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Maxadilan, the vasodilator/immunomodulator from *Lutzomyia longipalpis* sand fly saliva, stimulates haematopoiesis in mice

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SUMMARY

Protozoal parasites of the genus Leishmania *are transmitted to their vertebrate host within the saliva of the sand fly during a blood meal. The saliva of the sand fly* Lutzomyia longipalpis *contains maxadilan, a potent vasodilator and immunomodulator. Maxadilan has been shown to enhance the virulence of* L. major *in all strains of laboratory mice when injected along with the organism. Increased haematopoiesis has been associated with enhanced susceptibility to* Leishmania *organisms. Here, we show that maxadilan alone stimulates bone marrow haematopoiesis through its ability to stimulate interleukin-6 production by bone marrow stromal cells. Moreover, these effects of maxadilan are mediated through the interaction of maxadilan with the pituitary adenylate cyclase activating polypeptide receptor. These data suggest that increasing haematopoiesis may be yet another way that maxadilan enhances susceptibility of mice to* Leishmania *infection.*

Keywords *sand flies*, *maxadilan*, *haematopoiesis*, *IL-6*, *pituitary adenylate cyclase activating polypeptide*

INTRODUCTION

Leishmaniasis is a sand fly borne parasitic disease. Studies using experimental models have most commonly focused on immune responses to the parasite inoculated in medium. Sand fly saliva, which is injected along with the parasite in naturally occurring transmission, contains maxadilan (MAX), one of several potent biologically active components (1). MAX is both a potent vasodilator and a modulator of inflammatory/immune functions (1), actions that are mediated through the pituitary adenylate cyclase activating polypeptide receptor (PACAP-R) (2,3). The macrophage (MØ), the host cell of *Leishmania* spp., is a target cell of MAX in which it modulates cytokine production. Perhaps its most striking effect is its ability to markedly enhance secretion of interleukin (IL)-6 by MØs in the absence of any other stimulus (3). PACAP receptors have been identified on rat bone marrow-derived stromal cells, and PACAP binding results in increased IL-6 production by these cells (4).

IL-6 has been shown to enhance haematopoiesis (5–9). Because increased myelopoiesis has been associated with increased susceptibility to *Leishmania* infection (10) and as MAX exacerbates infection with the parasite (11), we investigated whether MAX would stimulate haematopoiesis. We assessed the effect of MAX on bone marrow haematopoiesis both by injecting MAX into mice and incubating it with bone marrow mononuclear cells *in vitro* and, subsequently, by measuring the number of haematopoietic progenitors using *in vitro* clonogenic assays. In addition, we explored the mechanism of the effects of MAX by determining the role of IL-6 and PACAP-R, and the involvement of various bone marrow stromal cell types (MØs, fibroblasts), in the effects of MAX on haematopoiesis. We found that MAX stimulates granulocyte-monocyte progenitors [colony-forming units-granulocyte/monocyte (CFU-GM)], early erythroid progenitors [burst forming units-erythroid (BFU-E)] and late erythroid progenitors (CFU-E) both *in vivo* and *in vitro.* We also found that this stimulation is mediated through

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PACAP receptors in bone marrow stromal cell populations and that this results in the production of IL-6 both by bone marrow stromal fibroblasts and peritoneal macrophages. These data show that stimulation of haematopoiesis may be another mechanism whereby MAX enhances infectivity of *Leishmania* parasites.

MATERIALS AND METHODS

Mice

Six to 8-week-old-female BALB/cByJ and CBA/J mice were obtained from the National Cancer Institute (Bethesda, MD, USA) or were bred at Colorado State University Laboratory Animal Resources.

Peptide and antibodies

Synthetic MAX was prepared by Dr Charles E. Dahl, Biopolymers Laboratory, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School. The 63-mer amino acid sequence used was based on the predicted sequence of mature, secreted MAX (CDATCQFRKAIEDCRKKAHHSDVLQTSVQTTATFT-SMDTSQLPGSGVFKECMKEKAKEFKAGK) (12). A monoclonal antibody (MP5 20F3) (13) against murine IL-6 (hereafter referred to as anti-IL-6) was produced from ascites. A control antibody (GL113) specific for β-galactosidase, produced from same species and of the same isotype as the anti-IL-6 antibody (14), was also used in some experiments.

Isolating bone marrow cells and counting reticulocytes

Bone marrow cells were flushed out of both tibias of each mouse, a single-cell suspension was prepared in Minimal Essential Medium-α (MEM-α) (Gibco BRL, Grand Island, NY, USA) and the concentration of the cells was determined (15). Reticulocytes were enumerated by counting their number out of 1000 total red blood cells in a thin blood film stained with New Methylene Blue (Sigma, St Louis, MO, USA).

Colony progenitor assays

Colony progenitor assays were performed to assess haematopoiesis. CFU-E are late erythroid progenitors, which form small colonies after 48–72 h in semisolid culture. Both BFU-E (early erythroid progenitors) and CFU-GM (progenitors of granulocytes and MØs) form colonies after 6–7 days. Suspensions of bone marrow cells from infected mice or cells harvested from cultures *in vitro* were all adjusted to 2×10^6 cells/ml in MEM-α. Cells were then further diluted 1 : 10 into methylcellulose culture medium (Methocult GF3434, StemCell Technologies, Vancouver, BC, Canada) and were plated in a volume of 200 µl in wells of 48-well plates (40 000 cells/well). Plates were incubated at 37°C. CFU-E, BFU-E and CFU-GM were enumerated using an inverted phase microscope. Counts from the duplicate wells were averaged and numbers are reported per 40 000 cells plated or per tibia.

Long-term bone marrow culture (LTBMC) experiments

Bone marrow cells from BALB/c or CBA mice were seeded at 3×10^6 cells/ml in Myelocult (StemCell Technologies), a liquid medium, supplemented with hydrocortisone 21-hemisuccinate (Sigma) at a concentration of 10^{-6} M in 48-well plates (16). Cells were incubated for 3 days at 37°C and then at 33°C for the duration of the experiment. After the first week, weekly feedings were performed. Half of the medium from each well was removed, the plate was gently shaken to allow nonadherent cells to escape from the adherent layer, and the remaining medium was removed and saved. Half of the volume of the saved cells and medium were reintroduced in each well and the same volume of fresh medium was added. After 4 weeks, the LTBMC were established and could be treated with MAX. At that time, stromal cells were confluent and exhibited a cobblestone appearance that is typical for a mixture of fibroblasts, adipocytes, macrophages and endothelial cells (16,17). Clusters of haematopoietic cells could also be seen. During the last feeding, four wells from each LTBMC culture plate were treated with either MAX at a concentration of 4 ng/ml in Myelocult medium, MAX at 4 ng/ml and anti-IL-6 at a concentration of 5 mg/ml in Myelocult medium, anti-IL-6 at a concentration of 5 mg/ml in Myelocult medium, or Myelocult medium alone, with the latter being used as control. Medium alone is a standard control in experiments involving maxadilan. After 3 days, cells were removed with trypsin-EDTA (0·25%, Sigma). The cells were washed and clonogenic progenitor assays were performed.

