed, AcSDKP was up-regulated postulating the idea that the level of IL-10 reflects the proceeding AcSDKP response which suggests a counter balance between IL-10 and AcSDKP towards improving survival of the host. However, this study did not measure this mechanistic response between AcSDKP and IL-10 and we suggest further studies to look into this aspect.

Conclusions

AcSDKP was seen to be lowered from its normal physiological levels during the presence of high IL-10 in trypanosome induced anaemia. In normal body physiology AcSDKP is observed in high titres and IL-10 is normally low or undetected. We have also observed an increase in bone marrow nucleated cells numbers which corresponded to the increase of AcSDKP concentration in the blood. Later as the infection progressed towards the terminal stage, both

AcSDKP and bone marrow nucleated cells were seen to be increasing while IL-10 was decreasing. The IL-10/AcSDKP pathway needs to be further investigated.

Competing interests

All authors declare that they have no competing interests related to this work.

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