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DISSERTATION

LEISHMANIA MAJOR AND HEMATOPOIESIS: EFFECT OF THE PARASITE OR SAND FLY SALIVA PEPTIDE MAXADILAN

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

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Fort Collins, Colorado

Summer 2000

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY Valérie O. Guilpin ENTITILED *Leishmania major* and hematopoiesis: Effect of the parasite or sand fly salivary peptide maxadilan BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR DEGREE OF DOCTOR OF PHILOSPHY.

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Abstract of Dissertation

Leishmania major and hematopoiesis:
Effect of the parasite or sand fly salivary peptide maxadilan

Experimental models of leishmaniasis tend to overlook the vector role in transmission of the parasite. Saliva clearly exacerbates *Leishmania* infection. The vasodilatory peptide maxadilan has been isolated from the saliva of the sand fly *Lutzomyia longipalpis*. This molecule exhibits immunomodulatory properties by acting as an analogue of the neuropeptide pituitary adenylate cyclase activating-peptide (PACAP). PACAP receptors have been found in rat bone marrow-derived stromal cells and PACAP has been shown to induce interleukin-6 (IL-6) (an important cytokine in hematopoiesis) production by these cells. Maxadilan has been shown to increase IL-6 production. Moreover, increased hematopoiesis has been associated with increased susceptibility to *L. major* infection. Thus, we were interested in studying hematopoiesis in the murine model of leishmaniasis. Clonogenic progenitor assays were used to assess hematopoiesis.

We hypothesized that hematopoiesis would be increased in BALB/c mice and decreased in CBA mice due to their differences in cytokine profiles produced during the course of the immune response. We found that bone marrow hematopoiesis three days after *L. major* infection, was increased in BALB/c mice and decreased in CBA mice. We used feeder layer-based assays to assess the importance of IL-4 and TNF-α on the hematopoietic response to the parasite in both strains of mice. We found that IL-4 was responsible for the increased hematopoiesis seen in BALB/c mice and TNF-α for the decreased hematopoiesis seen in CBA mice.

Due to its effects on IL-6 production and PACAP receptors, we hypothesized that maxadilan will increase hematopoiesis in both susceptible and resistant mice. We found that maxadilan increased bone marrow hematopoiesis in both strains of mice. This increase in hematopoiesis was further supported by increased circulating reticulocytes in maxadilan-treated mice. Incubation of hematopoietic progenitors with maxadilan resulted in stimulation of hematopoiesis. Using long-term bone marrow cultures and feeder layer-based assays, we found that maxadilan stimulated hematopoiesis in both strains of mice through PACAP receptor binding and IL-6 production, and that maxadilan acted on bone-marrow stromal cells, more particularly macrophages and fibroblasts. Thus, maxadilan might exacerbate *L. major* infection by stimulating hematopoiesis.

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This dissertation is dedicated to my parents, I could not have wish for better ones, Serge et Mauricette Guilpin, who believe in me more than I believe in myself.

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Introduction

Leishmaniasis is a vector-borne zoonotic disease with a worldwide distribution and a wide range of vertebrate hosts; it is difficult to control due to both vectors and reservoirs (1). According to the World Health Organization (2), approximately 12 million people are affected with an incidence of 2 million new cases each year. The disease presents in three main clinical forms. These forms include cutaneous, mucocutaneous and visceral leishmaniasis, depending on the *Leishmania* species involved and the host immune response (3). Cutaneous leishmaniasis, caused mainly by *L. major*, is characterized by the presence of ulcers in an area easily accessible to sand fly bites. Ulcers usually resolve in a few months and leave scars. Mucocutaneous leishmaniasis is mostly seen in South America and is usually most often caused by *L. braziliensis*. Lesions on mucous membranes occur within two years after the appearance of cutaneous ulcers. The nose is primarily involved and lesions can extend from the nasal septum to the mouth and lips, and beyond. Mortality is often secondary to pulmonary infections and malnutrition. Visceral leishmaniasis is the diffuse form of the disease in which the parasite, usually *L. donovani*, spreads throughout the host. Cachexia, fever, splenomegaly, lymphadenopathy, and mucocutaenous lesions are often present. Laboratory abnormalities include pancytopenia, anemia, and hyperglobulinemia (4). Therapy with pentavalent antimonial compounds usually is successful. Diagnosis is based on cytological identification of the parasite in bone marrow, lymph nodes or spleen aspirates as well as indirect laboratory tests such as enzyme-linked immunosorbent assay (ELISA), direct agglutination test, immunoblot and immunofluorescence (1,5)

The life cycle of *Leishmania* sp. is composed of two distinct phases. Flagellated promastigotes, variable in shape, reside extracellularly in the midgut of the invertebrate vector, a phlebotomine sand fly. Amastigotes, on the other hand, are non-flagellated forms that reside within the macrophages of a vertebrate host. Transformation from the amastigote to promastigote form occurs in the midgut of the sand fly within the peritrophic membrane that contains the infective blood meal. After a few days of active division, promastigotes spread to the anterior midgut. Once the organisms reach the stomodeal valve and foregut, they strategically position for transmission to a vertebrate host during the sand fly blood meal, and they divide actively to form highly infective metacyclic promastigotes. This colonization process takes approximately five days (6,7). Transmission to a vertebrate occurs during a blood meal. Promastigotes enter macrophages through binding with macrophage surface molecules, which vary depending on *Leishmania* sp. For example *L. major* uses complement to enter macrophages. Once in the macrophages, promastigotes reside in parasitophorus vacuoles. In this hostile environment, transformation into amastigotes and replication occur (8). Containment at the infection site or dissemination of the parasite to other tissues is dependent upon the host immune response, which has been extensively reviewed (9, 10).

Most experimental models used to study leishmaniasis overlook the role of the vector in transmission of the parasite and how the vector may help the parasite establish infection (11). Recently, renewed interest has been given to sand fly saliva and its role in infection and host immune response. Exacerbation of lesions and modulation of the immune response are seen when parasites are injected with sand fly saliva (12-15).

Moreover, saliva lowers the numbers of parasites required to establish infection (13,16). Clearly saliva plays a role in *Leishmania* infection, yet the elucidation of that role is difficult given the complexity of saliva and the small size of the molecules present therein (17, 18). Progress has been realized through the isolation of a small peptide, maxadilan, from the saliva of the sand fly *Lutzomyia longipalpis* (19-21). This peptide is a vasodilator that also exhibits immunomodulatory properties (22), primarily by acting as an analogue of the neuropeptide pituitary adenylate cyclase activating-peptide (PACAP) (23).

Interactions between the immune and neuroendocrine systems are common (24, 25) thus, it is interesting that a peptide that mimics the effects of a neuropeptide can be found in sand fly saliva. The connection between hematopoiesis and the immune and neuroendocrine systems is also interesting. The connection between the immune system and hematopoiesis is well documented. For example, chronic inflammatory processes lead to anemia through an increase in interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) levels (26). The connection between the neuroendocrine system and control of hematopoiesis however is more recent (27). PACAP receptors have been found in rat bone marrow-derived stromal cells and the ability of PACAP to induce interleukin-6 (IL-6) production by these cells also is documented (28). IL-6 is an important cytokine in hematopoiesis, acting on both early and committed progenitors (29). Cells involved in inflammation and immune response are produced within the bone marrow. Adequate cell production and maturation is essential to insure efficient immune response. Reports on the hematopoietic response using the *Leishmania* model are very few, and only describe increased hematopoiesis in susceptible mice in response to *L. major* infection (30). The hematopoietic response has not been studied in resistant animals. Moreover, differences in macrophage maturation between susceptible and resistant animals have been noted. Thus, decreased macrophage maturation and increased hematopoiesis have been associated with increased susceptibility to leishmaniasis.

We wanted to study hematopoiesis in response to *L. major* infection in both susceptible and resistant mice. We also wanted to determine if maxadilan, which has been shown to increase IL-6 production by macrophages, would have an effect on hematopoiesis (31). We found that resistant and susceptible mice had an opposite hematopoietic response in the face of infection, and that this response was driven by the cytokines produced during the immune response. We found also that the sand fly salivary peptide maxadilan was able to stimulate hematopoiesis in both resistant and susceptible mice through PACAP receptor binding and IL-6 production. Maxadilan appears to exert its action on bone-marrow stromal cells, more particularly macrophages and fibroblasts.

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Chapter I: Hematopoiesis and *L. major* infection

Introduction

Murine experimental leishmaniasis provides an excellent model in which to study the immune response to a protozoan parasite. Most strains of mice are resistant to *Leishmania sp.* infection and are able to contain and eliminate the infection within a few weeks. In most mice, the disease takes the cutaneous form characterized by self-healing ulcers (1). However, BALB/c mice are highly susceptible to the parasite and die of visceral leishmaniasis (2). This dichotomy, seen in the disease course, is reflected in the immune response of these mice. Resistant mice, such as CBA strain, have a Th1 immune response to the infection and are able to contain the parasite within regional lymph node limits, whereas BALB/c mice exhibit a Th2 immune response, and parasites spread rapidly throughout the body (3, 4). Early immunological events are decisive in the outcome of the infection (5, 6). Macrophages are important for immunity and are also the target of the parasites. Adequate macrophage functions are required for both killing of the parasite as well as antigen presentation that leads to acquired immunity. In resistant mice, mature and fully functional macrophages appear more rapidly at the site of infection and also appear more rapidly in bone marrow cultures. In contrast, immature macrophages are seen in susceptible mice (7). Killing of the parasite requires production of nitrite oxide (NO). Macrophages from resistant mice have a greater NO production than those from susceptible mice (8). Tumor necrosis factor

(TNF-α) is essential in macrophage activation leading to NO production and killing of the parasite (9). Resistant animals produce more TNF-α than do susceptible mice (10, 11).

Numerous cytokines and chemokines also are involved in the control of hematopoiesis. In the case of leishmaniasis, increased hematopoiesis is seen in susceptible animals, however this study was not performed in resistant animals (12). One of the reasons for increased hematopoiesis in susceptible mice might reside in the cytokines produced in response *to L. major* infection. BALB/c mice are high producers of interleukin-4 (IL-4), which is involved in up-regulation of hematopoiesis (13). In contrast, cytokines produced in resistant animals, such as TNF-α or interferon-γ (IFN-γ), are involved mainly in the inhibition of hematopoeisis (14,15). Given these data, we were interested in the early hematopoietic response to *L. major* infection in both resistant and susceptible mice. We hypothesized that hematopoiesis would be increased in BALB/c mice and decreased in CBA mice, due to their differences in cytokine profiles produced during the course of the immune response. We found that bone marrow hematopoiesis in response to *L. major* infection was indeed increased in BALB/c mice and decreased in CBA mice and that the underlying mechanisms appear to reside within the cytokines produced during the immune response.

Materials and methods

- Mice:

Most of the six to eight week-old female BALB/cByJ mice used in these experiments were bred at Colorado State University (CSU) Laboratory Animal Resources (LAR). Small numbers were obtained from National Cancer Institute (NCI) (Bethesda, MD), an external provider. Six to eight week-old female CBA/J mice were obtained from NCI and housed at LAR. These mice were used primarily to provide bone marrow stromal feeder layer cells and/or bone marrow progenitor cells. Both tibias from these mice were aseptically removed and bone marrow cells were isolated as described below. Some mice were infected with *Leishmania major* parasites.

- *L. major* infection experiments:

Experiments were performed in both BALB/c and CBA strains of mice. Two groups of four mice within each strain were used, with one group serving as control. Prior to parasite inoculation, mice received 50 μl, intraperitonealy (i.p.) of a combination of xylazine (6 mg/ml) and ketamine (30 mg/ml) in minimal essential medium (MEM)-α (Gibco BRL, Grand Island, NY). The infected group received $5\text{x}10^6$ *L. major* LV39 organisms in 50 μl of (MEM)-α in the left footpad, whereas the control group received 50 μl of (MEM)-α only, also in the left footpad. Mice were euthanized three days later. The left tibia and spleen were aseptically removed and processed as follows.

- Bone marrow and spleen cell isolation:

After euthanasia, tibias were removed and muscle was cleaned off the bone. If needed, the tibial distal metaphysis was cut off. Tibias were flushed with 1 ml of (MEM)-α. Bone marrow cells were pipetted vigorously to separate cell clusters and brought to 2 x 10^6 cells/ml. The peritoneal cavities were opened and spleens were removed. Spleens were homogenized in 5 ml of (MEM)-α using sterile metal screens. Spleen cells were pipetted vigorously to disrupt cell clusters. After a wash with (MEM)-α, red blood cells were removed from the spleen cell preparation with ACK lysing buffer (16). Spleen cells were washed once and brought to 2x10^6 cells/ml.

- Feeder layer establishment:

Bone marrow cells obtained from either BALB/c or CBA were brought to 2 x 10^6 cells/ml in Myelocult® (StemCell Technologies Inc., Vancouver, BC). Cells were plated in 48 well plates in a volume of 500 µl per well and cultured for 8 to 12 days at 37°C. After this incubation period, trypsin-EDTA (0.25%) (Sigma, St Louis, MO) was applied to the wells to remove the cells. The cells were irradiated at 1500 cGy and washed once in (MEM)-α. Cells were plated in 48 well plates at 2 x 10^6 cells/well and incubated at 37°C for 24h to form a confluent monolayer (17).

- *Leishmania major* antigen preparation:

Parasites were frozen and thawed rapidly 10 times by alternating between a liquid nitrogen bath and a 37°C water bath. Soluble antigen was obtained from supernatant after centrifugation at 3400 rpm for 1 min and protein content was determined by micro-BCA protein assay (Pierce, Rockford, IL) performed according to manufacturer's directions.

- Feeder layer treatment with *Leishmania major*:

Once confluence was reached, cells were treated either with *L. major* soluble antigen at a concentration of 5 µg/ml in Myelocult®, or with Myelocult® only, for three days at 37°C. After three days, supernatants were removed and bone marrow cells were seeded on the feeder layer at 2 x 10^6 cells/ml. In some cases, antibodies directed against either IL-4 or TNF-α or a control antibody were added to assess the effect of these cytokines on hematopoiesis in response to *L. major* antigenic stimulation. After a three day incubation period, cells were removed from wells with trypsin-EDTA (0.25%), washed and colony progenitor assays were performed.

- Colony progenitor assays:

Colony progenitor assays were performed to assess hematopoiesis. Colony Forming Unit-Erythroid (CFU-E) cells are late erythroid progenitors which form small colonies after 48-72h in semi-solid culture, Burst Forming Unit-Erythroid (BFU-E) cells are early erythroid progenitors which form large colonies after 6-7 days, and Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) cells are progenitors of granulocytes and macrophages which form colonies after 6-7 days. Bone marrow and spleen cells were brought to 2 x 10^6 cells/ml. After dilution, cells were recounted and the definitive cell number, N, was determined. Cells were diluted in methyl-cellulose culture medium (StemCell Technologies) at 200,000 cells/ml. Cells were plated in a volume of 200 μl in wells of a 48-well plate at 20,000 cells/well. Plates were incubated at 37°C. After 48-72h incubation, CFU-E were counted using an inverted phase microscope. BFU-E and CFU-GM were evaluated similarly after 6-7 days. Counts from the duplicate wells were averaged and, as there is a linear relationship between colony numbers and cells plated (18), this result was multiplied by $2 \times 10^6/N$ to compensate for variations between actual cell number, N, and the desired concentration of 2×10^6/ml. For *in vitro* experiments, this calculated number was the final data entry from these duplicate wells. For the *in vivo* experiments, absolute numbers per tibia and spleen were calculated.

- Statistical analysis:

The Kolmogorov-Smirnov test was performed to ensure of normal distribution of data. As data were normally distributed, parametric statistical tests were indicated. A t-test was used to analyze *in vivo* data. ANOVA factorial analysis was used to analyze results from the *in vitro* experiments. Results were considered significant when p value < 0.05.

Results

- Effect of *Leishmania major* infection on hematopoietic response in mice

These experiments were performed twice in both BALB/c and CBA mice. The experimental design is illustrated in Figure 1.1. For each strain, there were two treatment groups composed of four mice each. The *L. major*-treated group received 5 x 10^6 organisms in 50 µl of (MEM)-α (Gibco) in the left footpad, whereas the medium-treated group received 50 µl of (MEM)-α only also in the left footpad. Three days later, mice were euthanized and bone marrow and spleen cells were isolated for clonogenic progenitor assays. As shown in Figures 1.2 and 1.3, CFU-E, BFU-E and CFU-GM were significantly increased in *L. major*-infected BALB/c mice, whereas these were significantly decreased in infected CBA mice. These results suggest that *L. major*-infected BALB/c and CBA mice have a different hematopoietic response with increased hematopoiesis in BALB/c mice and decreased hematopoiesis in infected CBA mice. The effect of infection on the different types of progenitors in the spleen in both strains of mice, as seen on Figures 1.4 and 1.5, was variable. Moreover, results were inconsistent from experiment to experiment. This might be due to the fact that in the spleen of rodents, hematopoiesis is activated during a stress situation (20, 21). Lower numbers of progenitors and a different hematopoietic microenvironment in the spleen may account for the difference in the hematopoietic response between bone marrow and spleen we saw in our experiments (19, 20).

- Importance of Th1 vs., Th2 cytokines in the hematopoietic response to *L. major* infection

BALB/c and CBA mice provide an interesting model to study leishmaniasis, each having a strikingly different outcome in the course of the disease, probably as a result of having a very different immune response. These differences in the immune response are reflected in the set of cytokines produced by each strain of mice. BALB/c mice produce Th2 cytokines in response to *L. major* infection whereas CBA mice produce Th1 cytokines. Interestingly, Th2 cytokines such as IL-4 have been involved in stimulation of hematopoiesis whereas Th1 cytokines such as TNF-α have been associated with decreased hematopoiesis. Since both BALB/c and CBA mice had markedly different hematopoietic responses early in the course of infection, we were interested in evaluating the importance of some of the Th1 and Th2 cytokines on hematopoiesis in response to *L. major* infection. Blocking experiments targeting IL-4 and TNF-α were performed to assess the effect of these cytokines on the hematopoietic response to the parasite in each strain of mice. All experiments were performed twice in each strain of mice. The experimental design is illustrated in Figure 1.6. We used *L. major* antigen-treated bone marrow feeder layers, seeded with bone marrow hematopoietic progenitors with or without antibody against the cytokine of interest. After three days, clonogenic progenitors were quantified. As seen in Figure 1.7, there was a significant increase in CFU-E, BFU-E and CFU-GM in response to *L. major* antigen in BALB/c mice. These results indicated stimulation of hematopoiesis in response to *L. major* stimulation and were consistent with the *in vivo* effect of *L. major* infection on hematopoiesis. Moreover, it appeared that bone marrow-derived stromal cells were involved in mediating the effect of *L. major* on hematopoiesis. The stimulatory effect of *L. major* antigen on hematopoiesis was not seen, however when anti-IL-4 was present. There were no differences between the control group and the anti-IL-4 and *L. major* antigen-treated group, suggesting that IL-4 was involved in stimulating hematopoiesis in BALB/c mice in response to *L. major* infection. IL-4 did not appear to be involved in the regulation of normal hematopoiesis as indicated by the lack of differences between the control group and the anti-IL-4-treated control group. In CBA mice, as seen in Figure 1.8, there was a significant decrease in CFU-E, BFU-E and CFU-GM in response to *L. major* antigen treatment. These results were consistent with the *in vivo* effect of *L. major* infection in which a decrease in hematopoiesis was noted. The presence of anti-IL-4 however, had no effect on the hematopoietic response of these mice in both control and *L. major*-treated groups, which suggested that IL-4 was not involved in the control of the hematopoietic response to *L. major* antigen stimulation in CBA mice.