Establishing and treating irradiated bone marrow stromal layers *in vitro*

A stromal layer is a mixture of fibroblasts, adipocytes, macrophages, endothelial cells and a few haematopoietic precursors (17). Irradiated stromal layers differ from LTBMC in that the residual haematopoietic cells from donor mice are inactivated. Therefore, the effects of MAX on the stromal components only (as opposed to direct effects on haematopoietic progenitors) could be determined. BALB/c or CBA bone marrow cells were cultured in Myelocult at 1×10^6 cells per well in 48-well plates for 8–12 days at 37°C.

The cells were then detached with trypsin-EDTA, irradiated at 15 Gy to inactivate donor haematopoietic cells, replated in Myelocult at 10^6 cells/well in 48-well plates and incubated at 37°C for 24 h (16) so that a confluent monolayer of cells was achieved. The stromal layer cells were then treated with either MAX (4 ng/ml) in Myelocult or Myelocult only for 3 days at 37°C. In some cases, the PACAP antagonist PACAP 6–38 (Peninsula Laboratories, Belmont, CA, USA) was also added to the cultures (200 ng/ml). The supernatants of the cultures were removed, the cultures were rinsed and normal, nonirradiated, syngeneic bone marrow cells in Myelocult were seeded on the feeder layers at 1×10^6/well as a source of haematopoietic progenitors. In some cases, anti-IL-6 or control antibody (5 μg/ml) was also added to the cultures. Three days later, cells were removed from wells with trypsin-EDTA and washed in MEM-α. The cells were then tested in colony progenitor assays.

Producing and treating peritoneal-derived macrophages *in vitro*

Peritoneal cells were isolated from BALB/c or CBA mice as described (15,18). The cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) for 24 h at 3×10^6/ml in 24-well culture dishes (Costar 3524, Corning, NY, USA) to allow MØs to adhere. The cultures were then rinsed to remove nonadherent cells. The adherent cells were shown to be 95% $CD11b^+$ cells (18). The MØs were treated for 24 h with either MAX at a concentration of 4 ng/ml or culture medium alone as a control. Supernatants were removed, cultures rinsed and fresh medium was added for an additional 48 h. The supernatants were then removed and stored at –20°C until use. Macrophage supernatants were incubated with bone marrow mononuclear cells in medium at a 1 : 1 dilution for 2 h on ice with or without the addition of anti-IL-6 or control antibody at 5 μg/ml. After incubation, colony progenitor assays were performed.

Producing and treating bone marrow-derived fibroblasts *in vitro*

Fibroblasts were cultured by standard methods. Briefly, bone marrow cells were isolated from either BALB/c or CBA mice and cultured for 24 h. The fibroblast culture medium is composed of D-MEM, 15% fetal calf serum, L-glutamine and gentamycin. The nonadherent cells were removed and the cultures were fed by medium replacement every other day until confluence was reached. The cells were detached with trypsin-EDTA, washed and subcultured. Cells underwent three passages before being used. After the last trypsin treatment and wash, bone marrow-derived fibroblasts were irradiated at 1500 cGy and washed once in MEM-α. Cells were plated in 48-well plates at 1×10^6 cells/well and incubated at 37°C for 24 h to form a confluent monolayer (16). The fibroblasts were treated with either MAX at a concentration of 4 ng/ml in Myelocult or Myelocult only for 3 days at 37°C. The supernatants of the cultures were removed, the cultures were rinsed and normal, nonirradiated, syngeneic bone marrow cells in Myelocult were seeded on the fibroblast layers at 1×10^6 per well as a source of haematopoietic progenitors. In some cases, anti-IL-6 (5 μg/ml) was also added to the cultures. Three days later, cells were removed from wells with trypsin-EDTA and washed in MEM-α. Hematopoietic progenitors were then enumerated in clonogenic progenitor assays.

IL-6 ELISA

Supernatants from LTBMC were tested for IL-6 using a sandwich ELISA performed according to manufacturer's directions (Pharmingen, San Diego, CA, USA).

Statistical analysis

All experiments were repeated at least two times. The Kolmogorov–Smirnov test was performed to ensure the data from each experiment were normally distributed. All data were analysed by single factor ANOVA except reticulocyte experiments, which were analysed with a repeated measures ANOVA. A Fisher's PLSD was used as a post-hoc test. $P < 0{\cdot}05$ was considered statistically different.

RESULTS

Maxadilan stimulates haematopoiesis in mice

Susceptibility to *L. major* in mice is genetically controlled, and CBA mice are relatively resistant to cutaneous leishmaniasis compared with BALB/c mice (19). Maxadilan, when inoculated along with parasite, increases the susceptibility of both strains (11,20). We therefore asked whether MAX stimulates haematopoiesis in both strains of mice. We injected CBA and BALB/c mice with 4 ng of MAX subcutaneously in one hind footpad and enumerated bone marrow haematopoietic progenitors in clonogenic assays 3 days later. We chose the 4 ng dose because the results of previous experiments showed that this dose of MAX enhanced the infectivity of *L. major* for mice to the greatest extent (11). Figure 1 shows that MAX significantly enhanced the frequency of CFU-E, BFU-E and CFU-GM colonies in both CBA (Figure 1a) and BALB/c (Figure 1b) mice. Animals treated with medium alone developed colony frequencies that did not differ from untreated control mice.

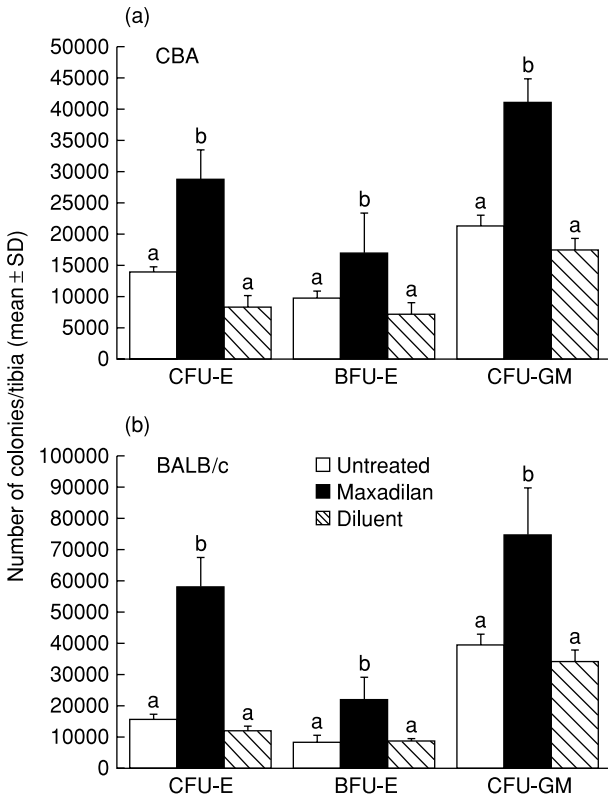

Figure 1 Maxadilan stimulates haematopoiesis in mice. Groups of four CBA (a) or BALB/c (b) mice were injected with 4 ng of MAX subcutaneously in a hind footpad and clonogenic progenitor assays were performed with bone marrow cells from the mice 3 days later. Controls were left untreated or were injected with medium (MEM-α) only. The figure depicts the number of CFU-E, BFU-E and CFU-GM colonies obtained per tibia (mean ± SD). Different letters (a and b) denote differences between treatment groups ($P < 0{\cdot}05$) for each progenitor evaluated.

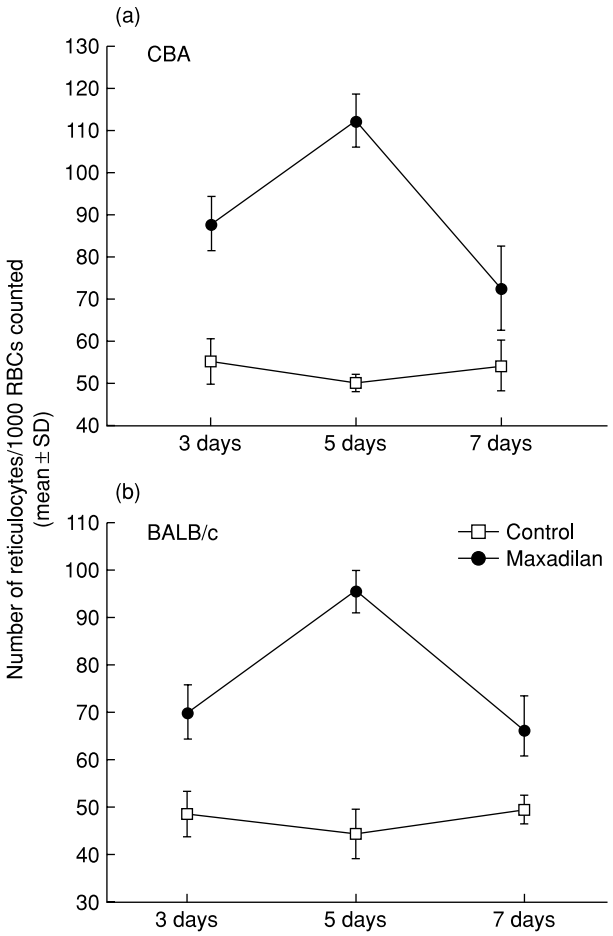

Figure 2 Maxadilan increases the number of circulating reticulocytes in mice. Groups of four CBA (a) or BALB/c (b) mice were injected with 4 ng of MAX subcutaneously in a hind footpad, and the number of circulating reticulocytes (mean ± SD) was determined at 3, 5 and 7 days later. Controls were injected with MEM-α. At all times, there were significantly more circulating reticulocytes in MAX-treated compared to control mice ($P < 0{\cdot}05$).

Maxadilan increases the number of circulating reticulocytes in mice

To determine whether the increased bone marrow erythropoiesis results in changes in peripheral blood, we treated mice with MAX and followed the number of circulating reticulocytes (immature red blood cells). Figure 2 demonstrates that MAX significantly increased the numbers of circulating reticulocytes in both CBA (Figure 2a) and BALB/c mice (Figure 2b). MAX treatment resulted in a significantly elevated number of circulating reticulocytes in both strains of mice, compared with controls at 3, 5 and 7 days post treatment. These results indicate that the erythropoietic effects of MAX result in an increase of all erythroid maturational stages. The decline in reticulocyte numbers seen over time in MAX-treated groups suggests that MAX has a transient stimulatory effect on erythropoiesis *in vivo*.

Maxadilan stimulates haematopoiesis in cultured bone marrow cells

In the previous section, our results showed that MAX, injected subcutaneously, stimulates haematopoietic progenitors from the bone marrow of treated mice. We were not able to conclude from these results whether MAX affects bone marrow directly, or indirectly through its effects on another tissue. To determine whether MAX has direct effects on bone marrow cells, we used both short-term progenitor assays and long-term bone marrow cultures (LTBMC). Using these assays, we ensured that we could recapitulate the *in vivo* effects, thus setting the stage to dissect the effects of MAX on cellular compartments of the