Similar experiments were performed using antibody directed against TNF-α. Results in BALB/c mice are shown in Figure 1.9. CFU-E were significantly increased in anti-TNF-α treated control group compared to controls, suggesting a regulatory function of anti-TNF-α on normal hematopoiesis. In *L. major* treated groups, CFU-E were increased even more with no effect of anti-TNF-α noted. CFU-GM and BFU-E were increased only in *L. major* Ag-treated groups. These results were consistent with the *in vivo* effect of *L. major* on hematopoiesis. For CBA mice, as seen in figure 1.10, CFU-E, BFU-E and CFU-GM were significantly decreased in the *L. major* antigen-treated group, as seen *in vivo*. When anti-TNF-α was added to *L. major* antigen-treated cultures, CFU-E, BFU-E and CFU-GM were comparable to controls, suggesting the involvement of TNF-α in down-regulating hematopoiesis in response to *L. major* treatment. The presence of anti-TNF-α in control cultures leaded to significant increases in CFU-E and CFU-GM, suggesting a regulatory function of TNF-α on normal hematopoiesis.

We used an isotype control directed against β-galactosidose (anti-βgal) in a similar setting to ensure that the effects seen in the previous experiments were not due to the antibody itself but rather to the targeted cytokines. Results are shown in Figure 1.11 and 1.12 for BALB/c and CBA mice respectively. There was a significant increase in CFU-E, BFU-E and CFU-GM in response to *L. major* antigen treatment in BALB/c mice, whereas in CBA mice these progenitors were decreased, as seen *in vivo*. The *L. major* antigen effect was not altered when anti-βgal was present. Therefore, it appeared that the *in vitro* system we used mimicked the *in vivo* hematopoietic response to *L. major* infection in both strains of mice, with hematopoiesis increased in BALB/c mice and decreased in CBA mice. Also, it appeared that IL-4 in BALB/c mice and TNF-α in CBA mice were involved in mediating the effect of the parasite on hematopoiesis.

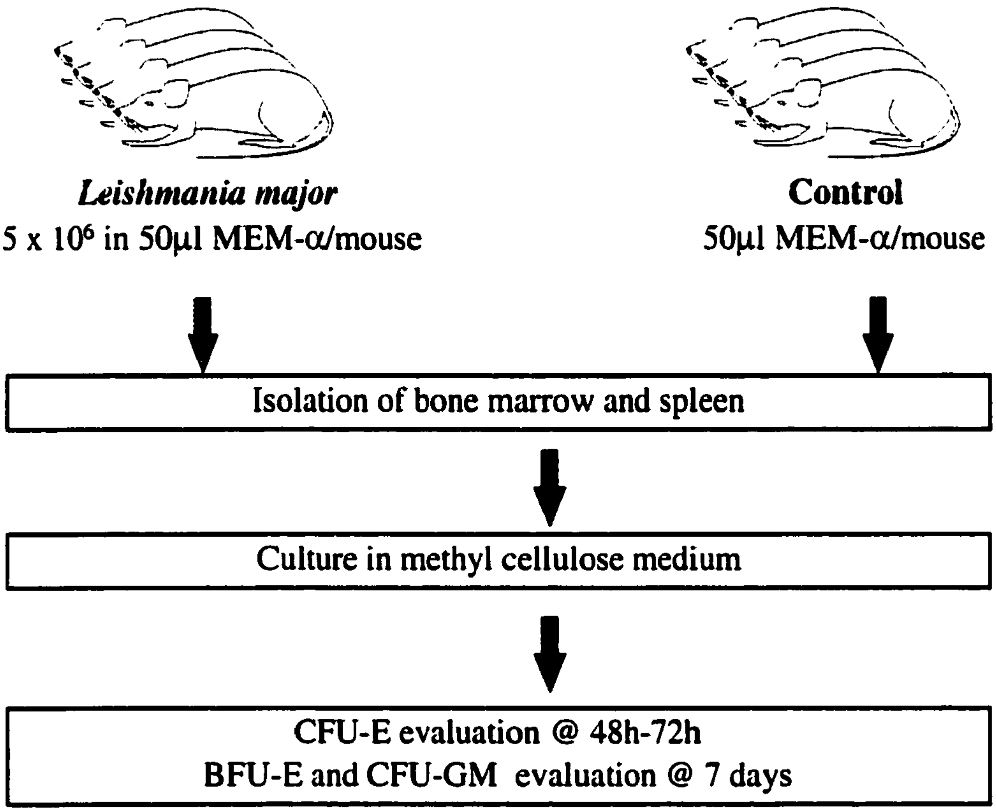

Figure 1.1: Effect of *Leishmania major* infection on hematopoiesis in mice

These experiments were performed in both BALB/c and CBA. For each strain, two treatment groups composed of four mice each were used. The *L. major*-treated group received 5 x 10^6 organisms in 50 µl of (MEM)-α in the left footpad, whereas the medium-treated group received 50 µl of (MEM)-α only also in the left footpad. Three days later, mice were euthanized and bone marrow and spleen cells were isolated for clonogenic progenitor assays.

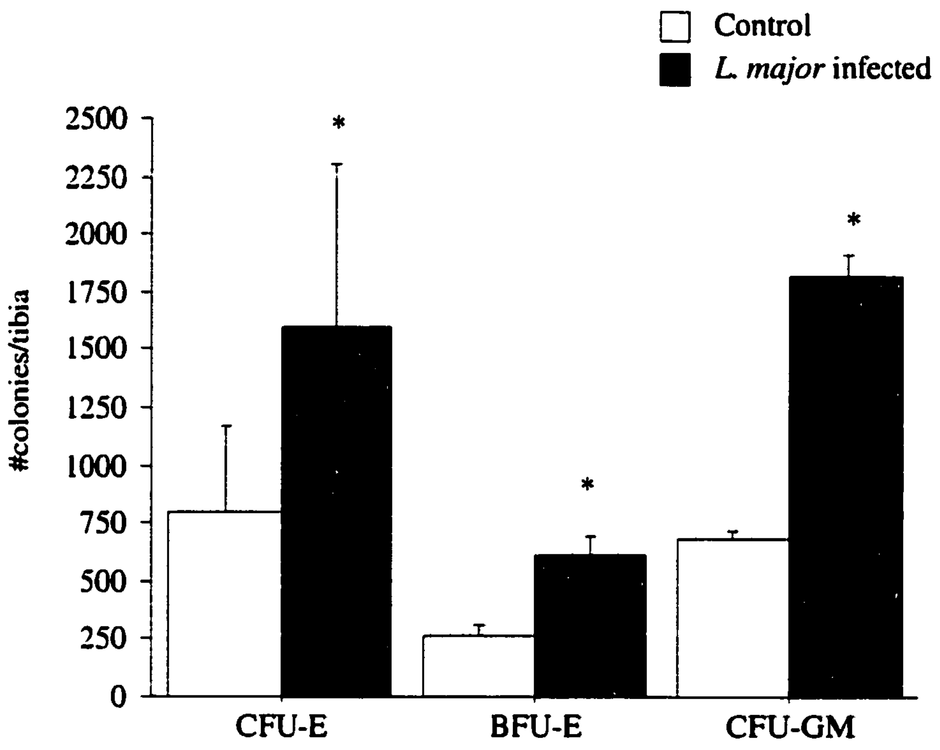

Figure 1.2: Effect of *L. major* infection on bone marrow hematopoietic responses in BALB/c mice

2 x 10^4 bone marrow cells were plated in duplicate wells in methylcellulose medium three days after infection with 5 x 10^6 *L. major* organisms. Results are expressed as numbers of colonies per tibia. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

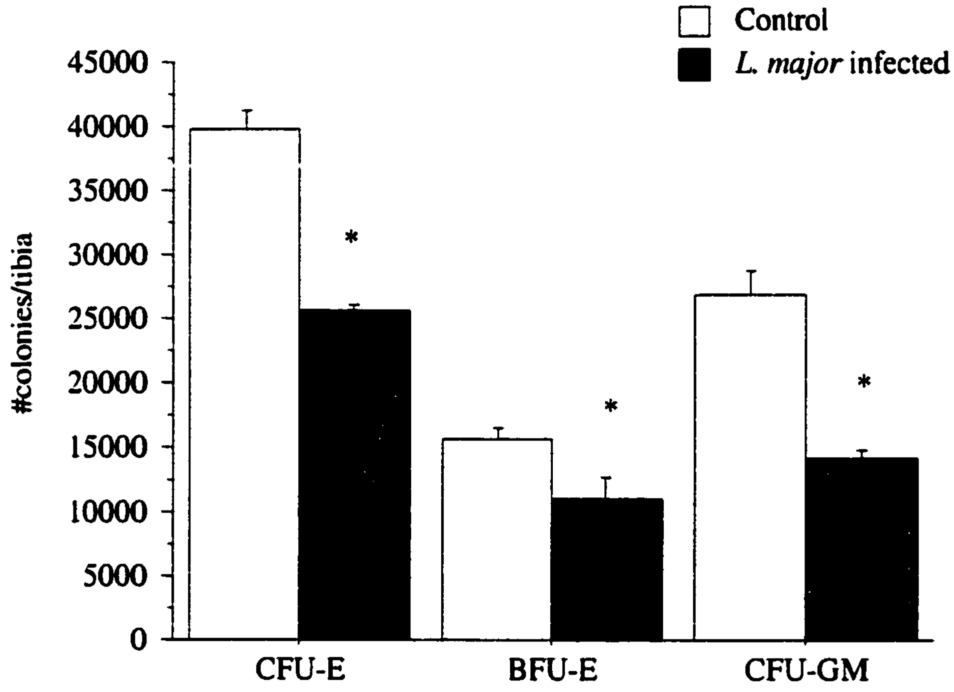

Figure 1.3: Effect of *L. major* infection on bone marrow hematopoietic responses in CBA mice.

2×10^4 bone marrow cells were plated in duplicate wells in methylcellulose medium three days after infection with 5×10^6 *L. major* organisms Results are expressed as numbers of colonies per tibia. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

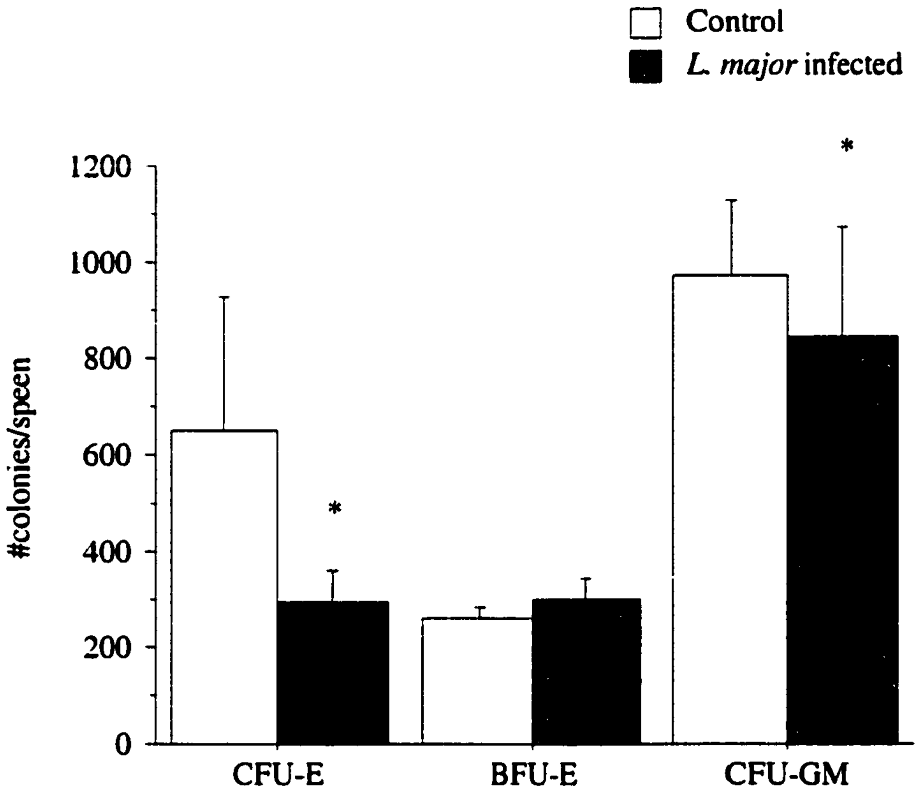

Figure 1.4: Effect of *L. major* infection on spleen hematopoietic responses in BALB/c mice.

2 x 10^4 spleen cells were plated in duplicate wells in methylcellulose medium three days after infection with 5 x 10^6 *L. major* organisms. Results are expressed as numbers of colonies per spleen. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

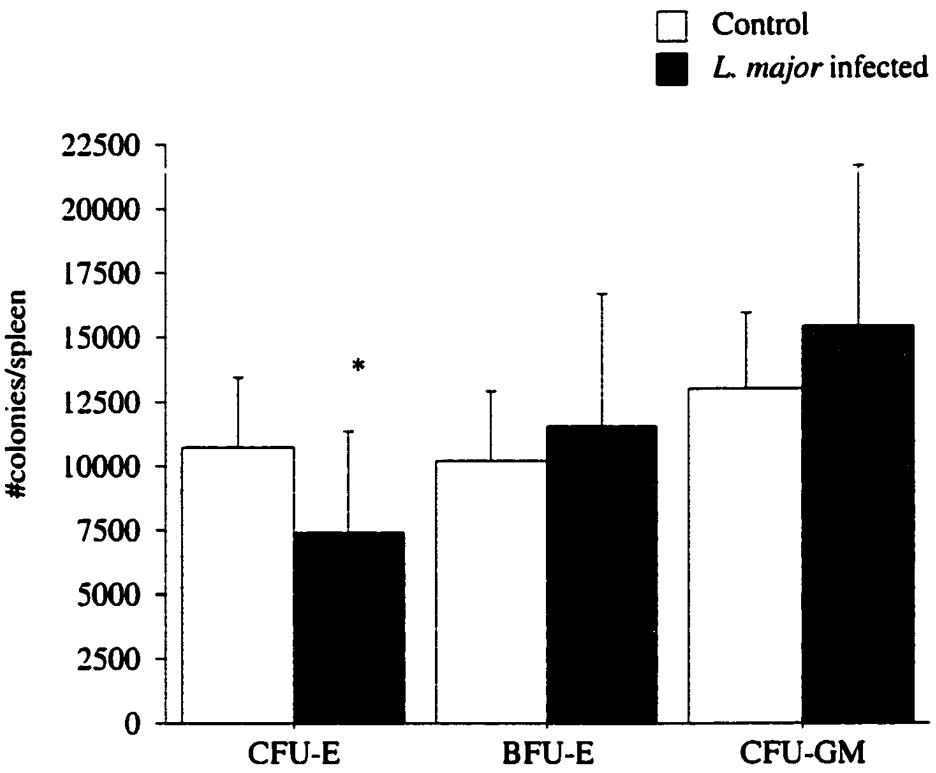

Figure 1.5: Effect of *L. major* infection on spleen hematopoietic responses in CBA mice.

2 x 10^4 bone marrow cells were plated in duplicate wells in methylcellulose medium three days after infection with 5 x 10^6 *L. major* organisms. Results are expressed as numbers of colonies per spleen. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

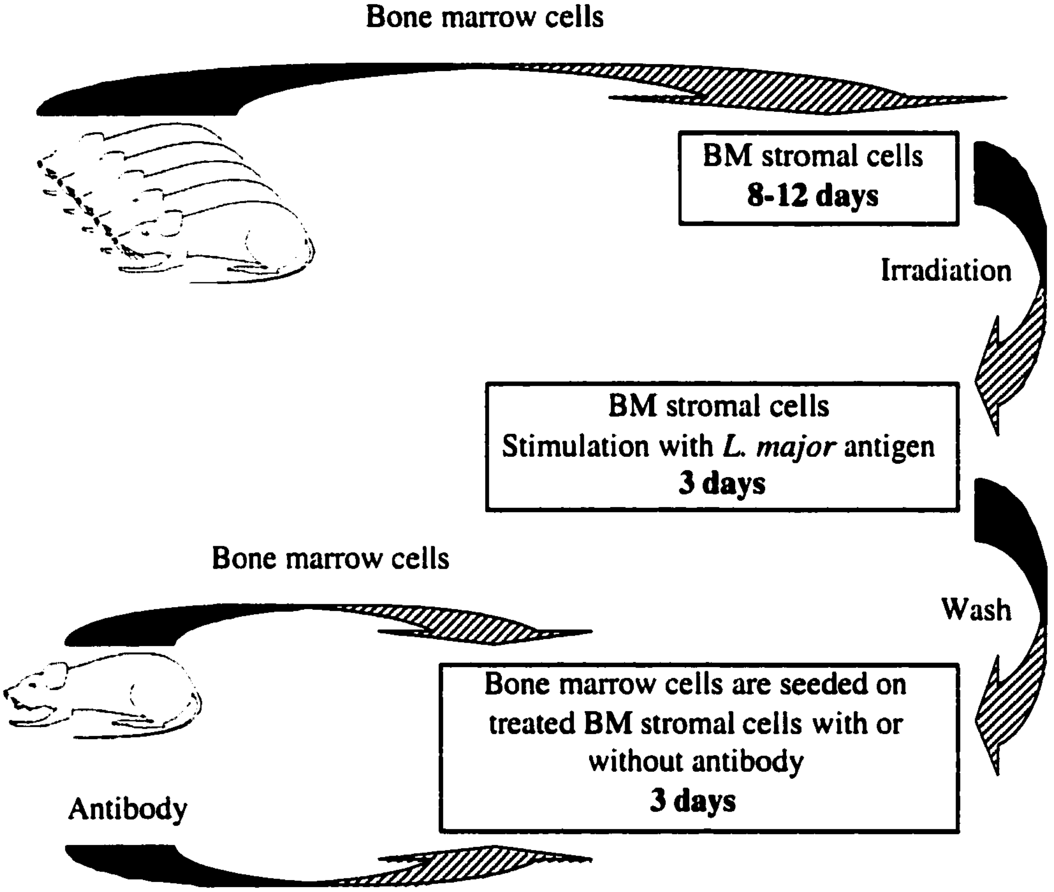

Figure 1.6: Importance of cytokines in hematopoietic responses to *L. major* infection Bone marrow cells obtained from either BALB/c or CBA mice were plated in 48-well plates (2 x 10^6 cells/ml) and cultured for 8 to 12 days. After this incubation period, cells were irradiated, washed, and plated in 48-well plates (2 x 10^6 cells/ml). After a 24h incubation, cells were treated for three days with either *L. major* soluble antigen (5 μg/ml) or culture medium only. Supernatants were removed after three days and bone marrow cells were seeded on the bone marrow stromal cells (2x10^6 cells/ml). In some cases, antibodies directed against either IL-4 or TNF-α or a control antibody (anti-βgal) were added to assess the effect of these cytokines on hematopoietic responses to *L. major* antigen stimulation. Three days later, colony progenitor assays were performed.