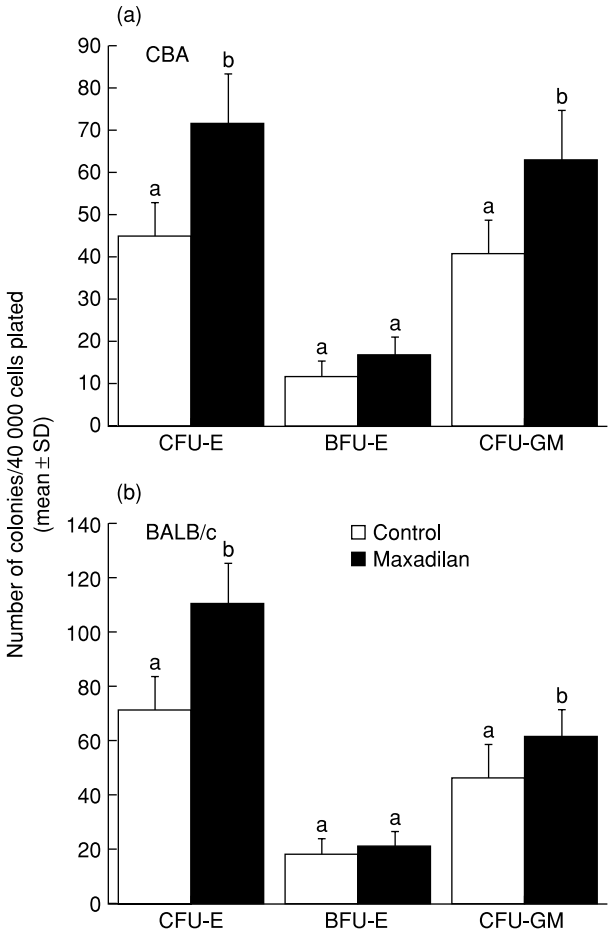

Figure 3 Maxadilan stimulates haematopoiesis in short-term progenitor assays *in vitro*. Bone marrow was collected from CBA (a) or BALB/c (b) mice (four mice per treatment group) and preincubated either with MAX (4 ng/ml) or with medium alone (control) for 2 h at 4°C prior to progenitor enumeration. The figure depicts the number of CFU-E, BFU-E and CFU-GM colonies obtained per 40 000 cells plated (mean ± SD). Different letters (a and b) denote differences between treatment groups ($P < 0{\cdot}05$).

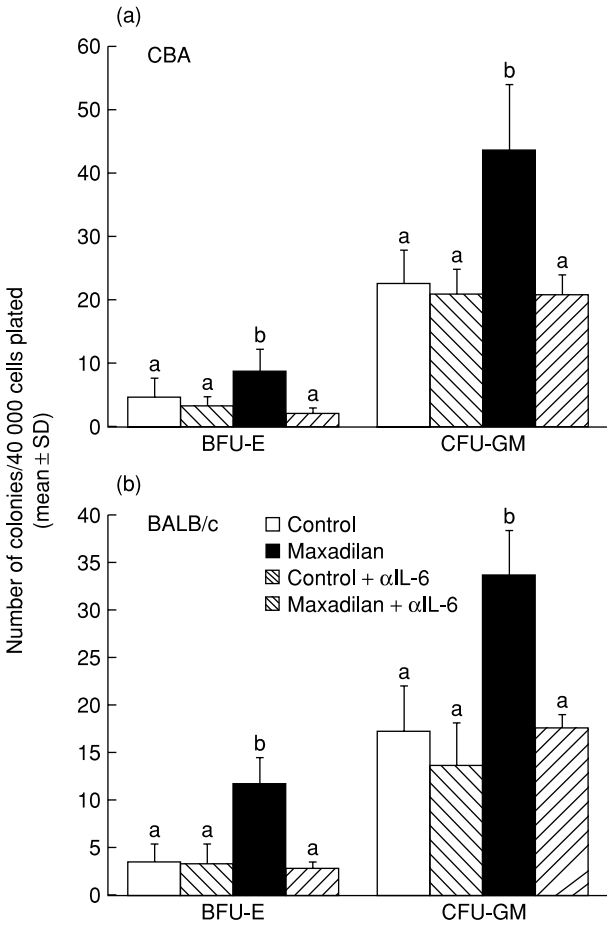

Figure 4 Maxadilan can stimulate haematopoiesis through IL-6 in long-term bone marrow cultures. LTBMCs were derived from CBA (a) and BALB/c (b) bone marrow cells. Four wells each were then treated with either MAX (4 ng/ml) or medium alone (control) in the presence or absence of a neutralizing anti-IL-6 antibody (5 μg/ml). Three days later, the cultures were harvested and washed and then clonogenic progenitor assays were performed. The figure depicts the number of BFU-E and CFU-GM colonies obtained per 40 000 cells plated (mean ± SD). Different letters (a and b) denote differences between treatment groups ($P < 0{\cdot}05$).

bone marrow. We also asked whether IL-6 played a role in progenitor stimulation by adding anti-IL-6 to the LTBMC.

For short-term assays, we incubated freshly isolated bone marrow cells from CBA and BALB/c mice with MAX (4 ng/ml) and performed clonogenic progenitor assays. The results in Figure 3 show that, under these culture conditions, MAX significantly stimulated CFU-E and CFU-GM in both CBA (Figure 3a) and BALB/c (Figure 3b) mice.

LTBMCs recapitulate all aspects of bone marrow haematopoiesis *in vivo* because the cultures contain both bone marrow stromal cells and haematopoietic progenitors (16,17,21,22). CFU-E were not enumerated because erythroid progenitors in LTBMC do not differentiate beyond BFU-E without erythropoietin (23,24). As seen in Figure 4, when LTBMCs from CBA and BALB/c were stimulated with MAX for 3 days, there was a significant increase compared to controls in BFU-E and CFU-GM in both CBA (Figure 4a) and BALB/c (Figure 4b) mice. The addition of anti-IL-6 eliminated MAX-induced haematopoietic progenitor stimulation, whereas it had no effect on control cultures. A control antibody (anti β-galactosidase) also had no effect on the activities of MAX. These data indicate that MAX mediates haematopoietic stimulation via IL-6 production. This conclusion was reinforced the observation that supernatants from LTBMC of CBA and BALB/c mice contained