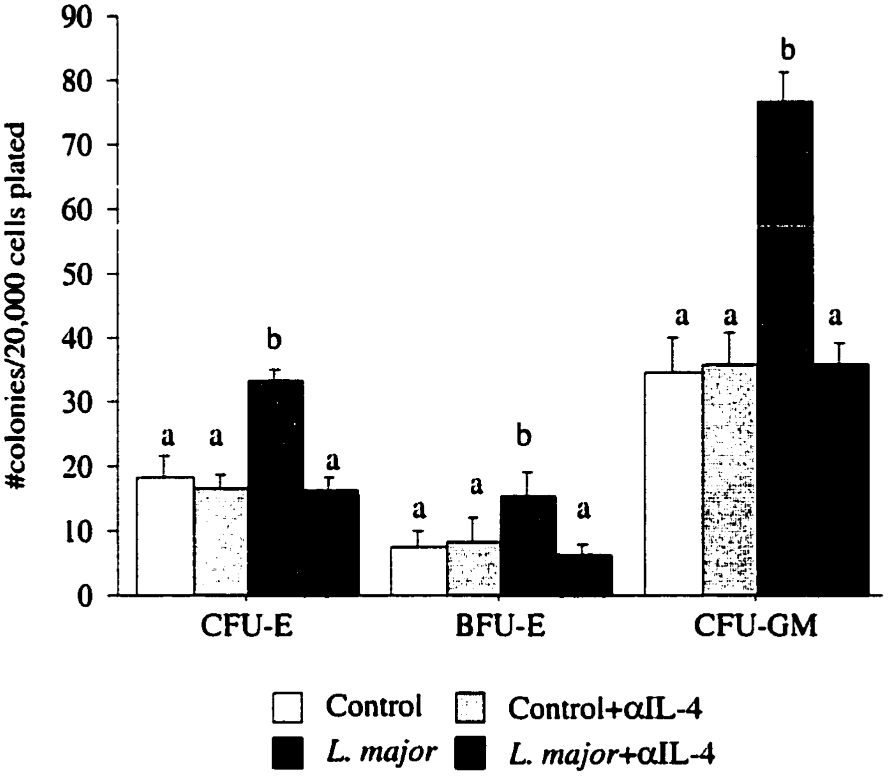

Figure 1.7: Effect of anti-IL- 4 antibody (αIL-4) on bone marrow responses to *L. major* soluble antigen (*L. major* Ag) in BALB/c mice

Bone marrow cells were plated with or without αIL-4 on irradiated bone marrow stromal cells stimulated with or without *L. major* Ag. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and B) denote significant differences between treatment groups (p value < 0.05).

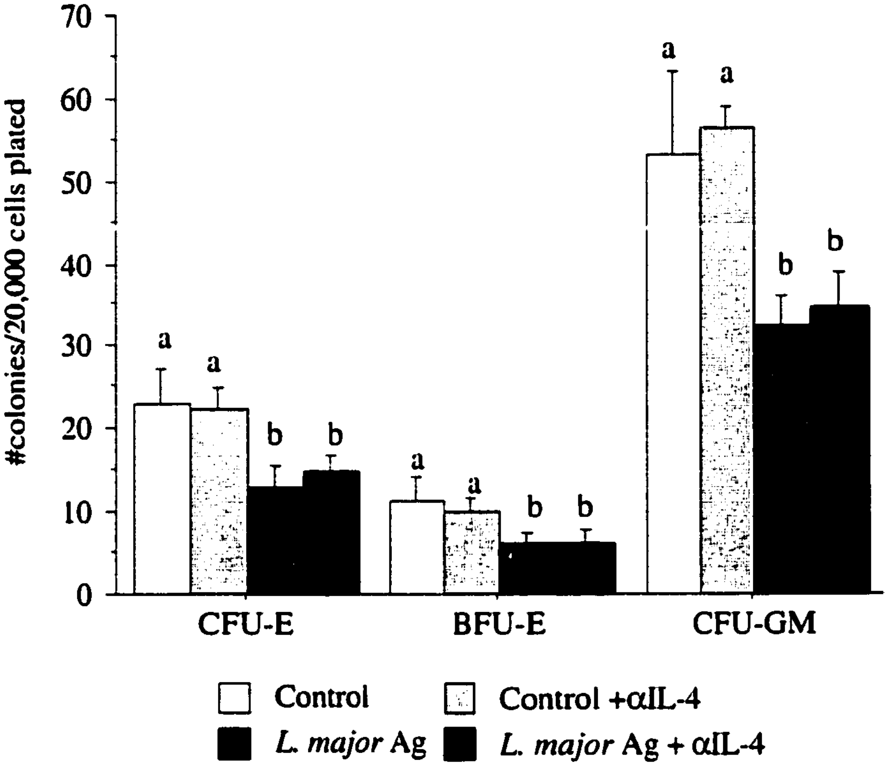

Figure1.8: Effect of anti-IL- 4 antibody (αIL-4) on bone marrow responses to *L. major* soluble antigen (*L. major* Ag) in CBA mice

Bone marrow cells were plated with or without αIL-4 on irradiated bone marrow stromal cells stimulated with or without *L. major* Ag. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant differences between treatment groups (p value < 0.05).

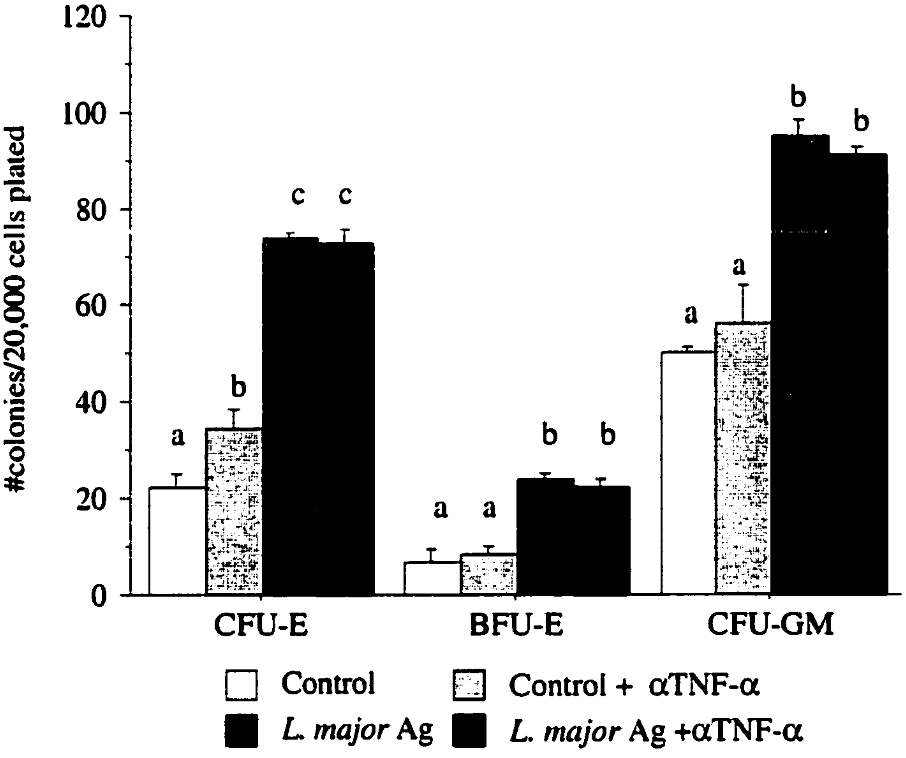

Figure1.9: Effect of anti- TNF-α antibody (αTNF-α) on bone marrow responses to *L. major* soluble antigen (*L. major* Ag) in BALB/c mice.

Bone marrow cells were plated with or without antibody directed against TNF-α on irradiated bone marrow stromal cells stimulated with or without *L. major* Ag. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a, b, and c) denote significant difference between treatment groups (p value < 0.05.

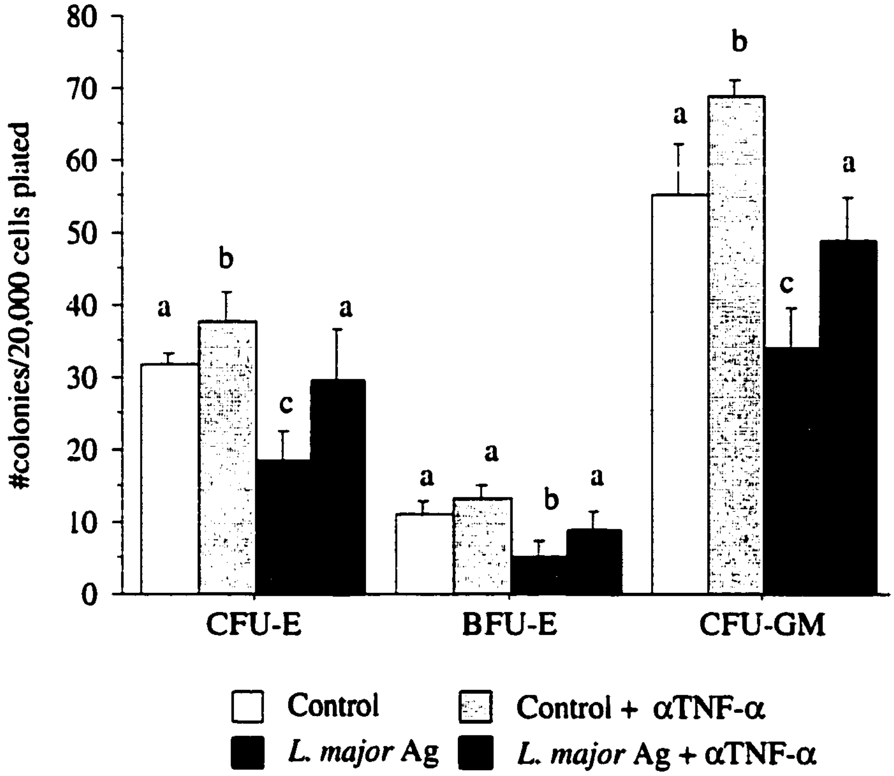

Figure 1.10: Effect of anti- TNF-α antibody (αTNF-α) on bone marrow responses to *L. major* soluble antigen (*L. major* Ag) in CBA mice

Bone marrow cells were plated with or without αTNF-α on irradiated bone marrow stromal cells stimulated with or without *L. major* Ag. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a, b and c) denote significant difference between treatment groups (p value < 0.05).

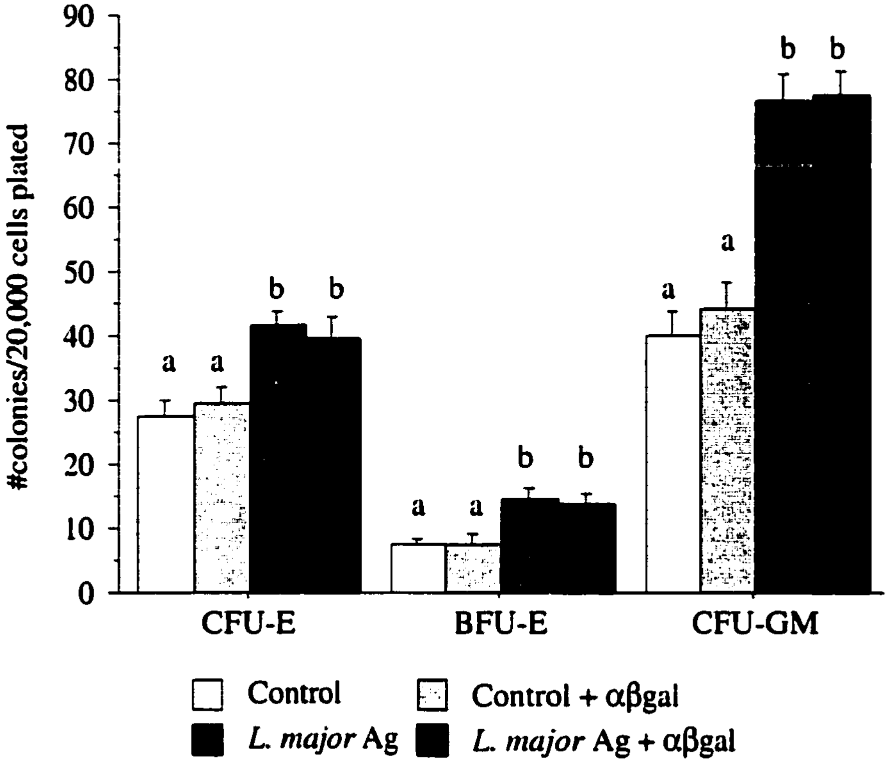

Figure 1.11: Effect of a control antibody specific for β-galactosidose (αβgal) on bone marrow responses to *L. major* soluble antigen (*L. major* Ag) in BALB/c mice Bone marrow cells were plated with or without αβgal on irradiated bone marrow stromal cells stimulated with or without *L. major* Ag. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

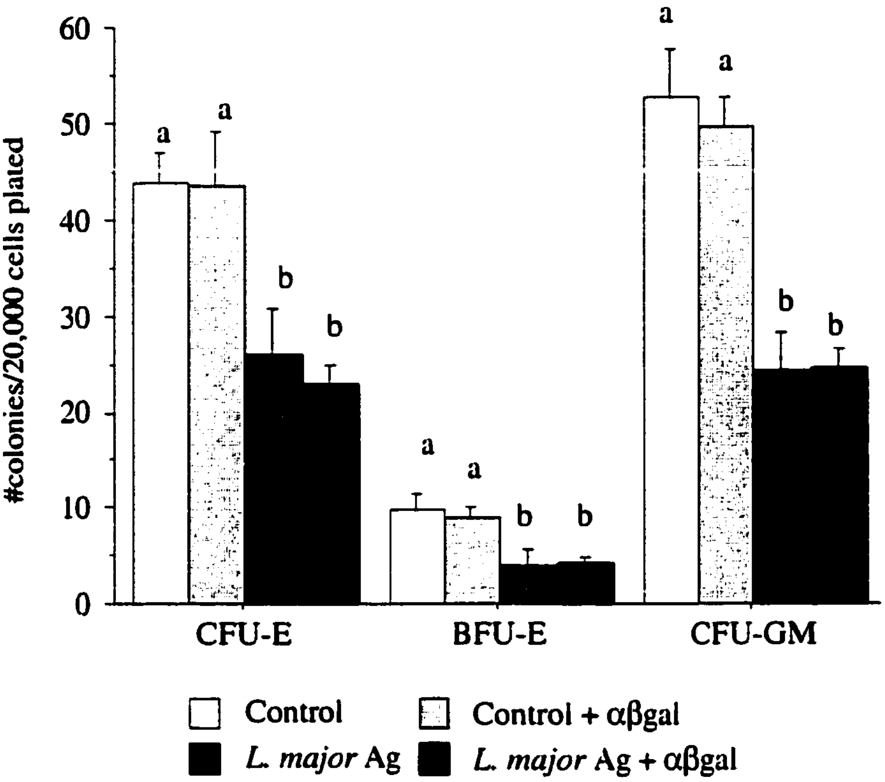

Figure 1.12: Effect of a control antibody specific for β-galactosidose (αβgal) on bone marrow responses to *L. major* soluble antigen (*L. major* Ag) in CBA mice

Bone marrow cells were plated with or without αβgal on irradiated bone marrow stromal cells stimulated with or without *L. major* Ag. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

Discussion

We were interested in studying the early hematopoietic in response to *L. major* infection in both resistant and susceptible mice. We hypothesized that they would have opposite hematopoietic responses due to the different sets of cytokines these mice produce during the course of infection.

Three days after infection, we found a significant increase in hematopoiesis in BALB/c mice affecting both early and late erythroid progenitors as well as myeloid progenitors. These results are consistent with the recent study of hematopoietic response *to L. donovani* infection in BALB/c mice (19). An increase in bone marrow and spleen Colony Forming Unit-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM), Burst Forming Unit–erythroid (BFU-E) and Colony Forming Unit - granulocyte, monocyte (CFU-GM) was found but not until 7 days after infection. In that model, 2 x 10^7 parasites were inoculated intravenously, whereas we used a lower concentration of parasites that were inoculated in the footpad. Differences between the strains *L. major* and *L. donovani* and the route of inoculation may account for the more rapid hematopoietic response in our model. We did not notice any consistent effect of *L. major* on spleen hematopoiesis. It is most likely that a three day time point is too early to see a response in spleen hematopoeisis. In the study on *L. donovani*, an increase in splenic hematopoeisis was not seen until 7 days after infection for CFU-GM and 14 days for BFU-E and CFU-GEMM. In the spleen of rodents, hematopoiesis is activated during a stress situation (20, 21). As in BALB/c mice, spleen hematopoietic response in CBA mice was inconsistent, most likely for the same reasons. Lower numbers of progenitors and a different hematopoietic microenvironment in the spleen may account for the difference in the hematopoietic response between bone marrow and spleen we saw in our experiments (19, 20). In CBA mice, bone marrow response to infection was strikingly different from that of BALB/c mice. Hematopoiesis in infected mice was markedly decreased.

This difference in the hematopoietic response between the two strains of mice may result from the cytokines produced in response to parasite invasion of macrophages. CBA and BALB/c mice have different immune responses to the *L. major* infection, with CBA mice exhibiting a Th1 immune response (characterized by a potent cellular response to parasite infection), whereas BALB/c mice produce a Th2 response (3). In BALB/c mice, IL-4 production occurs as early as 16 hours after infection (22). From a hematopoietic standpoint, IL-4 is a cytokine that supports proliferation and differentiation of lineage-committed and multipotential progenitors (13). Moreover, IL-4 has been shown to stimulate IL-6 production by bone marrow stromal cells (23). IL-6 is a molecule with a stimulatory role on stem cells and lineage-committed progenitors of both myeloid and erythroid lineage (24).

In contrast to the response of susceptible BALB/c mice, resistant mice produce TNF-α and IL-12 that trigger IFN-γ production by natural killer (NK) cells when the mice are infected with *L. major*. TNF-α in synergy with IFN-γ induces a nitric oxide (NO) burst and killing of *Leishmania* (25). TNF-α appears essential in macrophage activation leading to *Leishmania* killing (26, 27). Both resistant and susceptible mice are able to produce TNF-α, however it has been shown that resistant animals produce more TNF-α than susceptible mice do (10, 11). TNF-α has a dual action on hematopoiesis. At low levels, and in combination with IL-3 or GM-CSF, TNF-α has a stimulatory effect on hematopoiesis, whereas in combination with other cytokines such as IFN-γ, G-CSF or erythropoietin, it is a potent inhibitor of hematopoiesis (14). The cytokine environment in resistant mice, with production of IFN-γ and NO, supports the inhibitory functions of TNF-α (28, 14). On the other hand, susceptible mice produced cytokines such as IL-3 in response to infection (29), which in association with lower levels of TNF-α, will stimulate hematopoiesis.

We used a feeder layer-based assay to assess the importance of IL-4 and TNF-α on the hematopoietic response to the parasite in both strains of mice. The bone marrow microenvironment is essential to ensure self-renewal, proliferation, maturation and release within the circulation of hematopoietic progenitors (30). It is composed of fibroblasts, endothelial cells, macrophages, adipocytes, osteoclasts and osteoblasts. Bone marrow stroma provides both cell to cell interactions, as well as regulatory molecules, that control hematopoiesis (31). In the *in vitro* system we used, irradiated bone marrow stromal layers, which are composed of bone marrow-derived fibroblasts, adipocytes, macrophages and a few endothelial cells, provide the essential interaction to sustain adequate hematopoeisis. These cells also maintain their ability to produce cytokines (32, 33). When bone marrow cells are added on these feeder layers, clusters of hematopoietic progenitors can be seen after a few days. By incubating bone marrow cells for three days on feeder layers, we were evaluating mainly lineage-committed progenitors (34). We found that in BALB/c mice, stromal bone marrow feeder layers were able to stimulate hematopoiesis in response to *L. major* antigen-stimulation whereas in CBA mice the opposite was noted. These results were consistent with the effect of *L. major* infection in mice, indicating that our *in vitro* model offered a representation of the *in vivo* phenomenon. In one experiment in CBA mice (results not shown), BFU-E were not affected by *L. major* antigen treatment, this is most likely due to variation in the ability of the feeder layer to sustain hematopoiesis. Since the system is more efficient in supporting myelopoiesis, it is not surprising that a slightly less efficient feeder layer will affect early erythroid progenitor development (34).