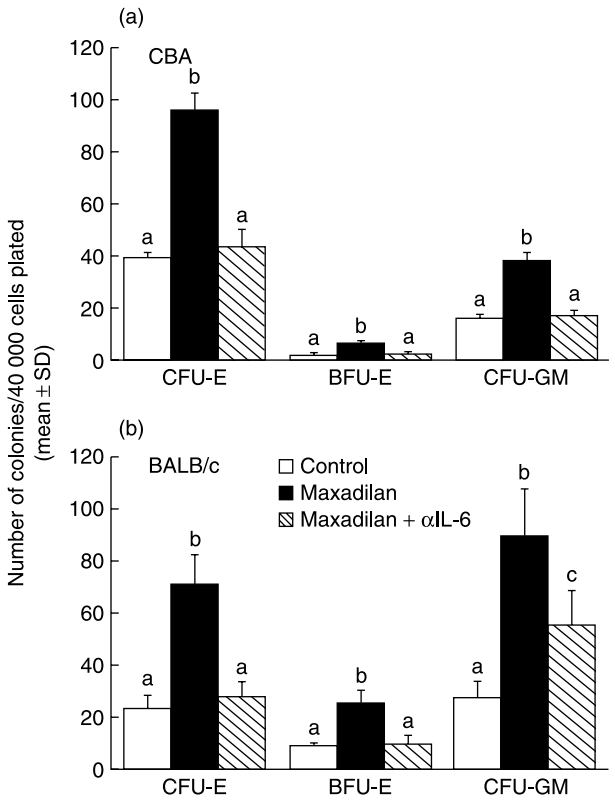

Figure 5 Maxadilan can stimulate haematopoiesis through IL-6 in stromal cell cultures. Stromal cell cultures were derived from CBA (a) and BALB/c (b) bone marrow cells and irradiated. Four wells of each feeder layer type (CBA or BALB/c) were then treated with either MAX (4 ng/ml) or medium alone (control) for 3 days. The cultures were rinsed and syngeneic bone marrow cells were added as a source of haematopoietic progenitors in the presence or absence of anti-IL-6 (5 μg/ml). Three days later, the cells were removed with trypsin-EDTA and progenitors were enumerated. The figure depicts the number of CFU-E, BFU-E and CFU-GM colonies obtained per 40 000 cells plated (mean ± SD). Different letters (a, b and c) denote differences between treatment groups ($P < 0{\cdot}05$).

increased (11·8-fold and five-fold, respectively) amounts of IL-6 compared to controls when stimulated with MAX for 3 days.

MAX-stimulated bone marrow stromal cells support increased haematopoiesis via IL-6

The above experiments, which reproduced the *in vivo* effects of MAX, show that cells that reside in the marrow can mediate the stimulation of haematopoiesis. The bone marrow mononuclear cell population that we used contains both stromal cells and haematopoietic progenitors. The stimulatory effect of MAX treatment on progenitor proliferation could therefore be a direct effect on haematopoietic progenitors or an indirect effect mediated by stromal cells. Maxadilan stimulates macrophages, via the PACAP receptor, to produce IL-6 (3) and IL-6 is capable of stimulating haematopoiesis (5–9). PACAP receptors have been identified on rat bone marrow derived stromal cells (4). In the next set of experiments, we sought to determine whether stromal cells (in the form of an irradiated stromal layer) could mediate the effects of MAX through PACAP-R, and whether these effects are due to IL-6 production.

We established stromal cell cultures (17) from CBA and BALB/c mice and measured their ability to support haematopoiesis after treatment with MAX. The results are shown in Figure 5. MAX-treated bone marrow-derived stromal cells were able to stimulate increased haematopoiesis in both strains of mice, as indicated by increased numbers of CFU-E, BFU-E and CFU-GM, in MAX-treated groups compared to controls. This effect was not seen when anti-IL-6 antibody was present, indicating that the effects of MAX are mediated at some level through IL-6 (Figure 5).

The PACAP antagonist PACAP 6-38 eliminates maxadilan-induced haematopoietic-stimulation by bone marrow stromal cells

MAX appeared to stimulate haematopoiesis through interaction with bone marrow stromal cells and IL-6 production (Figure 5). As MAX has been shown to act through PACAP receptors and as these have been identified on bone marrow stromal cells of rats, we sought to determine whether MAX acted on bone marrow-derived stromal cells through interaction with PACAP receptors by adding the PACAP antagonist, PACAP 6-38. PACAP 6-38 blocked the effects of MAX in both CBA (Figure 6a) and BALB/c (Figure 6b) stromal cell cultures. We conclude that MAX mediates its effects on bone marrow stromal cells through interaction with the PACAP receptor.

Supernatants from maxadilan-treated macrophages stimulate haematopoiesis *in vitro*

Stromal bone marrow cells, which interact with haematopoietic progenitors to regulate haematopoiesis, are composed of a mixture of fibroblasts, MØs, adipocytes and some endothelial cells (25). Because PACAP-R are expressed on both fibroblasts (26) and MØ (3), we sought to determine whether MAX could mediate its effects through macrophages, using the peritoneal cavity as a source of MØs. As seen in Figure 7, cell free supernatants from MAX-treated MØs were able to stimulate bone marrow haematopoietic progenitors as indicated by increased CFU-E, BFU-E and CFU-GM colonies in these groups compared to controls. Anti-IL-6 antibody inhibited the effect of MAX-treated MØ supernatants.

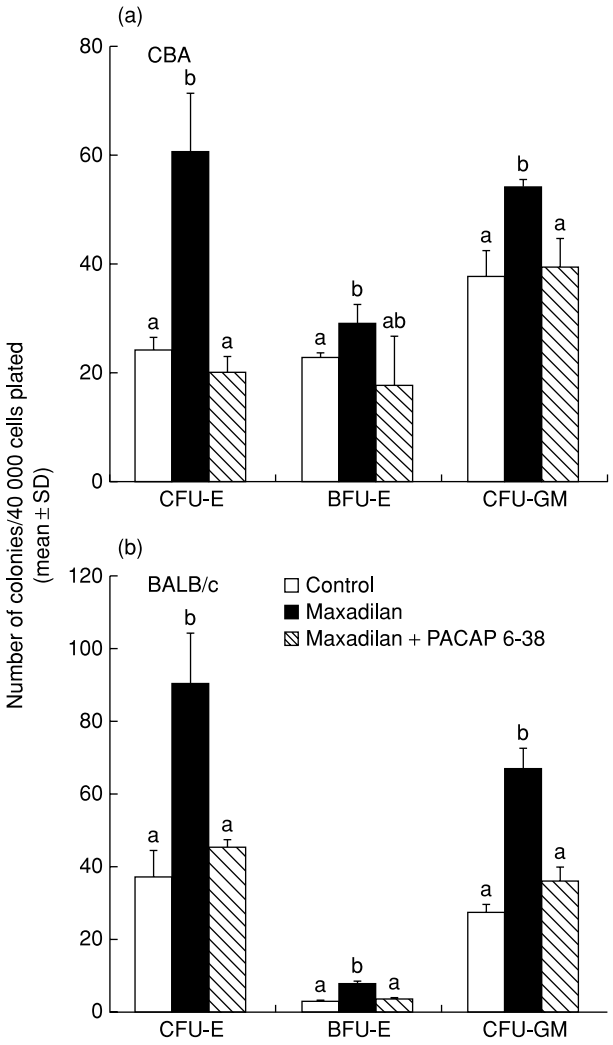

Figure 6 PACAP receptor antagonist abrogates the haematopoietic stimulation of maxadilan in stromal cell cultures. Stromal cell cultures were derived from CBA (a) and BALB/c (b) bone marrow cells and irradiated. Four wells of each feeder layer type (CBA or BALB/c) were then treated with either MAX (4 ng/ml), MAX plus the PACAP-R antagonist PACAP 6-38, or medium alone (control) for 3 days. The cultures were rinsed and syngeneic bone marrow cells were added as a source of haematopoietic progenitors. Three days later, the cells were removed with trypsin-EDTA and progenitors were enumerated. The figure depicts the number of CFU-E, BFU-E and CFU-GM colonies obtained per 40 000 cells plated (mean ± SD). Different letters (a and b) denote differences between treatment groups ($P < 0{\cdot}05$).

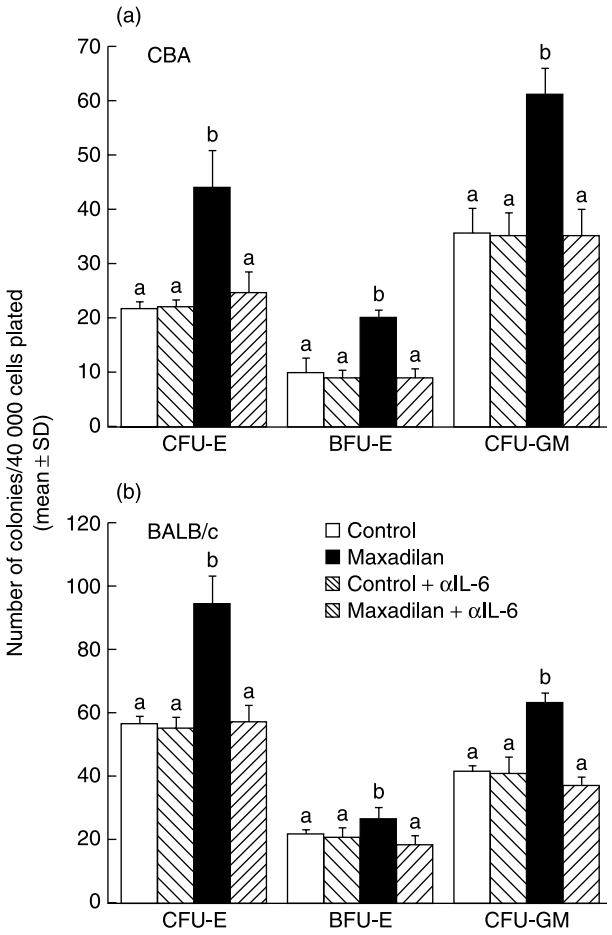

Figure 7 Maxadilan-treated macrophage supernatants can stimulate haematopoiesis through IL-6. Peritoneal MØs were treated with MAX (4 ng/ml) or medium alone (control) and the supernatants of these cultures were tested for their effect on haematopoiesis by incubating freshly isolated bone marrow cells with the supernatants (in the presence or absence of anti-IL-6, 5 μg/ml) and then testing the cells in colony progenitor assays. The figure depicts the number of CFU-E, BFU-E and CFU-GM colonies obtained per 40 000 cells plated (mean ± SD) using CBA (a) or BALB/c (b) bone marrow cells. Different letters (a and b) denote differences between treatment groups ($P < 0{\cdot}05$).

Maxadilan-treated bone marrow-derived fibroblasts stimulate haematopoiesis

In order to determine whether MAX can stimulate haematopoiesis through fibroblasts, we incubated bone marrow fibroblast cultures with MAX and measured their ability to support haematopoiesis. Maxadilan-treated fibroblast cultures stimulated increased haematopoiesis as indicated by significantly higher numbers of colonies compared to controls (Figure 8). Because fibroblasts can produce IL-6 (26), we tested the effects of anti-IL-6 in these fibroblast/haematopoietic cell cocultures. The presence of anti-IL-6 eliminated the effect of MAX treatment of fibroblasts, indicating that IL-6 is involved in mediating the stimulatory effect of MAX on haematopoiesis.

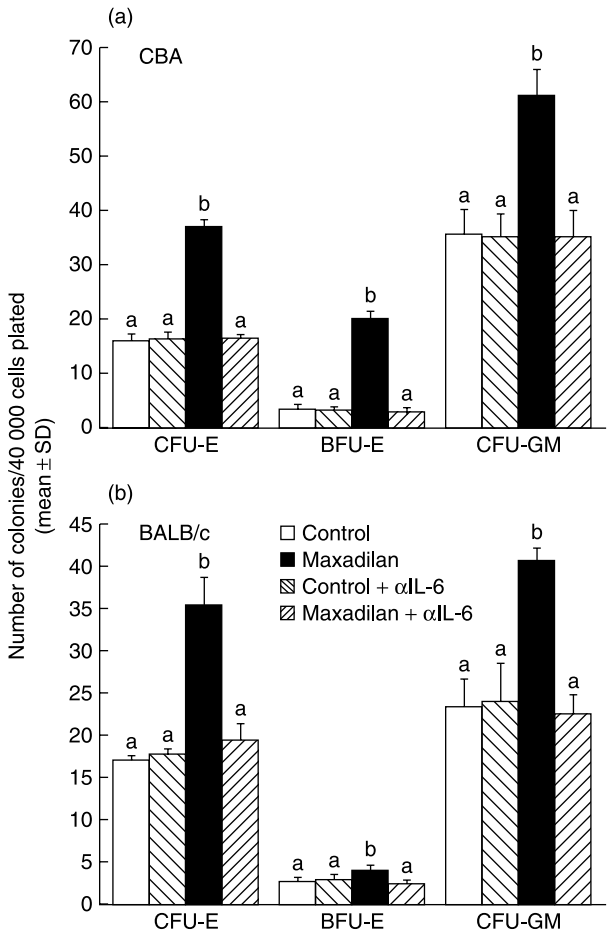

Figure 8 Maxadilan-treated, bone marrow-derived fibroblasts can stimulate haematopoiesis. Bone marrow fibroblasts were treated with MAX (4 ng/ml) or medium alone (control) for 3 days, the cultures were rinsed and bone marrow cells were added as a source of haematopoietic progenitors in the presence or absence of anti-IL-6. The cells were removed using trypsin-EDTA and the numbers of progenitors were enumerated in colony progenitor assays. The figure depicts the number of CFU-E, BFU-E and CFU-GM colonies obtained (mean ± SD) with CBA (a) or BALB/c (b) bone marrow haematopoietic progenitor cells. Different letters (a and b) denote differences between treatment groups ($P < 0{\cdot}05$).