Blocking experiments targeting IL-4 and TNF-α were performed to study the effect of these cytokines in both strains of mice. In BALB/c mice, anti-IL-4 was able to eliminate the hematopoietic stimulatory effect of *L. major* antigen treatment of the feeder layer. Thus, IL-4 appears essential in stimulating hematopoiesis in BALB/c mice in response to *L. major* antigen stimulation. IL-4 did not have any effect in regulation of hematopoiesis in CBA mice, which is not surprising given that these mice do not produce significant levels of IL-4 in response to *L. major* infection (28, 26). In both strains of mice, TNF-α appears to have a regulatory function on normal hematopoiesis as indicated by slightly higher levels of CFU-E, BFU-E, and CFU-GM in control cultures treated with anti-TNF-α, suggesting a regulatory function on lineage-committed progenitors. TNF-α appears to play a role in the down-regulation of hematopoiesis seen in CBA mice in response to *L. major* infection however, as evidenced by the fact the anti-TNF-α antibodies eliminated the suppressive effect of *L. major*. The fact that CFU-GM in anti-TNF-α and *L. major* antigen-treated cultures remain lower than in control cultures suggest that TNF-α is not solely responsible of the inhibition of hematopoiesis seen in these mice.

Resistant mice produce IFN-γ and NO, effector molecules of parasite killing, in response to parasite infection (26). Both molecules are potent inhibitors of hematopoiesis, with IFN-γ inducing apoptosis in both early and late hematopoietic progenitors (15) and NO at least in lineage-committed progenitors (35). In BALB/c mice, anti-TNF-α did not have any effect on hematopoietic response to *L. major* antigen stimulation suggesting that TNF-α is either not involved in the hematopoietic response to *L. major* infection or that compensatory mechanisms are present. This is likely given the complexity and redundancy present in the regulation of hematopoiesis (36).

From our experiments, it appears that both CBA and BALB/c mice have a completely opposite hematopoietic response to *L. major* infection driven by cytokines involved in the early immune response to the parasite. In BALB/c mice, increased hematopoiesis appears controlled mainly by IL-4 production. A direct action of IL-4 on hematopoiesis as well as induction of other cytokines such as IL-6 is possible. In CBA mice, TNF-α appears to be involved in the inhibition of hematopoiesis, however, it appears that it acts in synergy with other molecules. IFN-γ and NO are likely candidates but they were not investigated in this study. The implications of these effects on hematopoiesis are discussed in the summary of this dissertation.

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Chapter II: Effect of maxadilan on bone marrow and spleen hematopoiesis

Introduction

Leishmaniasis is a vector-borne disease that has been studied in experimental models focusing mainly on the effects of the parasite itself. However, natural infection occurs in a slightly different setting. An infected sand fly co-injects parasites along with saliva into a vertebrate host during a blood meal. In experimental infection, millions of parasites are needed for the host to develop disease, however in a natural infection mediated by a sand fly bite, only a few infective metacyclic forms of the parasite are inoculated (1). These few parasites are enough to establish infection and disease. The role of saliva in leishmaniasis has been overlooked for a long time, which is surprising given the myriad of pharmacological properties that saliva contains. This richness of saliva in pharmacological agents is shared between blood-feeding arthropods since they all encounter the same obstacles during a blood meal (2). They have to probe to reach blood vessels in the skin with their buccal parts. Once the blood barrier is broken, the host system reacts to the intrusion: platelet aggregation, the coagulation cascade, vasoconstriction, inflammation and immune responses are initiated. To fight the host defense mechanisms, a similar strategy has been developed: their saliva often induces effects similar (3). The composition of saliva varies between blood-feeding arthropods but the properties are similar (4), with molecules including anticoagulants and vasoconstrictors as well as molecules, which counter inflammation and immunity (2).

Over the last two decades, increasing interest has been given not only to the study of saliva's composition but also to its interaction with pathogens and vertebrate hosts. In the case of leishmaniasis, most of the studies have been conducted on the saliva of the New World sand fly *Lutzomyia longipalpis* (5), with fewer on the Old World sand fly *Phlebotomus papatasi*, the natural vector of *Leishmania major* (6). Saliva from either sand fly increases the severity of the disease, even in resistant animals in an experimental setting (7-10). Given the complexity of sand fly saliva (11), and the variations even within sand fly sibling species (12), most of its components are still to be discovered. A small peptide, named maxadilan, however, has been isolated and cloned from the saliva of *L. longipalpis* (13, 14). It was its vasodilatory properties (it was called at first erythema-inducing factor or EIF) that led to its discovery (15, 16). Interestingly, it appears that maxadilan and *L. longiplapis*' saliva shared numerous immunomodulatory properties (4). They both modulate macrophage functions in response to *L. major* infection (17, 18). Further studies of maxadilan showed that its action was mediated through pituitary adenylate cyclase-activating peptide (PACAP) receptor (PACAP-R) (19). Maxadilan was able to modulate macrophage cytokine production in response to LPS stimulation, more precisely by stimulating IL-6 production and reducing TNF-α levels (18). Interestingly, IL-6 is involved in stimulating proliferation and differentiation of hematopoietic progenitors (20). Moreover, increased hematopoeisis has been associated with increased susceptibility to leishmaniasis (21). Given these data, we were interested in studying the effect of maxadilan on hematopoeisis, with the hypothesis that maxadilan will increase hematopoiesis. To test this hypothesis, we injected maxadilan into mice at concentrations compatible with those administered by the sand fly during a blood meal

(13), and we assessed hematopoietic responses using clonogenic progenitor assays. We found that hematopoiesis was increased in the bone marrow but not in the spleen. Increased hematopoietic activity was further supported by increased circulating reticulocytes in maxadilan-treated mice. Incubation of hematopoietic progenitors with maxadilan also resulted in stimulation of hematopoietic activity.

Materials and methods

- Mice:

Most of six to eight week-old female BALB/cByJ mice used in these experiments were bred at Colorado State University (CSU) Laboratory Animal Resources (LAR). Small numbers were obtained from National Cancer Institute (NCI) (Bethesda, MD), an external provider. Six to eight week-old female CBA/J mice were obtained from NCI and housed at LAR. These mice were either injected with maxadilan or used as source of naïve bone marrow progenitor cells. In the latter cases, both tibia form these mice were aseptically removed and bone marrow cells were isolated as described below.

- *In vivo* treatment with maxadilan:

Experiments were performed in both BALB/c and CBA mice. Three treatment groups composed of four mice within each strain were used. Mice were anesthetized by i.p. injection with 50 µl of a combination of xylazine (6 mg/ml) and ketamine (30 mg/ml) in minimal essential medium (MEM)-α (Gibco BRL, Grand Island, NY). The maxadilan-treated group received 4 ng of maxadilan in 50 µl of (MEM)-α in the left footpad, whereas the medium-treated group received 50 µl of (MEM)-α only also in the left footpad. The control group did not receive any treatment and was used to assess baseline hematopoiesis in normal mice and the effect of the medium used as vector on hematopoiesis. Mice were euthanized three days later with CO_2. The left tibia and spleen were aseptically removed and processed as follows.

- Bone marrow and spleen cell isolation:

After euthanasia, tibias were removed and muscle was cleaned off the bone. If needed, the tibial distal metaphysis was cut off. Tibias were flushed with 1 ml of (MEM)-α. Bone marrow cells were pipetted vigorously to separate cell clusters and brought to 2×10^6 cells/ml. The peritoneal cavities were opened and spleens were removed. Spleens were homogenized in 5 ml of (MEM)-α using sterile metal screens. Spleen cells were pipetted vigorously to disrupt cell clusters. After a wash with (MEM)-α, red blood cells were removed from the spleen cell preparation with ACK lysing buffer (22). Spleen cells were washed once and brought to 2×10^6 cells/ml.

- Colony progenitor assays:

Colony progenitor assays were performed to assess hematopoiesis. Colony Forming Unit-Erythroid (CFU-E) cells are late erythroid progenitors which form small colonies after 48-72h in semi-solid culture, Burst Forming Unit-Erythroid (BFU-E) cells are early erythroid progenitors which form large colonies after 6-7 days, and Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) cells are progenitors of granulocytes and macrophages which form colonies after 6-7 days. Bone marrow and spleen cells were brought to 2 x 10^6 cells/ml. After dilution, cells were recounted and the definitive cell number, N, was determined. Cells were diluted in methyl-cellulose culture medium (StemCell Technologies) at 200,000 cells/ml. Cells were plated in a volume of 200 µl in wells of a 48-well plate at 20,000 cells/well. Plates were incubated at 37°C. After 48-72h incubation, CFU-E were counted using an inverted phase microscope. BFU-E and CFU-GM were evaluated similarly after 6-7 days. Counts from the duplicate wells were averaged and, as there is a linear relationship between colony numbers and cells plated (23), this result was multiplied by 2 x 10^6/N to compensate for variations between actual cell number, N, and the desired concentration of 2 x 10^6/ml. For *in vitro* experiments, this calculated number was the final data entry from these duplicate wells. For the *in vivo* experiments, absolute numbers per tibia and spleen were calculated.

- Effect of maxadilan on reticulocyte count *in vivo*:

Experiments were performed in both BALB/c and CBA mice. Two treatment groups composed of four mice within each strain were used, with one group serving as control. Mice were anesthetized by i.p. injection with 50 µl of a combination of xylazine (6 mg/ml) and ketamine (30 mg/ml) in minimal essential medium (MEM)-α. The maxadilan-treated group received 4 ng of maxadilan in 50 µl of (MEM)-α in the left footpad, whereas the medium-treated group received 50 µl of (MEM)-α only also in the left footpad. Reticulocytes were enumerated by counting their number out of

1,000 total red blood cells counted on a new methylene blue (Sigma) stained blood film. They were counted at 3, 5 and 7 days after stimulation with maxadilan.

- Effect of maxadilan on bone marrow and splenic hematopoietic progenitors *in vitro*:

Four mice from each strain were used in each experiment. Bone marrow and spleen cells from mice were obtained as described above. These cells were counted and brought to 2×10^6 cells/ml. The cells were then incubated with either (MEM)-α or maxadilan at a concentration of 4 ng/ml in (MEM)-α for 2h on ice. After incubation, colony progenitor assays were performed as described above.

- Statistical analysis

The Kolmogorov-Smirnov test was performed to ensure normal distribution of data. As data were normally distributed, parametric statistical tests could be used. ANOVA factorial analysis was used to analyze data from the effect of maxadilan on hematopoiesis *in vivo* and hematopoietic precursor experiments. A repeated measurement ANOVA was used to analyze data from reticulocyte experiments. Results were considered significant when p value < 0.05.

Results

- Effect of maxadilan on bone marrow and spleen hematopoiesis *in vivo*:

Clonogenic progenitor assays were performed in both BALB/c and CBA mice to assess hematopoiesis three days after maxadilan injection. Experimental design is illustrated in Figure 2.1. Experiments were performed twice in both strains of mice. As shown in Figures 2.2 and 2.3, with results expressed as numbers of CFU-E, BFU-E, and CFU-GM per tibia, bone marrow hematopoiesis was increased in both strains of mice in maxadilan-treated groups compared to controls. In the spleen, as seen on Figures 2.4 and 2.5, results are expressed as numbers of colonies per spleen. In maxadilan-treated BALB/c mice, only late erythroid progenitors were significantly increased compared to controls, whereas all hematopoietic progenitors were significantly increased in maxadilan-treated CBA mice. However, results in the spleen were not repeatable in other experiments. This might be due to the fact that in the spleen of rodents, hematopoiesis is activated during a stress situation. Lower numbers of progenitors and a different hematopoietic microenvironment in the spleen may account for the difference in the hematopoietic response between bone marrow and spleen we saw in our experiments (30, 32). The medium used as vector to inject maxadilan had no effect on hematopoiesis when compared to basal hematopoiesis in normal mice in both spleen and bone marrow.

- Effect of maxadilan on reticulocyte counts *in vivo*:

As bone marrow erythroid progenitors were increased in both BALB/c and CBA mice three days after maxadilan injection, we were interested to see if this increase in hematopoietic activity resulted in an increase in circulating reticulocytes. These young erythrocytes are an indicator of erythropoietic activity. To test the hypothesis that maxadilan will increase circulating reticulocytes, reticulocyte counts were performed 3, 5 and 7 days after maxadilan treatment. Experimental design is illustrated in Figure 2.6. Experiments were performed in both strains of mice and results are shown in Figures 2.7 and 2.8. Results are expressed as numbers of reticulocytes per 1,000 erythrocytes (RBCs) counted. Three days after treatment, there was a significant increase, compared to untreated controls, in reticulocyte counts in both CBA and BALB/c mice, this increase was even more marked on the fifth day. Reticulocyte numbers declined from thereon. However, reticulocyte counts were still significantly higher in maxadilan-treated groups, in both BALB/c and CBA mice, compared to controls on the seventh day. These results indicated that the increased erythropoiesis seen in the bone marrow as indicated by increased CFU-E and BFU-E was also reflected by an increase in circulating reticulocyte counts. The decline in reticulocyte numbers seen over time in maxadilan-treated groups suggested that maxadilan had a transient stimulatory effect on hematopoiesis.

- Effect of maxadilan on hematopoietic progenitors:

We were interested to see if maxadilan was able to act on cells and hematopoietic progenitors isolated from the bone marrow and spleen of both BALB/c and CBA mice. Experimental design is illustrated in Figure 2.9. Experiments were performed twice in both strains of mice. After 2h incubation with maxadilan, clonogenic progenitor assays were performed. Results are expressed as number of colonies per number of cells plated. As shown in Figures 2.10 and 2.11, maxadilan acted on bone marrow hematopoietic progenitors to significantly stimulate CFU-E and CFU-GM formation in both strains of mice. It appeared that maxadilan was able to stimulate hematopoietic progenitors differentiation and proliferation without intervention of the bone marrow microenvironment. Maxadilan's effect was only seen in CFU-GM on spleen hematopoietic progenitors as seen in Figures 2.12 and 2.13. Lower numbers of progenitors in the spleen may account for the difference in the hematopoietic response between bone marrow and spleen we saw in our experiments (30, 32).

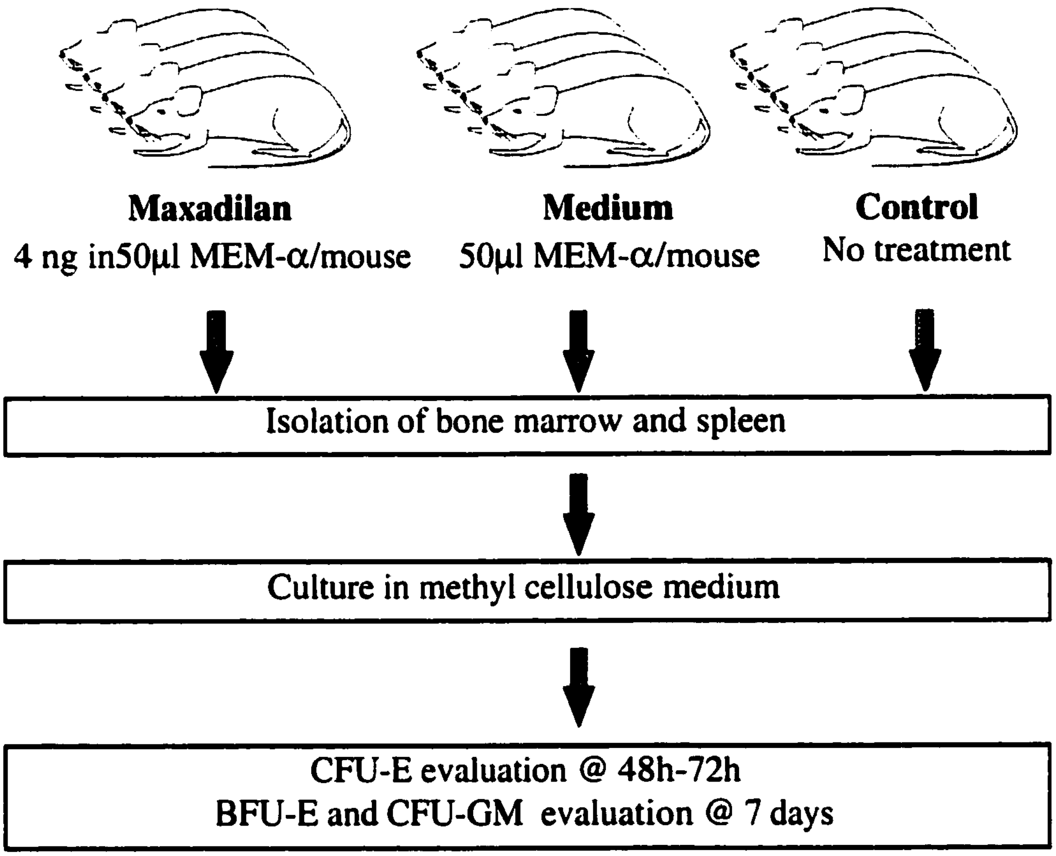

Figure 2.1: Effect of maxadilan on hematopoietic responses in mice

These experiments were performed in both BALB/c and CBA mice. Three treatment groups composed of four mice within each strain were used. The maxadilan-treated group received 4 ng of maxadilan in 50 µl of (MEM)-α in the left footpad, whereas the medium-treated group received 50 µl of (MEM)-α only also in the left footpad. The control group did not receive any treatment and was used to assess baseline hematopoiesis in normal mice and the effect of the medium used as vector on hematopoiesis. Three days later, mice were euthanized and bone marrow and spleen cells were isolated for clonogenic progenitor assays.

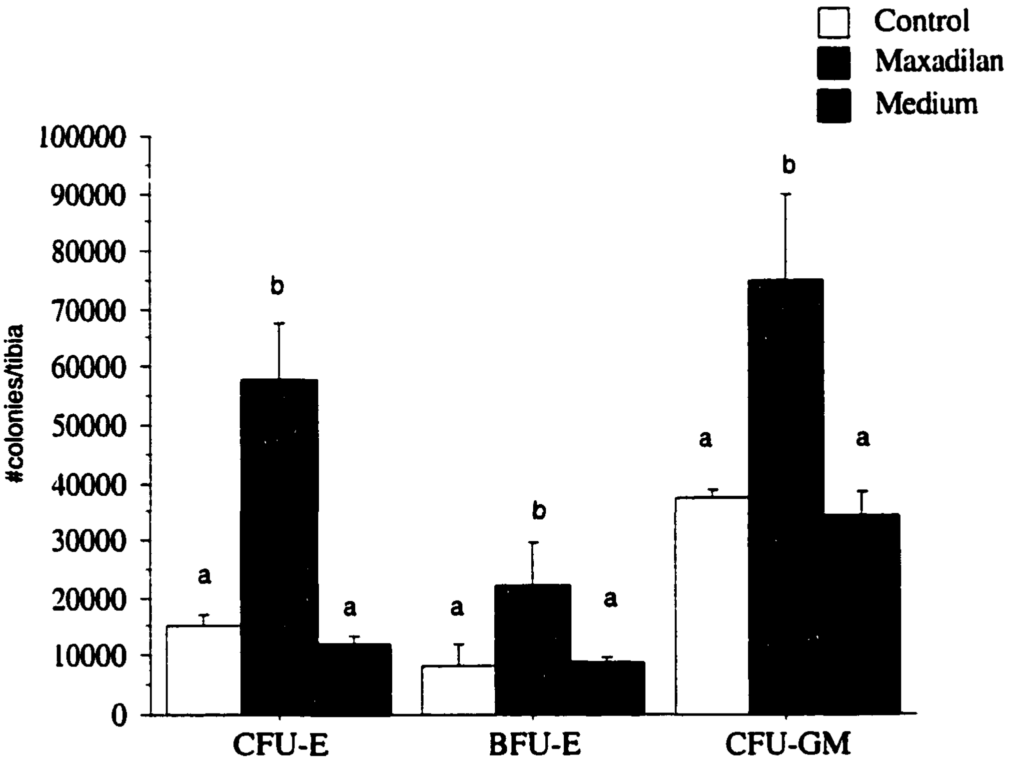

Figure 2.2: Effect of maxadilan on bone marrow hematopoiesis in BALB/c mice

2 x 10^4 bone marrow cells were plated in duplicate wells in methylcellulose medium three days after maxadilan injection. Results are expressed as numbers of colonies per tibia. Values represent means ± SD (bar). Different letters (a and b) denote differences between treatment groups (p value < 0.05).

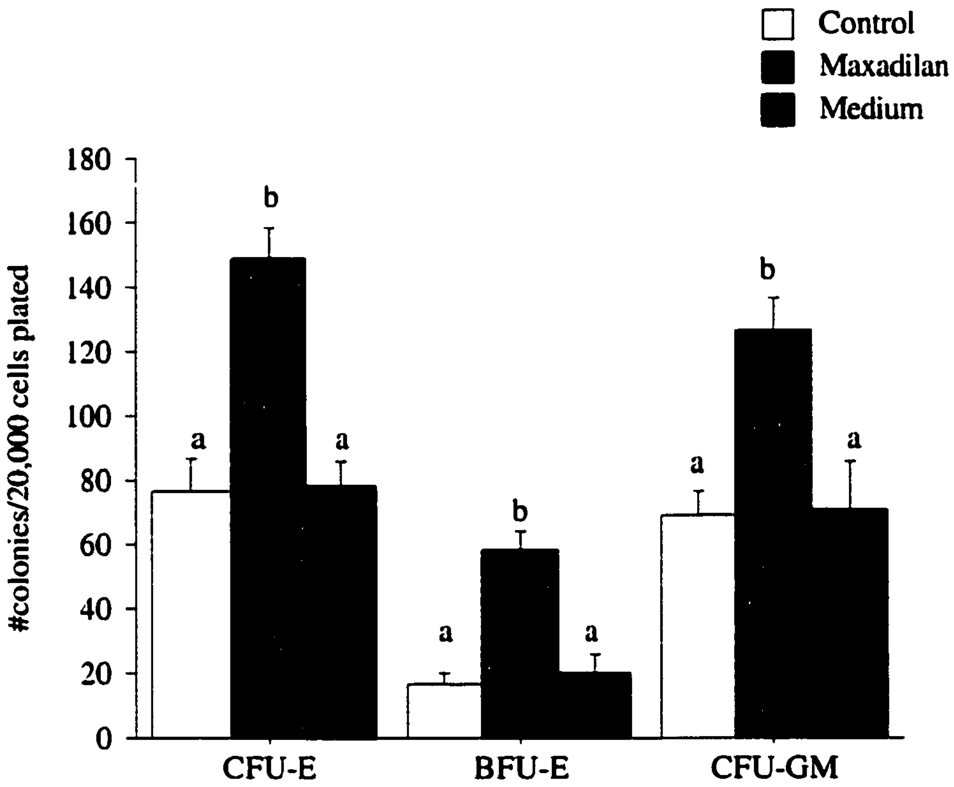

Figure 2.3: Effect of maxadilan on bone marrow hematopoiesis in CBA mice

2×10^4 bone marrow cells were plated in duplicate wells in methylcellulose medium three days after maxadilan injection. Results are expressed as numbers of colonies per tibia. Values represent means ± SD (bar). Different letters (a and b) denote differences between treatment groups (p value < 0.05).

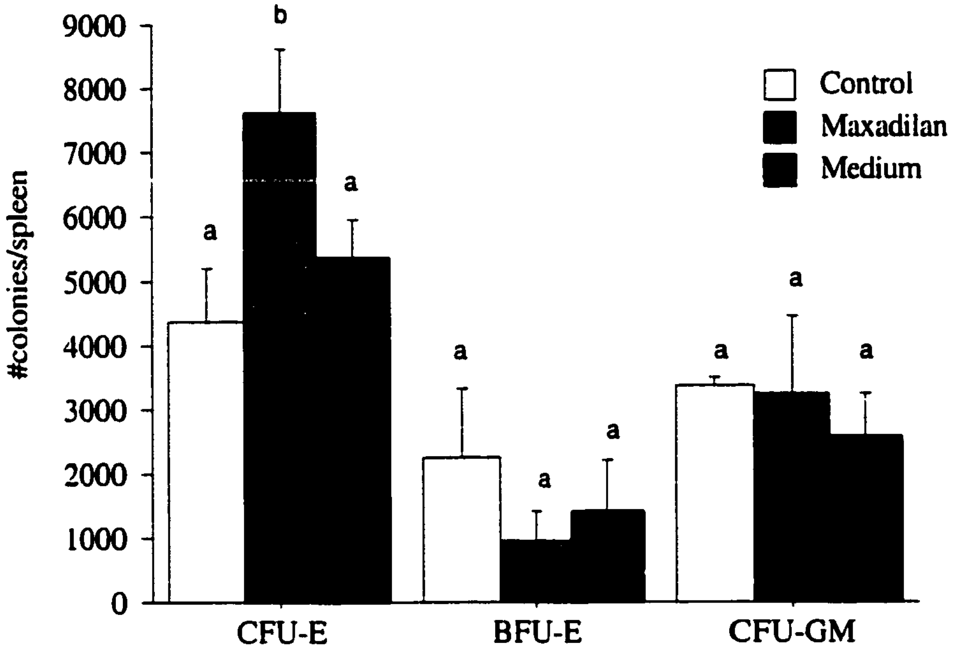

Figure 2.4: Effect of maxadilan on spleen hematopoiesis in BALB/c mice

2 x 10^4 spleen cells were plated in duplicate wells in methylcellulose medium three days after maxadilan injection. Results are expressed as numbers of colonies per spleen. Values represent means ± SD (bar). Different letters (a and b) denote differences between treatment groups (p value < 0.05).

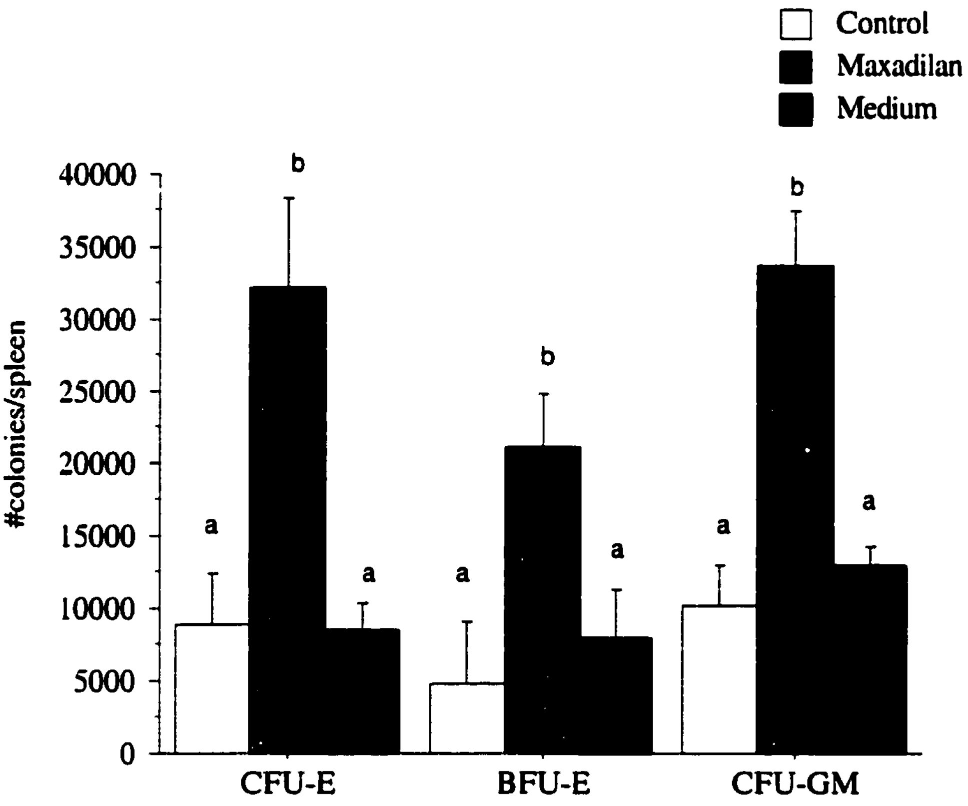

Figure 2.5: Effect of maxadilan on spleen hematopoiesis in CBA mice

2 x 10^4 spleen cells were plated in duplicate wells in methylcellulose medium three days after maxadilan injection. Results are expressed as numbers of colonies per spleen. Values represent means ± SD (bar). Different letters (a and b) denote differences between treatment groups (p value < 0.05).

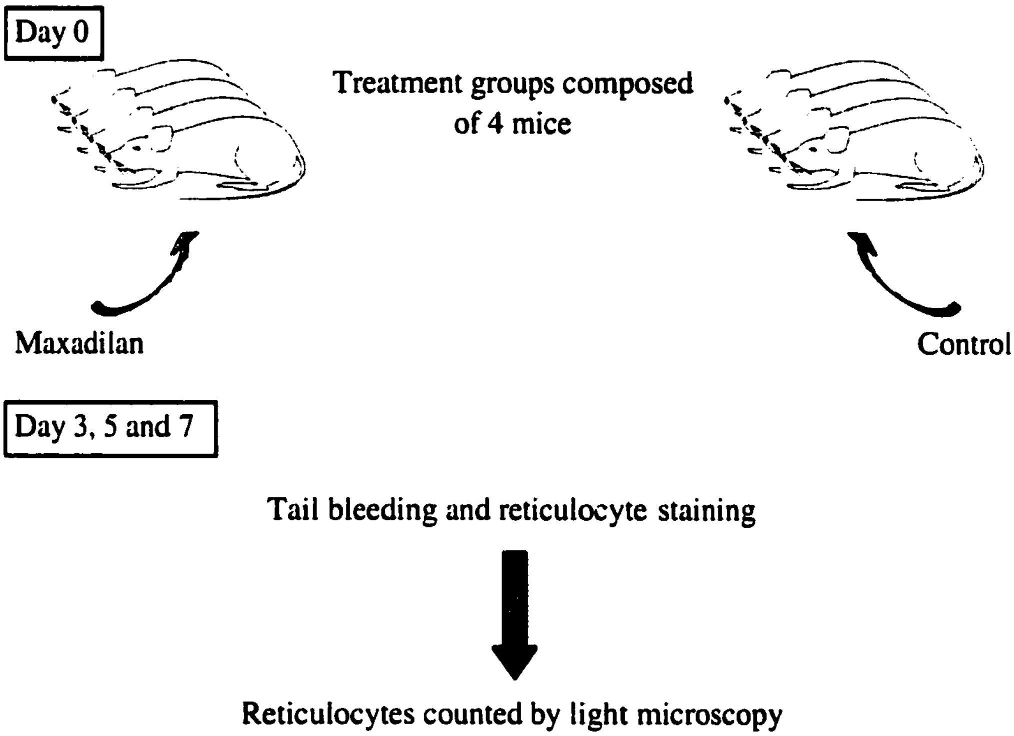

Figure 2.6: Maxadilan effect on reticulocyte counts

These experiments were performed in both BALB/c and CBA mice. Two treatment groups composed of four mice within each strain were used. The maxadilan-treated group received 4 ng of maxadilan in 50 μl of (MEM)-α in the left footpad, whereas the medium-treated group received 50 μl of (MEM)-α only also in the left footpad. Reticulocytes were stained with new methylene blue and counted at 3, 5 and 7 days after treatment.

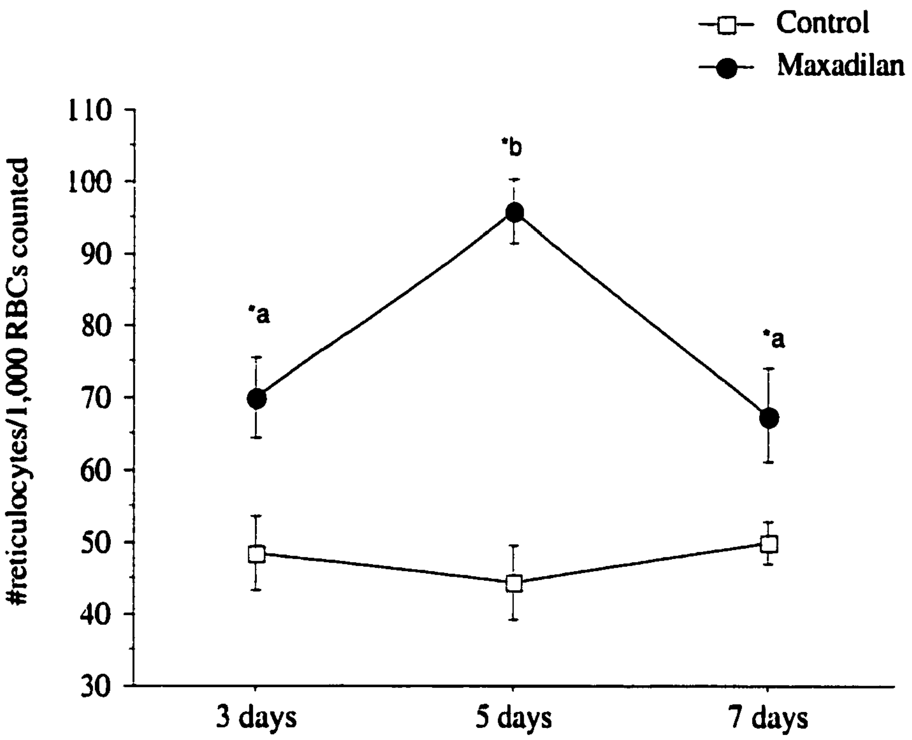

Figure 2.7: Effect of maxadilan on reticulocyte counts in BALB/c mice

Reticulocyte counts were performed 3, 5 & 7 days after maxadilan injection. Results are expressed as number of reticulocytes per 1,000 erythrocytes (RBCs) counted Values represent means ± SD (bar). Different letters (a, b and c) denote differences between treatment groups (p value < 0.05). * indicates a significant difference compared to control (p value < 0.05)..

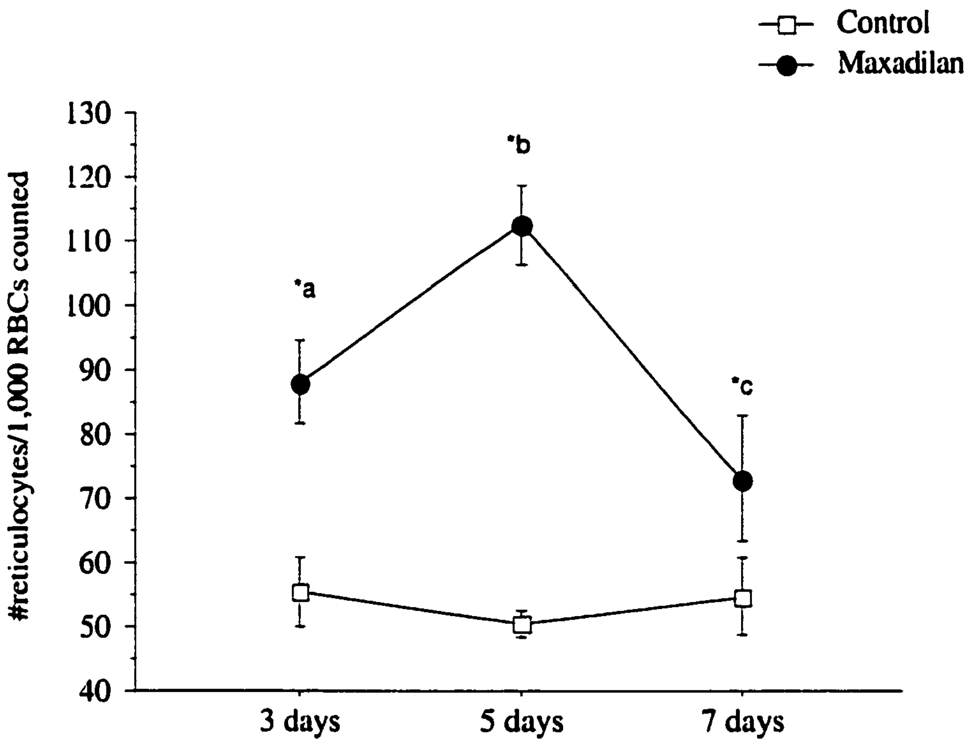

Figure 2.8: Effect of maxadilan on reticulocyte counts in CBA mice

Reticulocyte counts were performed 3, 5 & 7 days after maxadilan injection. Results are expressed as number of reticulocytes per 1,000 erythrocytes (RBCs) counted. Values represent means ± SD (bar). Different letters (a, b and c) denote differences between treatment groups (p value < 0.05). * indicates a significant difference compared to control (p value < 0.05).

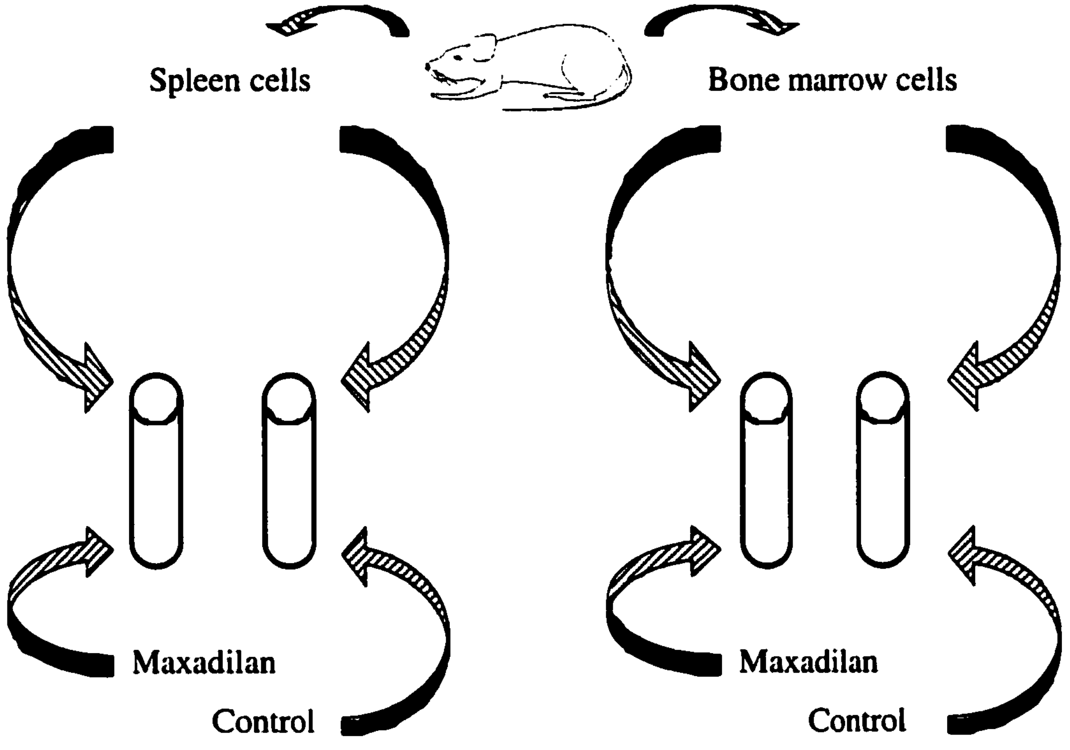

Figure 2.9: Maxadilan effect on bone marrow progenitors

Four mice from each strain were used in each experiment. Bone marrow and spleen cells from mice were isolated. These cells were counted and brought to 2 x 10^6 cells/ml. The cells were incubated with either (MEM)-α or maxadilan at a concentration of 4 ng/ml for 2h on ice. After incubation, colony progenitor assays were performed to assess effect of maxadilan on hematopoietic precursors.