DISCUSSION

It is now clear that the saliva of both Old World as well as New World sand flies contains several molecules that modulate blood flow, inflammation and the development of an immune response (1,27). Saliva profoundly exacerbates infection with *L. major* in many strains of mice (20,28) and this is associated with marked changes in the immune response to the parasite (29,30). Vaccination with salivary gland components, including maxadilan, has been shown to protect mice from infection with *L. major* (11,31). It was recently shown that MAX is the major component of *L. longipalpis* saliva responsible for exacerbation of *L. major* infection (11). The ability of MAX to exacerbate *L. major* infection may be linked to its ability to alter the activities of antigen presenting cells, principally MØs. For example, MAX stimulates IL-6 production by MØs while it inhibits TNF-α (3). Here, we show that MAX, injected subcutaneously, can stimulate bone marrow haematopoiesis.

We showed that the stimulatory effects of MAX on haematopoiesis were mediated through the production of IL-6, an effect that was abrogated by blocking PACAP receptors. Although some studies show that IL-6 can suppress erythropoiesis (32), the preponderance of evidence suggests that IL-6 has stimulatory effects on early progenitors and progenitors of both the granulocyte-monocyte and erythroid lineages (5–8,32). Both MAX and PACAP have been shown to stimulate IL-6 production by MØs (3,33), probably through activation of PACAP-R (34,35). PACAP-R is found on bone marrow-derived stromal cells (4), as well as in the pituitary gland, pancreas, adrenal medulla and testes (36).

We used LTBMC, a method that most closely resembles the *in vivo* haematopoietic environment, to ensure that the effects of MAX could be reproduced *in vitro*. Hematopoiesis in this system is dependent upon formation of a confluent stromal layer, composed of fibroblasts, MØs, endothelial cells and adipocytes (17), which will provide cell-to-cell interactions and adequate growth factors to sustain renewal, proliferation and differentiation of early and committed progenitors (21). After stimulation with MAX, both early erythroid and myeloid progenitors were increased, which was consistent with the effect of MAX seen *in vivo*.

MØs and fibroblasts are major components of the bone marrow stroma, both *in vivo* and *in vitro* (25), and both cell types are important in supporting the development of haematopoietic progenitor cells. Supernatants from MAX-treated MØs were able to stimulate CFU-E, BFU-E and CFU-GM formation after incubation with bone marrow progenitors. This stimulation was dependent upon IL-6 production because anti-IL-6 eliminated the stimulatory effect of MAX-treated MØ supernatants. Bone marrow-derived fibroblasts were also able to support haematopoiesis, and pretreatment with MAX led to an increase in haematopoiesis. As with MØs, IL-6 appeared to have a major role in mediating the effect of MAX. Therefore, we know that MAX acts to stimulate progenitor growth indirectly through cells of the bone marrow stroma. Whether MAX can stimulate progenitor cells directly remains to be determined.

In all the experimental settings we examined, IL-6 was involved in MAX-induced haematopoietic stimulation. Indeed, the ability of MAX to stimulate IL-6 production in MØs is one of its most striking effects; MAX stimulates IL-6 in the absence of any other stimuli (3). However, it is also

possible that MAX modulates the production of cytokines other than IL-6 and that this in turn affects haematopoiesis. For example, MAX has been shown to down-regulate TNF-α production by MØs (3), and TNF-α can be a potent inhibitor of haematopoiesis, inducing apoptosis of haematopoietic progenitors produced by endothelial cells and MØs, which are both elements of the bone marrow stroma (17,37). The role of TNF-α in our system requires further investigation.

To the best of our knowledge, this is the first report that a sand fly salivary gland molecule has been shown to affect haematopoiesis. Moreover, this is an example of what appears to be extensive cross talk between the immune and neural systems. Increased haematopoiesis and decreased MØ maturation have been associated with susceptibility to *L. major* infection (10,38), and *L. donovani* has been shown to increase myelopoiesis is mice (39). By stimulating haematopoiesis, MAX may favour parasite survival, and this may contribute to the overall enhancing effect of MAX/saliva on infection with *L. major*.