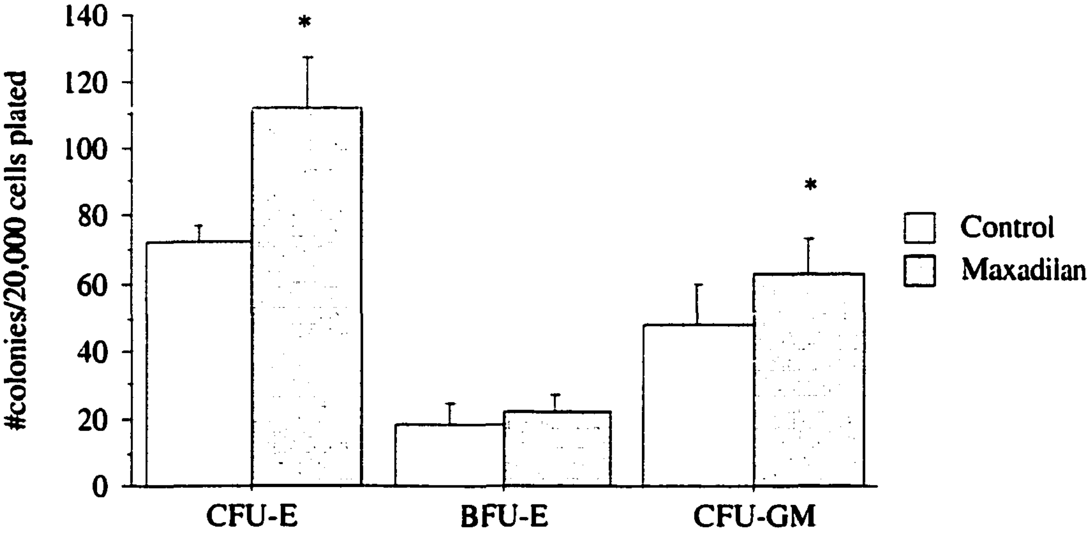

Figure 2.10: Direct effect of maxadilan on bone marrow progenitors in BALB/c mice 2 x 10^4 bone marrow cells from each mouse were plated in duplicate wells in methylcellulose medium 2 hours after incubation with maxadilan. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

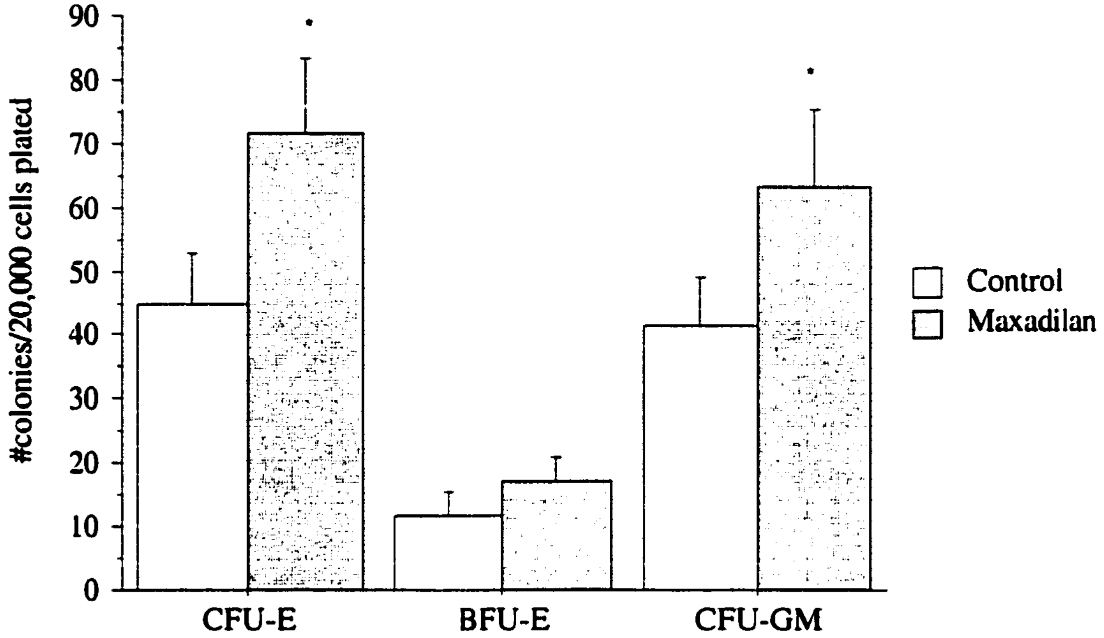

Figure 2.11: Direct effect of maxadilan on bone marrow progenitors in CBA mice

2 x 10^4 bone marrow cells from each mouse were plated in duplicate wells in methylcellulose medium 2 hours after incubation with maxadilan. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent mean ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

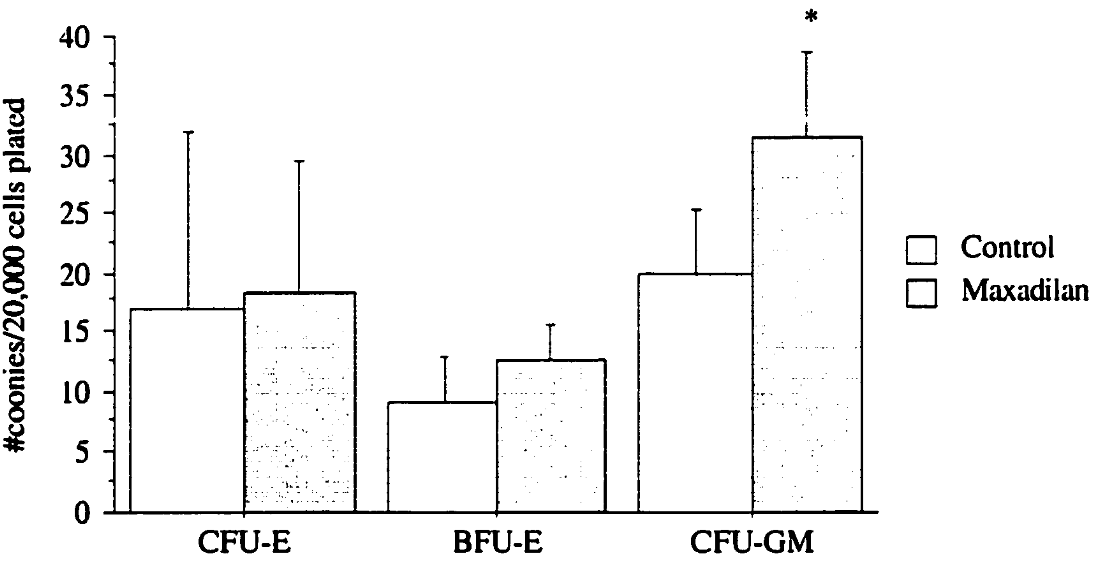

Figure 2.12: Direct effect of maxadilan on spleen hematopoietic progenitors in BALB/c mice

2 x 10^4 spleen cells from each mouse were plated in duplicate wells in methylcellulose medium 2 hours after incubation with maxadilan. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

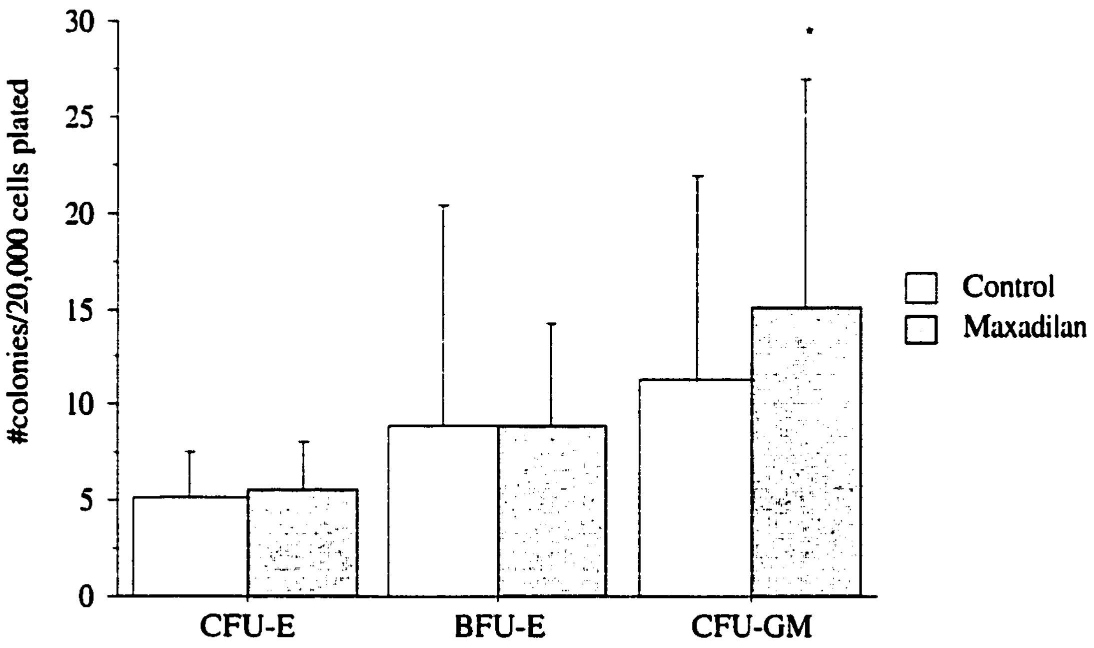

Figure 2.13: Direct effect of maxadilan on spleen hematopoietic progenitors in CBA mice

2 x 10^4 spleen cells from each mouse were plated in duplicate wells in methylcellulose medium 2 hours after incubation with maxadilan. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

Discussion

Maxadilan, a sand fly saliva peptide, had been shown to act through interactions with PACAP receptors. These have been identified in numerous tissues such as pituitary, pancreas, and adrenal medulla, testes (24), and bone marrow (25) as well as on macrophages and lymphocytes (26, 27). Both maxadilan and PACAP have been shown to stimulate macrophages' production of IL-6 (18, 28). This cytokine is essential in regulation of normal and inducible hematopoiesis (20). IL-6 synergizes with numerous others such as stem cell factor (SCF) to provide the adequate stimuli necessary for the development of early and lineage-committed progenitors (29). IL-6 is required for the development of both erythroid and myeloid progenitors (20, 30, 31). In our laboratory, maxadilan has been shown to exacerbate lesions due to *L. major* infection. Moreover increased hematopoiesis has been associated with susceptibility to the disease (21). Given the potential effect of maxadilan on hematopoiesis through its action on IL-6 production, we hypothesized that maxadilan will increase hematopoiesis. Three days after treatment with maxadilan, we found that both early and late erythroid progenitors as well as myeloid progenitors were significantly increased compared to controls in the bone marrow of both BALB/c and CBA mice. In the spleen, results were less consistent, however a general trend towards increased hematopoiesis could be seen in maxadilan treated mice. Differences in the spleen and bone marrow hematopoietic response to maxadilan treatment may reside in lower numbers of progenitors in the spleen and a different hematopoietic microenvironment (32, 33). Moreover, the spleen is not involved in normal hematopoiesis. Splenic hematopoiesis is stimulated during a stress situation such as anemia. Evidence of the stimulatory effect of maxadilan on hematopoiesis was the increase in circulating reticulocytes with a maximum at day 5 in maxadilan-treated mice of both strains and a decrease thereafter indicating that maxadilan had a transient effect on hematopoiesis. Tail bleeding did not affect reticulocyte counts in control groups, suggesting that increased reticulocyte numbers were due to maxadilan itself and not a response to the tail bleeding technique we used in these experiments.

Incubation of maxadilan with bone marrow hematopoietic progenitors lead to increases in late erythroid and myeloid progenitors in both BALB/c and CBA mice. Early erythroid progenitors were not affected. The stimulatory effect of maxadilan on spleen hematopoietic progenitors was only noted on CFU-GM. The different response between spleen and bone marrow progenitors is most likely due to lower frequency of hematopoietic progenitors in the spleen. Our hypothesis (investigated in chapter III) is that maxadilan action on hematopoiesis is due to its action on IL-6 production. Indeed, maxadilan has been shown to stimulate IL-6 production by binding PACAP receptors on macrophages (18). Macrophages are a normal inhabitant of the bone marrow stroma, which is comprised of about 1% of macrophages and 10% of immature monocytic cells (35). Macrophages, upon stimulation with maxadilan, are a likely source of IL-6. This hypothesis will be further investigated in Chapter III. IL-6 has been shown to be essential in control of hematopoiesis, acting on control of differentiation and proliferation of pluripotential and committed hematopoietic progenitors. Both erythroid and myeloid lineages are affected by IL-6 (20, 31). Myeloid progenitors however, appear to be more responsive to IL-6 than erythroid as shown in experiments with human bone marrow progenitors treated with anti IL-6 (36). IL-6 receptors have been identified on granulocytes, macrophages and their progenitors but not on red blood cells and erythroid progenitors (37), thus myeloid progenitors are more likely to respond to the stimulatory effect of IL-6 and maxadilan. IL-6 is necessary for the development of both erythroid and myeloid progenitors, however, it is likely to have an indirect effect on erythroid progenitors through stimulation of the bone marrow stroma to produce molecules such SCF, which is essential for BFU-E formation (38, 39). Also, differentiation of progenitors is more efficient with a combination of cytokines. A two hour incubation is most likely too short to allow signal exchanges and synthesis of all the molecules necessary to support BFU-E formation (40). Thus, this may explain the lack of response to maxadilan treatment of BFU-E. Lower numbers of monocytic cells in the spleen may also account for the differences in the hematopoietic responses seen.

We showed that maxadilan was able to stimulate hematopoiesis in both CBA and BALB/c mice. This is the first time that a sand fly salivary molecule has been shown to affect hematopoiesis. Increased hematopoiesis and decreased macrophage maturation have been associated with susceptibility to *L. major* infection (21, 41). By stimulating hematopoiesis, it appears that maxadilan will favor parasite development and infection establishment, however, the underlying mechanisms for maxadilan action need to be further investigated.

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Chapter III: How is the effect of maxadilan mediated?

Introduction

Saliva from blood-feeding arthropods contains a large array of molecules with various pharmacological activities (1). Saliva from the sand fly *Lutzomyia longipalpis*, vector of leishmaniasis, is no exception. The bite of a sand fly is able to produce a non-itching long lasting erythema without edema. This characteristic of the sand fly bite is due to the presence of a potent vasodilator present in small quantities in its saliva (2). This vasodilator, named maxadilan, has been isolated, cloned and a functional recombinant produced. Sand fly saliva contains a total amount of about 10ng of this small 7kb vasodilator. Maxadilan is one of the most potent vasodilators known to date (3, 5). This peptide has been shown to bind to the pituitary adenylate-cyclase activating peptide (PACAP) type 1 receptor (6, 7). Maxadilan is not only a vasodilator, this peptide also possesses immunomodulatory properties targeting both T-cells and macrophages (8-10).

As we showed in Chapter II, maxadilan is able to stimulate hematopoiesis in mice. We were interested in the mechanisms underlying this observation. Hematopoietic homeostasis is a very complex and redundant system with numerous cell interactions and molecules involved. Interestingly, both maxadilan and PACAP stimulates IL-6 production of macrophages (11). IL-6 is a cytokine that is involved in stimulating proliferation and differentiation of hematopoietic progenitors (12). Moreover, PACAP receptors have been identified on rat bone marrow-derived stromal cells, and PACAP binding results in increased IL-6 production by these cells (13). Given this evidence, we hypothesize that maxadilan stimulates hematopoiesis through IL-6 production, acting on bone marrow stromal cells (most likely fibroblasts and macrophages) through interaction with PACAP receptors. We found that maxadilan appeared to stimulate hematopoiesis through IL-6 production and PACAP receptor binding. It appeared to act on bone marrow stromal cells, more particularly macrophages and fibroblasts, to mediate its action.

Materials and methods

- Mice:

Most of the six to eight week-old female BALB/cByJ mice used in these experiments were bred at Colorado State University (CSU) Laboratory Animal Resources (LAR). Small numbers were obtained from the National Cancer Institute (NCI) (Bethesda, MD), an external provider. Six to eight week-old female CBA/J mice were obtained from NCI and housed at LAR. These mice were used primarily to provide bone marrow stromal feeder layer cells and/or bone marrow progenitor cells. Both tibias from these mice were aseptically removed and bone marrow cells were isolated as described below.

- Bone marrow cell isolation:

After euthanasia, tibias were removed and muscle was cleaned off the bone. If needed, the tibial distal metaphysis was cut off. Tibia were flushed with 1 ml of minimal essential medium (MEM)-α (Gibco BRL, Grand Island, NY). Bone marrow cells were then pipetted vigorously to separate cell clusters and brought to 2 x 10^6 cells/ml.

- Long-term bone marrow culture experiments:

Long term bone marrow cultures (LTBMC) were initiated from murine bone marrow cells isolated as described above. Bone marrow cells were seeded at 3 x 10^6 cells/ml in Myelocult® (StemCell Technologies Inc, Vancouver, BC) supplemented with hydrocortisone sodium succinate (Sigma, St Louis, MO) at a concentration of 10^{-6} M in 48-well plates in a 500 µl volume. 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 and saved, the plate was gently shaken to allow non-adherent cells to escape from the adherent layer, and the remaining medium was removed and saved. A volume of 250 µl of these removed cells and old medium were reintroduced in each well and fresh medium was added to return the volume to 500 µl. After 4 weeks, the LTBMC were established and could be treated with maxadilan (14). At that time, stromal cells were confluent and composed of a mixture of fibroblasts, adipocytes and mononuclear cells. Clusters of hematopoietic cells could also be seen. During the last feeding, four wells from each LTBMC culture plate were treated with either maxadilan at a concentration of 4 ng/ml in Myelocult® medium, maxadilan at 4 ng/ml and anti-IL-6 at a concentration of 5 µg/ml in Myelocult® medium, anti-IL-6 at a concentration of 5 µg/ml in Myelocult® medium, or Myelocult® medium alone, the latter being used as control. After three days, cells were removed with trypsin-EDTA (0.25%) (Sigma). The cells were washed and clonogenic progenitor assays were performed as described below.

- Feeder layer establishment:

Bone marrow cells obtained from either BALB/c or CBA mice were brought to 2 x 10^6 cells/ml in Myelocult®. Cells were plated in 48-well plates in a volume of 500 µl and cultured for 10 to 12 days at 37°C. After this incubation period, trypsin-EDTA (0.25%) was applied to the wells to remove the cells. The cells were irradiated at 1500 cGy and washed once in (MEM)-α. Cells were then plated in 48-well plates at 2 x 10^6 cells/well and incubated at 37°C for 24h to form a confluent monolayer (14).

- Feeder layer treatment with maxadilan:

After a 24h incubation, feeder layer cells were stimulated with either maxadilan at a concentration of 4 ng/ml in Myelocult® or Myelocult® only for 3 days at 37°C. In some cases, the pituitary adenylate cyclase-activating peptide (PACAP) inhibitor P6-38 (Peninsula Laboratories, Belmont, CA) was added to the cultures. Supernatants were removed and wells were rinsed once with (MEM)-α. Bone marrow cells then were seeded on the feeder layer at 2 x 10^6 cells/well. In some cases, anti-IL-6 was added to the cultures. An isotype-matched control antibody was added to control cultures.

- Production of peritoneal macrophages:

Mice were injected intraperitonealy (i.p.) with 3 ml of a sterile starch solution (ACS in saline, Sigma, catalogue number S-9765). Four days later, macrophages were harvested as follows: ice-cold wash medium (DMEM with amino acid supplement and Hepes), supplemented with 5% FBS, was injected i.p. with a 27g needle. The fluid then was withdrawn with a 21g needle (15, 16). Macrophages were pooled and washed once. They were plated in 24-well plates, at 3 x 10^6 cells/ml in DMEM medium. Macrophages were cultured for 24h to allow them to attach to the bottom of the wells. Non-adherent cells were removed and wells were washed before stimulation with maxadilan as described below.

- Treatment of macrophages with maxadilan:

After 24h incubation, non-adherent cells were removed and macrophages were treated for 24h with either maxadilan at a concentration of 4 ng/ml in DMEM medium or culture medium alone, the latter being used as control. Supernatants were removed and fresh medium was added to macrophage cultures for an additional 48h. The supernatant was then removed and stored at -20°C until use. When used, the supernatants were incubated for 2h on ice with bone marrow hematopoietic progenitor cells from four mice (each cultured separately) with or without addition of anti-IL-6 at 5 µg/ml. After incubation, colony progenitor assays were performed.

- Production of bone marrow (BM)-derived fibroblasts:

BM-derived fibroblasts were initiated from bone marrow cells isolated from both tibias of a pool of five mice. Cells were cultured in a flask under a volume of 10 ml of DMEM complemented with 15% FBS, L-glutamine and gentamycin. Non-adherent cells were removed after 24h incubation and adherent cells refed. Cultures were fed by medium replacement every other day. Once confluence was reached, cells were detached with trypsin-EDTA (0.25%), washed and sub-cultured. Cells underwent three passages before being used. After the last trypsin treatment and wash, BM-derived fibroblasts were irradiated at 1500 cGy and washed once in (MEM)-α. Cells were plated in 48-well plates at 2 x 10^6 cells/well and incubated at 37°C for 24h to form a confluent monolayer (14).

- Treatment of BM-derived fibroblasts with maxadilan:

After 24h incubation, BM-derived fibroblasts were stimulated with either maxadilan at a concentration of 4 ng/ml in Myelocult® or Myelocult® only for three days at 37°C. Supernatants were removed and wells were rinsed once with MEM-α. Bone marrow cells were then seeded on the feeder layer at a concentration of 2 x 10^6 cells/well. In some cases, anti-IL-6 was added to the cultures.

- Colony progenitor assays:

Colony progenitor assays were performed to assess hematopoiesis. Colony Forming Unit-Erythroid (CFU-E) cells are late erythroid progenitors which form small colonies after 48-72h in semi-solid culture, Burst Forming Unit-Erythroid (BFU-E) cells are early erythroid progenitors which form large colonies after 6-7 days, and Colony Forming Unit-Granulocyte/Macrophage (CFU-GM) cells are progenitors of granulocytes and macrophages which form colonies after 6-7 days. Bone marrow and spleen cells were brought to 2 x 10^6 cells/ml. After dilution, cells were recounted and the definitive cell number, N, was determined. Cells were diluted in methyl-cellulose culture medium (StemCell Technologies) at 200,000 cells/ml. Cells were plated in a volume of 200 μl in wells of a 48-well plate at 20,000 cells/well. Plates were incubated at 37°C. After 48-72h incubation, CFU-E were counted using an inverted phase microscope. BFU-E and CFU-GM were evaluated similarly after 6-7 days. Counts from the duplicate wells were averaged and, as there is a linear relationship between colony numbers and cells plated (17), this result was multiplied by 2 x 10^6/N to compensate for variations between actual cell number, N, and the desired concentration of 2 x 10^6/ml. For *in vitro* experiments, this calculated number was the final data entry from these duplicate wells. Results are expressed as numbers of cells plated.

- IL-6 determination:

Supernatants from some cultures were tested for IL-6 using a sandwich ELISA performed according to manufacturer's directions. Results are expressed in pg/ml. Antibodies reactive with IL-6 and recombinant mouse IL-6 were obtained from PharMingen (San Diego, CA).

- Statistical analysis:

Kolmogorov-Smirnov test was performed to ensure normal distribution of data. As data were normally distributed, parametric statistical tests were used. ANOVA factorial analysis was used to analyze data. Results were considered significant when p value < 0.05.

Results

- The effect of maxadilan is mediated through IL-6 production:

With long-term bone marrow cultures, we are able to study hematopoiesis in an *in vitro* environment, which allows more flexibility than an *in vivo* model. This system is more efficient at sustaining the formation of myeloid progenitors (14, 20). Erythroid progenitors do not differentiate beyond BFU-E due to the lack of erythropoietin (EPO) production by the stromal layer and the strict requirement for EPO of erythroid progenitors after the BFU-E stage (21, 22). Experimental design is illustrated in Figure 3.1. Experiments were performed twice in both strains of mice. As seen in Figures 3.2 and 3.3, when long-term bone marrow culture (LTBMC) from BALB/c and CBA mice were stimulated with maxadilan for three days, there was a significant increase, compared to control, in BFU-E and CFU-GM in both BALB/c and CBA mice. When anti-IL-6 antibody was present, maxadilan was not able to stimulate hematopoiesis. This antibody in itself did not appear to affect normal hematopoiesis since there was no significant difference between control cultures and control cultures with anti-IL-6 antibody. From these experiments, it appeared that the effect of maxadilan was mediated through IL-6 production. This result was reinforced by higher levels of IL-6 detected by ELISA in supernatants from LTBMC stimulated for three days with maxadilan as shown in Figures 3.4 and 3.5.

- Effect of maxadilan on bone marrow stromal cells:

Bone marrow stromal cells are important for hematopoietic homeostasis. Interestingly, PACAP receptors have been identified on bone marrow stromal cells in rats, and given the functional similarity of PACAP and maxadilan, we were interested in studying the effect of maxadilan on bone marrow derived-stromal cells in our system. Experimental design is illustrated in Figure 3.6. Experiments were performed twice in both BALB/c and CBA mice and results are shown in Figures 3.7 and 3.8. Maxadilan-treated bone marrow-derived stromal cells were able to stimulate hematopoiesis in both strains of mice, as indicated by increased numbers of CFU-E, BFU-E and CFU-GM in maxadilan-treated groups compared to controls. This effect was not seen when anti-IL-6 antibody was present. It appeared that maxadilan effect was mediated through IL-6 production and that, as seen in Figures 3.9 and 3.10, it was not an artifact due to the antibody used, since a control antibody specific for β-galactosidose had no effect on maxadilan stimulation of hematopoiesis. CFU-E, BFU-E and CFU-GM numbers in anti-β-galactosidose and maxadilan-treated groups were similar to these in maxadilan-treated groups. Moreover, anti-β-galactosidose treatment did not affect normal hematopoiesis as indicated by the lack of differences between anti-β-galactosidose-treated control groups and controls.

Maxadilan appeared to stimulate hematopoiesis through interaction with bone marrow stromal cells and IL-6 production. As maxadilan has been shown to act through PACAP receptors and since these have been identified on bone marrow stromal cells of rats, we were interested to know if maxadilan acted on bone marrow-derived stromal cells through interaction with PACAP receptors. To test this hypothesis, we used a potent PACAP inhibitor P6-38. Experimental design is illustrated in Figure 3.11. Experiments were performed twice in both strains of mice. Results are shown in Figures 3.12 and 3.13. When P6-38 was present, maxadilan was not able to stimulate hematopoiesis, as indicated by the lack of differences between P6-38 and maxadilan-treated groups and controls. Moreover, P6-38 did not affect normal hematopoiesis as colony numbers were similar between P6-38-treated controls and controls. Thus, it appeared that maxadilan stimulated hematopoiesis through interaction with PACAP receptors.

- Maxadilan effect is mediated by macrophages and bone marrow derived fibroblasts through IL-6 production:

Stromal bone marrow cells are composed a mixture of fibroblasts, macrophages, endothelial cells and adipocytes. These cells interact with hematopoietic progenitors to regulate hematopoiesis. As fibroblasts and macrophages are easy to obtain and culture, we studied the effect of maxadilan on both of these bone marrow components. Experimental design is illustrated in Figure 3.14. These experiments were performed twice in both BALB/c and CBA mice. As seen in Figures 3.15 and 3.16, cell free supernatants from maxadilan-treated macrophages were able to stimulate bone marrow hematopoietic progenitors as indicated by increased CFU-E, BFU-E and CFU-GM in these groups compared to controls. When present, anti-IL-6 antibody inhibited the effect of maxadilan-treated macrophage supernatants on hematopoietic progenitors as indicated by the lack of differences in colony numbers between these groups and controls. Thus, IL-6 appeared to be involved in stimulating hematopoiesis in response to maxadilan treatment. Bone marrow-derived fibroblasts were also involved in maxadilan stimulation of hematopoiesis. Experimental design is illustrated in Figure 3.17. Experiments were performed twice in both strains of mice. Maxadilan-treated fibroblast cultures were able to stimulate hematopoiesis as indicated by significantly higher numbers of colonies compared to controls. The presence of anti-IL-6 eliminated the effect of maxadilan's treatment of fibroblasts, indicating that IL-6 was involved in mediating the stimulatory effect of maxadilan on hematopoiesis. Results from these experiments are shown in Figures 3.18 and 3.19.

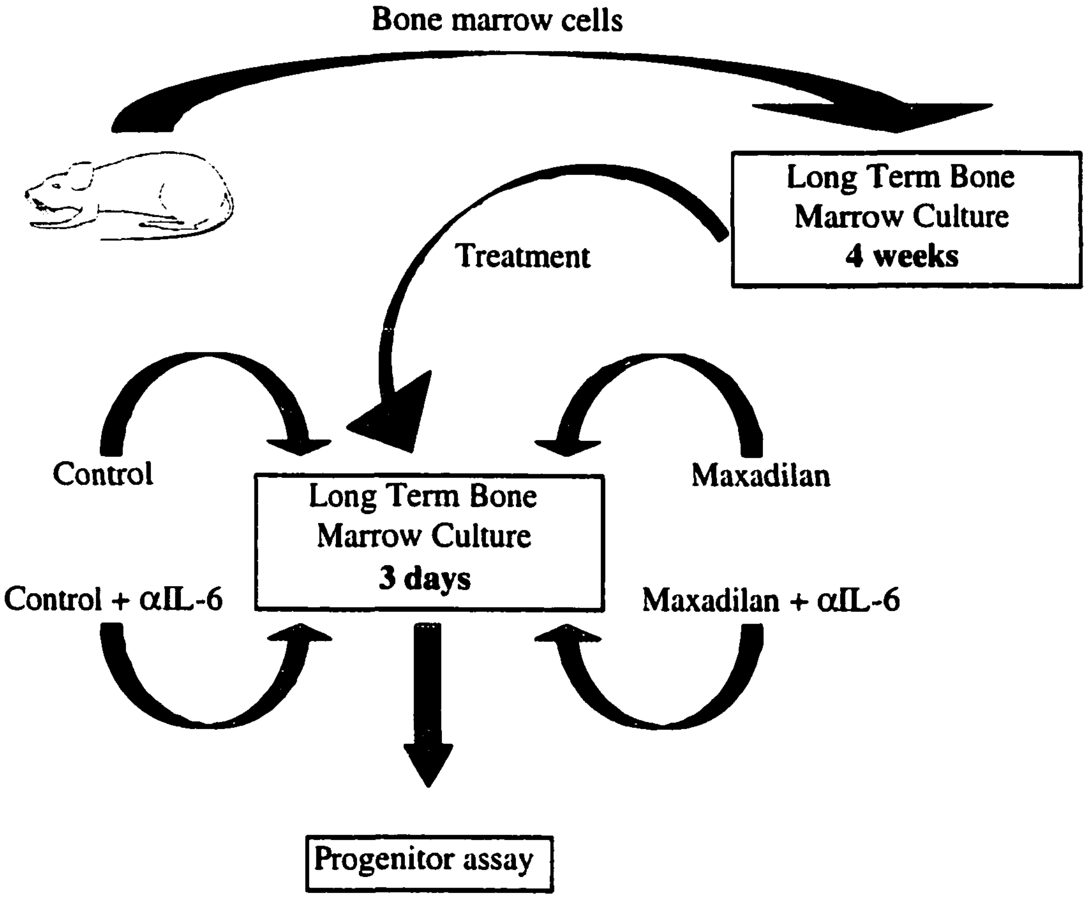

Figure 3.1: Long-term bone marrow culture (LTBMC) experiments

LTBMC were initiated with murine bone marrow cells. Bone marrow cells were seeded at 3 x 10^6 cells/ml in 48-well plates. Cells were incubated for 3 to 5 days at 37°C and then at 33°C for the duration of the experiment. After the first week, weekly feedings were performed. After four weeks, the LTBMC were established and could be treated with maxadilan. During the last feeding, four wells from each LTBMC culture plates were treated with either maxadilan (4 ng/ml), maxadilan (4 ng/ml) and αIL-6 (5 μg/ml), culture medium and αIL-6 (5 μg/ml), or culture medium alone, the latter being used as control. After three days, cells were removed with trypsin-EDTA (0.25%), the cells were washed and clonogenic progenitor assays were performed.

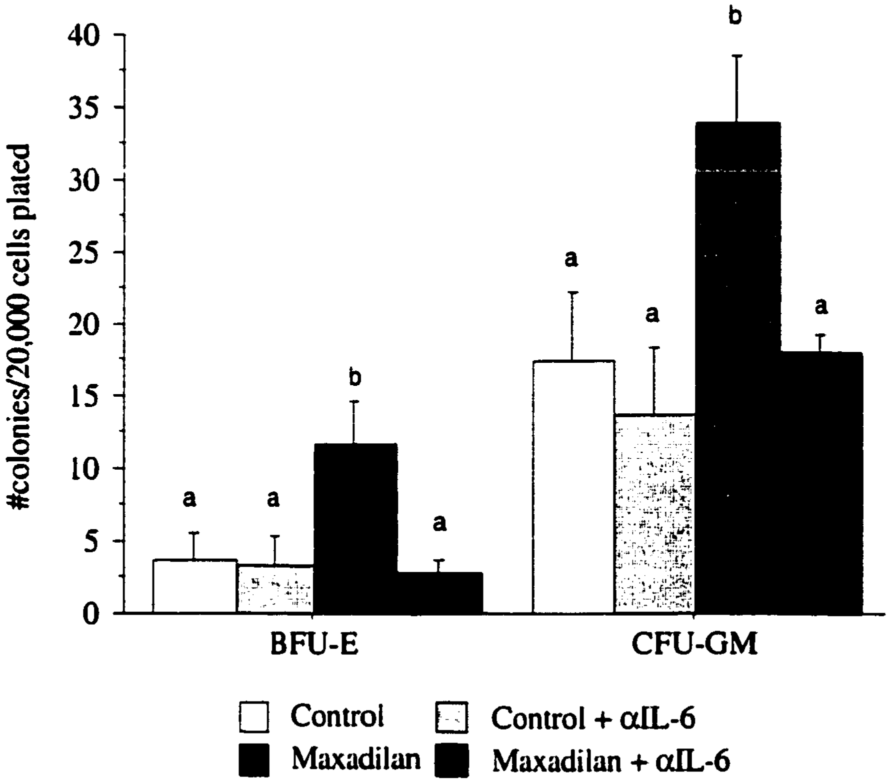

Figure 3.2: Effect of anti-IL-6 antibody (αIL-6) on Long-Term Bone Marrow Culture (LTBMC) stimulated with maxadilan in BALB/c mice

LTBMC were stimulated with maxadilan with or without αIL-6. Three days later, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

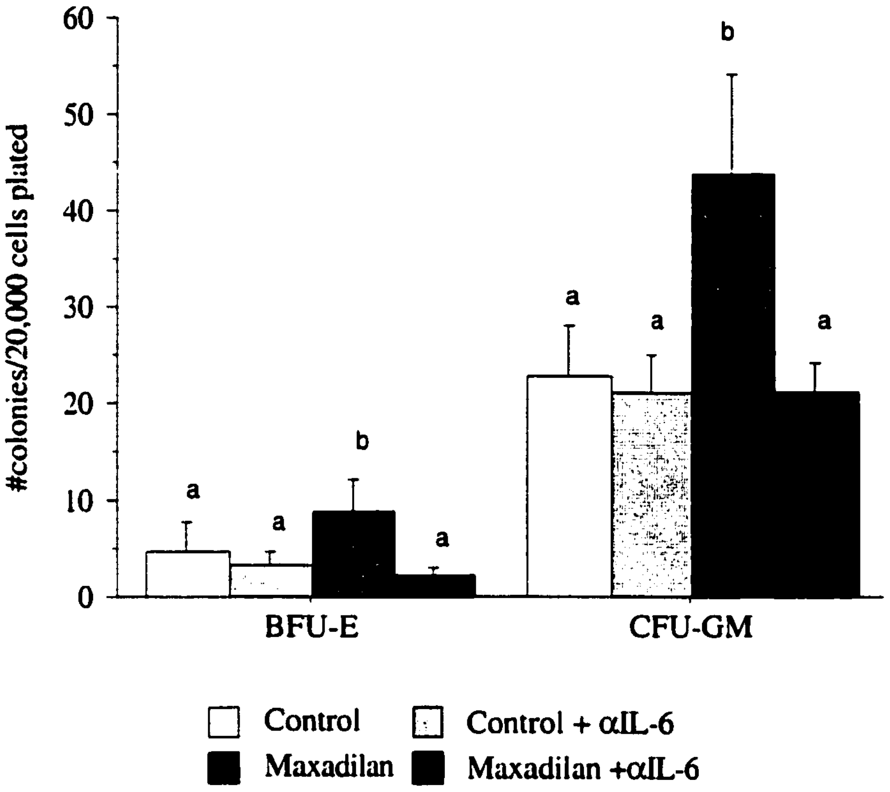

Figure 3.3: Effect of anti-IL-6 antibody (αIL-6) on Long-Term Bone Marrow Culture (LTBMC) stimulated with maxadilan in CBA mice

LTBMC were stimulated with maxadilan with or without of αIL-6. Three days later, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

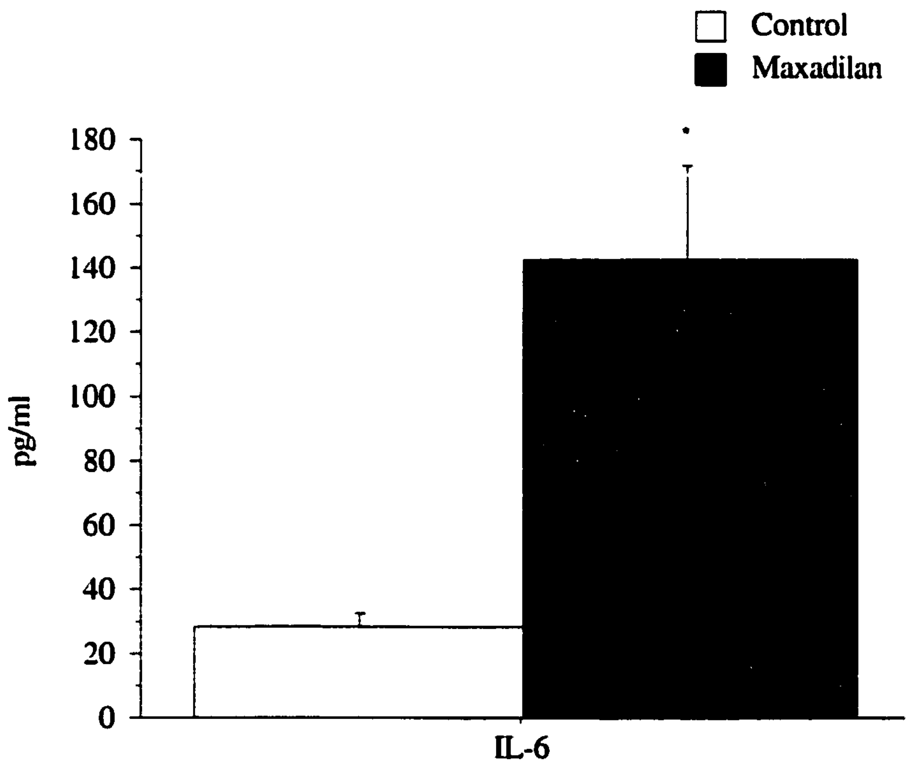

Figure 3.4: IL-6 production in supernatant from Long Term Bone Marrow Culture (LTBMC) stimulated with maxadilan in BALB/c mice

After a three-day stimulation with or without maxadilan, ELISA determination of IL-6 levels was performed on LTBMC cell-free supernatants. Results are expressed in pg/ml. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

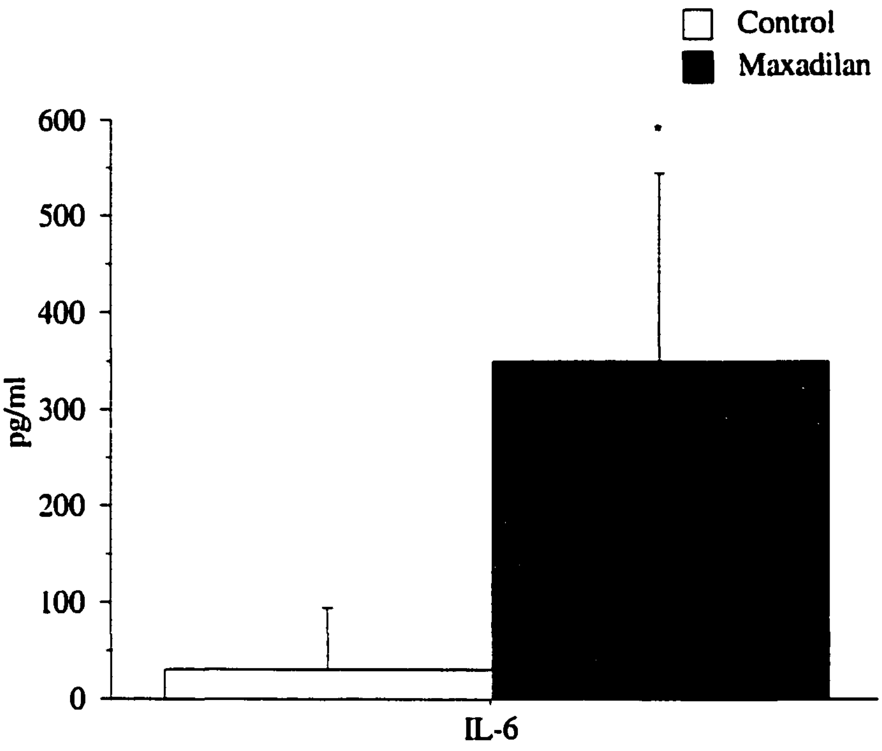

Figure 3.5: IL-6 production in supernatant from Long Term Bone Marrow Culture (LTBMC) stimulated with maxadilan in CBA mice

After a three-day stimulation with or without maxadilan, ELISA determination of IL-6 levels was performed on LTBMC cell-free supernatants. Results are expressed in pg/ml. Values represent means ± SD (bar). * indicates a significant difference compared to control (p value < 0.05).