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REFERENCES

1 Gillespie RD, Mbow ML, Titus RG. The immunomodulatory factors of bloodfeeding arthropod saliva. *Parasite Immunol* 2000; **22**: 319–331.
2 Moro O, Lerner EA. Maxadilan, the vasodilator from sand flies, is a specific pituitary adenylate cyclase activating peptide type I receptor agonist. *J Biol Chem* 1997; **272**: 966–970.
3 Soares MBP, Titus RG, Shoemaker CB, David JR, Bozza M. The vasoactive peptide Maxadilan from sand fly saliva inhibits TNF-a and induces IL-6 by mouse macrophages through interaction with the PACAP receptor. *J Immunol* 1998; **160**: 1811–1816.
4 Cai Y, Xin X, Shim G *et al.* Pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) stimulate interleukin-6 production through the third subtype of PACAP/VIP receptor in rat bone marrow-derived stromal cells. *Endocrinology* 1997; **138**: 2515–2520.
5 Chiu CP, Moulds C, Coffman RL, Rennick D, Lee F. Multiple biological activities are expressed by a mouse interleukin 6 cDNA clone isolated from bone marrow stromal cells. *Proc Natl Acad Sci USA* 1988; **85**: 7099–7103.
6 Ikebuchi K, Wong GG, Clark SC, Ihle JN, Hirai Y, Ogawa M. Interleukin 6 enhancement of interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. *Proc Natl Acad Sci USA* 1987; **84**: 9035–9039.
7 Ulich TR, del Castillo J, Yin SM, Egrie JC. The erythropoietic effects of interleukin 6 and erythropoietin in vivo. *Exp Hematol* 1991; **19**: 29–34.
8 Wong GG, Witek-Giannotti JS, Temple PA *et al.* Stimulation of murine hematopoietic colony formation by human IL-6. *J Immunol* 1988; **140**: 3040–3044.
9 Sui X, Tsuji K, Tajima S *et al.* Erythropoietin-independent erythrocyte production: signals through gp130 and c-kit dramatically promote erythropoiesis from human CD34+ cells. *J Exp Med* 1996; **183**: 837–845.
10 Mirkovich AM, Galelli AC, Modabber FZ. Increased myelopoiesis during *Leishmania major* infection in mice. generation of 'safe targets', a possible way to evade the effector immune mechanism. *Clin Exp Immunol* 1986; **64**: 1–7.
11 Morris RV, Shoemaker CB, David JR, Lanzaro GC, Titus RG. Sand fly maxadilan exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. *J Immunol* 2001; **167**: 5226–5230.
12 Lanzaro GC, Lopes AHCS, Ribeiro JMC *et al.* Variation in the salivary peptide, maxadilan, from species in the *Lutzomyia longipalpis* complex. *Insect Mol Biol* 1999; **8**: 267–275.
13 Coffman RL, Varkila K, Scott P, Chatelain R. Role of cytokines in the differentiation of CD4+ T-cell subsets *in vivo*. *Immunol Rev* 1991; **123**: 189–207.
14 DeKrey GK, Lima HC, Titus RG. Analysis of the immune responses of mice to infection with *Leishmania braziliensis*. *Infect Immun* 1998; **66**: 827–829.
15 Titus RG, Kelso A, Louis JA. Intracellular destruction of *Leishmania tropica* by macrophages activated with macrophage activating factor/interferon. *Clin Exp Immunol* 1984; **55**: 157–165.
16 Eaves CJ, Cashman JD, Eaves AC. Methodology of long-term culture of human hematopoietic cells. *J Tissue Culture Meth* 1991; **13**: 55–62.
17 Chen Z, Van Bockstaele DR, Buyssens N *et al.* Stromal populations and fibrosis in human long-term bone marrow cultures. *Leukemia* 1991; **5**: 772–781.
18 Chakkalath HR, Titus RG. *Leishmania major*-parasitized macrophages augment Th2-type T cell activation. *J Immunol* 1994; **153**: 4378–4387.
19 Rogers KA, DeKrey GK, Mbow ML, Gillespie RD, Brodskyn CI, Titus RG. Type 1 and type 2 responses to *Leishmania major*. *FEMS Microbiol Lett* 2002; **209**: 1–7.
20 Titus RG, Ribeiro JMC. Salivary gland lysates from the sand fly *Lutzomyia longipalpis* enhance *Leishmania* infectivity. *Science* 1988; **239**: 1306–1308.
21 Stark R, Andre C, Thierry D, Cherel M, Galibert F, Gluckman E. The expression of cytokine and cytokine receptor genes in long-term bone marrow culture in congenital and acquired bone marrow hypoplasia. *Br J Haematol* 1993; **83**: 560–566.
22 Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of haematopoietic stem cells *in vitro*. *J Cell Physiol* 1977; **91**: 335–344.
23 Migliaccio AR, Migliaccio G. The making of an erythroid cell. *Biotherapy* 1998; **10**: 251–268.
24 Michurina TV, Khrushchov NG. Experimental analysis of cell interactions during hematopoiesis. *Int J Dev Biol* 1997; **41**: 817–833.
25 Mayani H, Guilbert MH, Janowska-Wieczorek A. Biology of the hematopoietic microenvironment. *Eur J Haematol* 1992; **49**: 225–233.
26 Samardzic T, Jankovic V, Stosic-Grujicic S, Trajkovic V. STAT1 is required for iNOS activation, but not IL-6 production in murine fibroblasts. *Cytokine* 2001; **13**: 179–182.
27 Kamhawi S. The biological and immunomodulatory properties of sand fly saliva and its role in the establishment of *Leishmania* infections. *Microbes Infect* 2000; **2**: 1765–1773.
28 Theodos CM, Ribeiro JMC, Titus RG. Analysis of enhancing effect of sand fly saliva on *Leishmania* infection in mice. *Infect Immun* 1991; **59**: 1592–1598.

29 Mbow ML, Bleyenberg JA, Hall LR, Titus RG. *Phlebotomus papatasi* sand fly salivary gland lysate downregulates a Th1, but upregulates a Th2, response in mice infected with *Leishmania major*. *J Immunol* 1998; **161**: 5571–5577.
30 Belkaid Y, Kamhawi S, Modi G *et al.* Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. *J Exp Med* 1998; **188**: 1941–1953.
31 Valenzuela JG, Belkaid Y, Garfield MK *et al.* Toward a defined anti-Leishmania vaccine targeting vector antigens: characterization of a protective salivary protein. *J Exp Med* 2001; **194**: 331–342.
32 Bernad A, Kopf M, Kulbacki R, Weich N, Koehler G, Gutierrez-Ramos JC. Interleukin-6 is required *in vivo* for the regulation of stem cells and committed progenitors of the hematopoietic system. *Immunity* 1994; **1**: 725–731.
33 Martinez C, Delgado M, Pozo D *et al.* VIP and PACAP enhance IL-6 release and mRNA levels in resting peritoneal macrophages: *in vitro* and *in vivo* studies. *J Neuroimmunol* 1998; **85**: 155–167.
34 Delgado M, De la Fuente M, Martinez C, Gomariz RP. Pituitary adenylate cyclase-activating polypeptides (PACAP27 and PACAP38) inhibit the mobility of murine thymocytes and splenic lymphocytes: comparison with VIP and implication of cAMP. *J Neuroimmunol* 1995; **62**: 137–146.
35 Delgado M, Garrido E, De la Fuente M, Gomariz RP. Pituitary adenylate cyclase-activating polypeptide (PACAP-38) stimulates rat peritoneal macrophage functions. *Peptides* 1996; **17**: 1097–1105.
36 Tornoe K, Hannibal J, Fahrenkrug J, Holst JJ. PACAP-(1-38) as neurotransmitter in pig pancreas: receptor activation revealed by the antagonist PACAP-(6-38). *Am J Physiol* 1997; **273**: G436–G446.
37 Bendtzen K. Cytokines and natural regulators of cytokines. *Immunol Lett* 1994; **43**: 111–123.
38 Sunderkotter C, Kunz M, Steinbrink K *et al.* Resistance of mice to experimental leishmaniasis is associated with more rapid appearance of mature macrophages *in vitro* and *in vivo*. *J Immunol* 1993; **151**: 4891–4901.
39 Cotterell SE, Engwerda CR, Kaye PM. *Leishmania donovani* infection of bone marrow stromal macrophages selectively enhances myelopoiesis, by a mechanism involving GM-CSF and TNF-α. *Blood* 2000; **95**: 1642–1651.