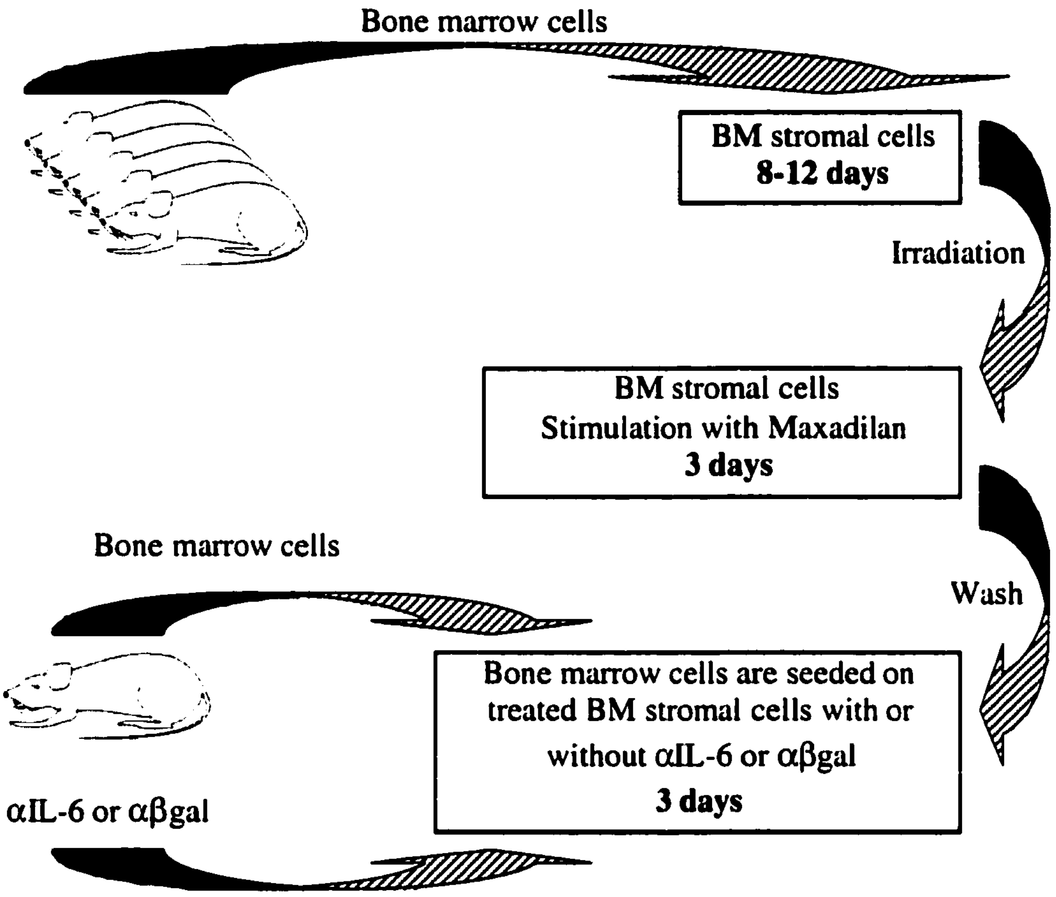

Figure 3.6: Effect of an anti-IL-6 antibody (αIL-6) on maxadilan-treated bone marrow-derived stromal cells

Bone marrow cells obtained from either BALB/c or CBA mice were brought to 2 x 10^6 cells/ml. Cells were plated in 48-well plates and cultured for 8 to 12 days. After a trypsin-EDTA (0.25%) treatment to remove them from the wells, cells were irradiated and washed. Cells were plated in 48-well plates (2 x 10^6 cells/ml) and incubated for 24h to form a confluent monolayer. Feeder layer cells were stimulated after this incubation with either maxadilan (4 ng/ml) or culture medium only for three days. Bone marrow cells (2 x 10^6 cells/ml) then were seeded on the feeder layer. In some cases, αIL-6 (5 μg/ml) was added to the cultures. An isotype-matched control antibody (αβgal) was added at the same concentration to control cultures.

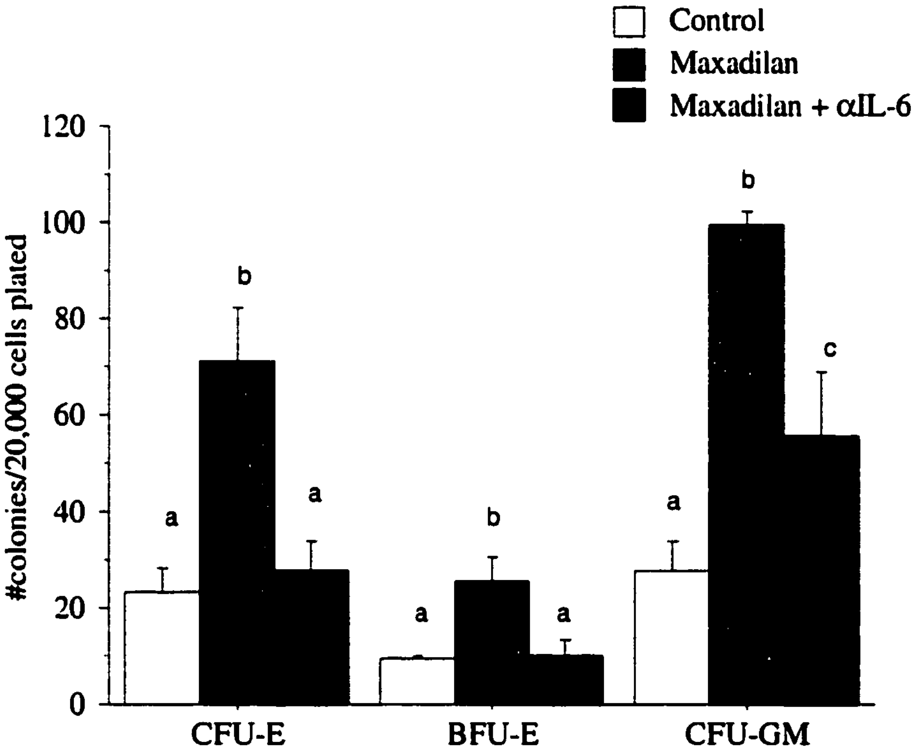

Figure 3.7: Effect of an antibody directed against IL-6 (αIL-6) on bone marrow response to maxadilan in BALB/c mice

Bone marrow cells were plated with or without αIL-6 on irradiated bone marrow stromal cells that had been stimulated with or without maxadilan. After three days, colony progenitors assay were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a, b and c) denote significant difference between treatment groups (p value < 0.05).

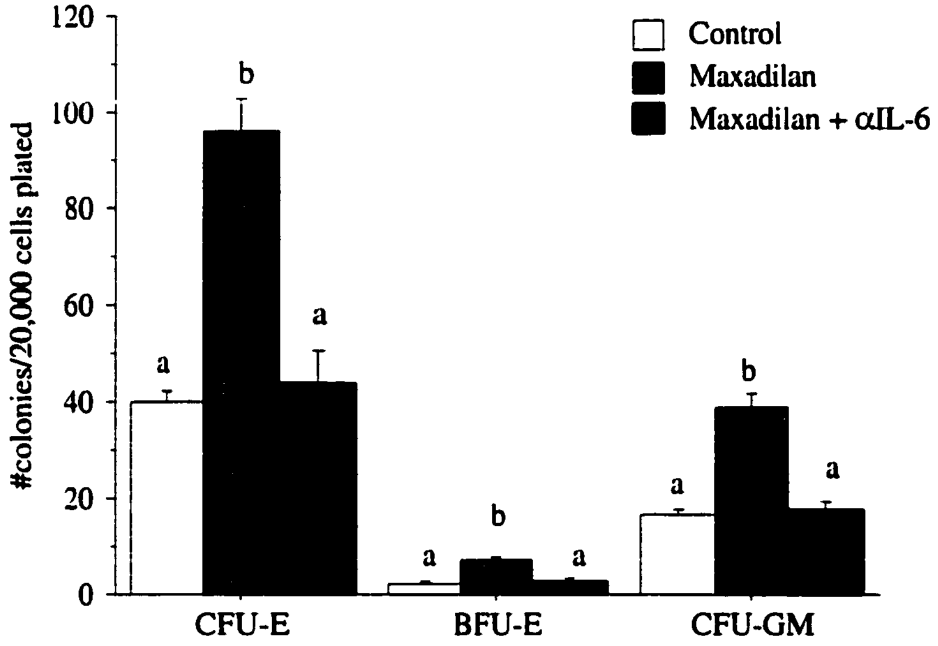

Figure 3.8: Effect of an antibody directed against IL-6 (αIL-6) on bone marrow response to maxadilan in CBA mice

Bone marrow cells were plated with or without αIL-6 on irradiated bone marrow stromal cells that had been stimulated with or without maxadilan. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

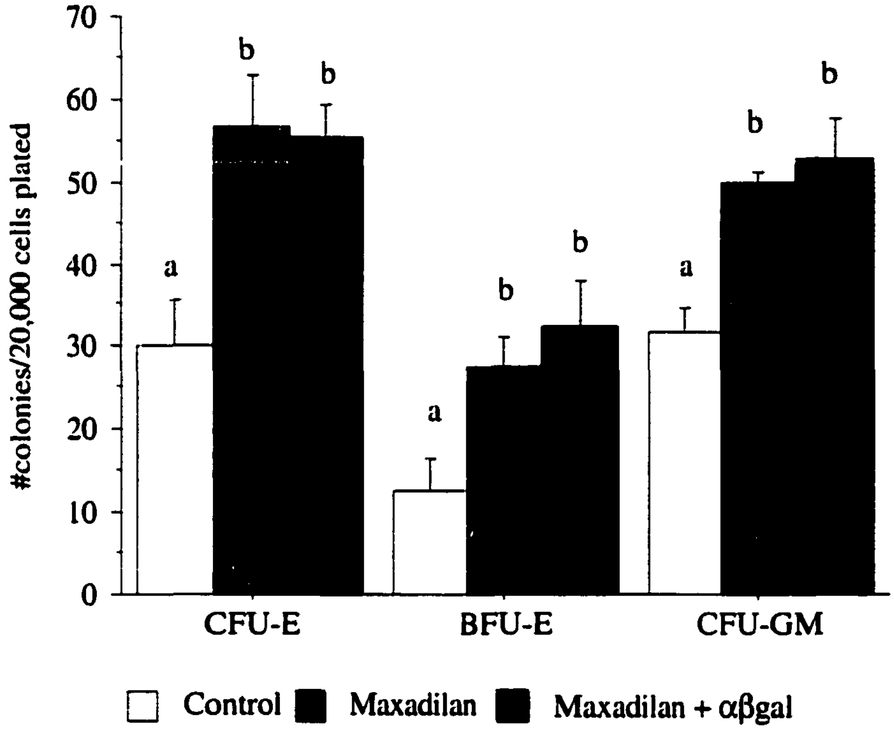

Figure 3.9: Effect of a control antibody specific for β-galactosidose (αβgal) on the bone marrow response to maxadilan in BALB/c mice

Bone marrow cells were plated with or without αβgal on irradiated bone marrow stromal cells that had been stimulated with or without maxadilan. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

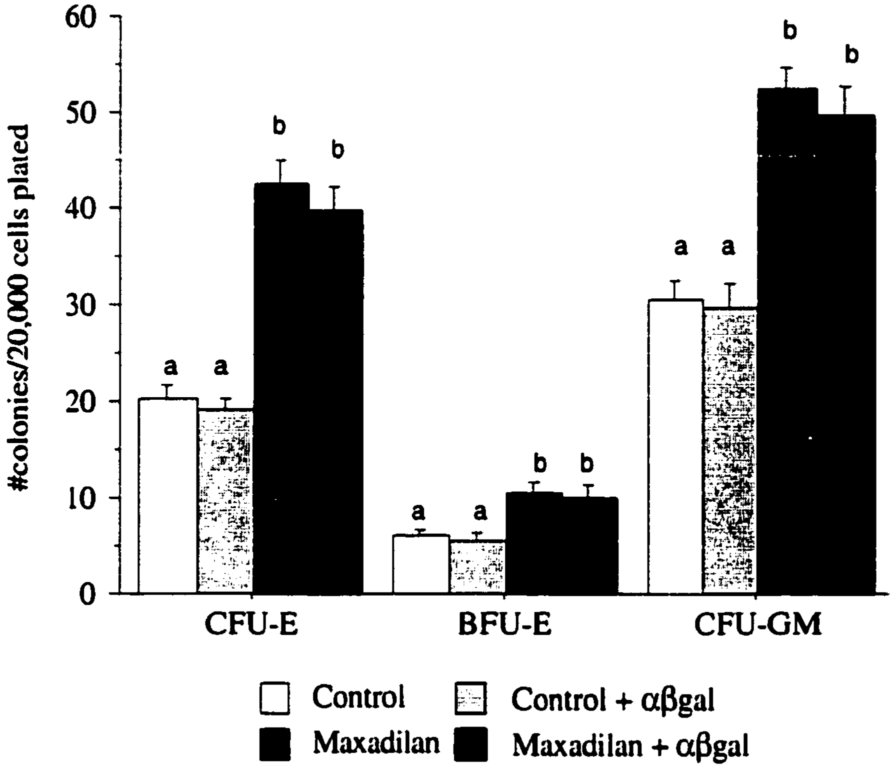

Figure 3.10: Effect of a control antibody specific for β-galactosidose (αβgal) on the bone marrow response to maxadilan in CBA mice

Bone marrow cells were plated with or without αβgal on irradiated bone marrow stromal cells that had been stimulated with or without maxadilan. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

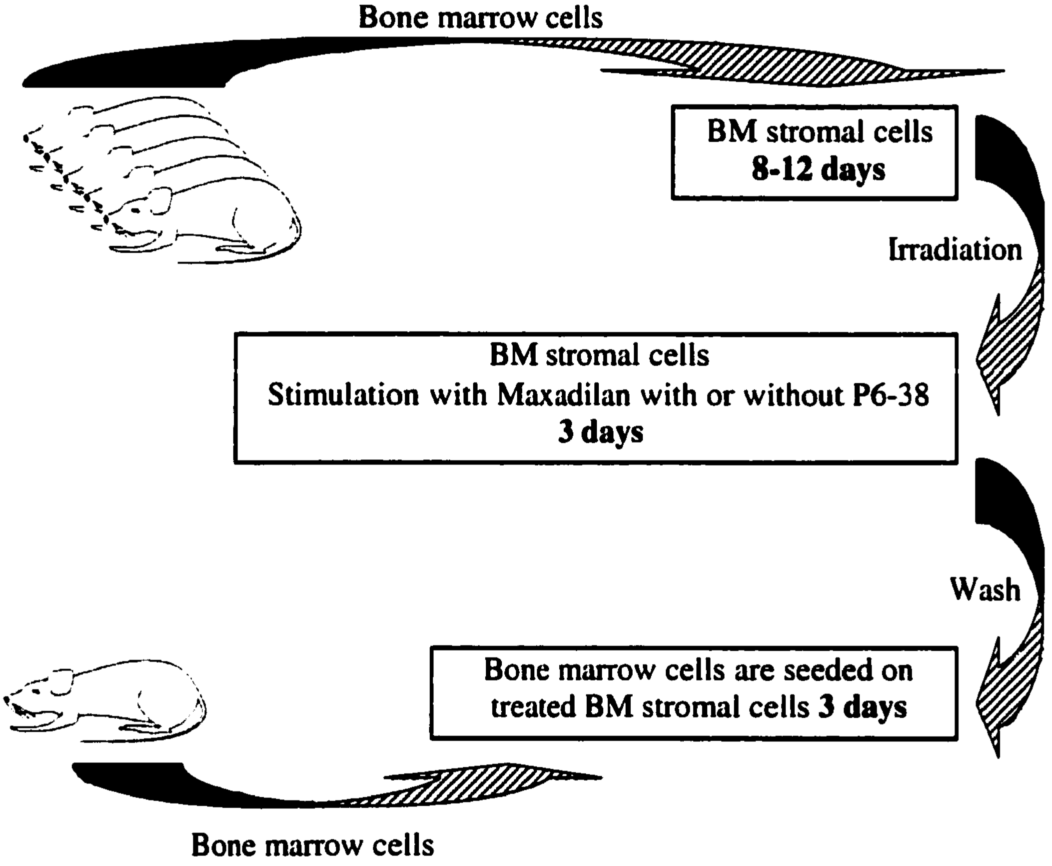

Figure 3.11: Effect of PACAP inhibitor P6-38 on maxadilan treated-bone marrow derived-stromal cells

Bone marrow cells obtained from either BALB/c or CBA mice were brought 2 x 10^6 cells/ml. Cells were plated in 48-well plates and cultured for 8 to 12 days. After a trypsin-EDTA (0.25%) treatment to remove them from the wells, cells were irradiated and washed. Cells were plated in 48-well plates (2 x 10^6 cells/ml) and incubated for 24h to form a confluent monolayer. Feeder layer cells were stimulated after this incubation with either maxadilan (4 ng/ml), maxadilan with P6-38 (200 ng/ml), culture medium only (control) or culture medium and P6-38 in the case of CBA for three days. Bone marrow cells (2 x 10^6 cells/ml) then were seeded on the feeder layer. Three days later, clonogenic progenitor assays were performed.

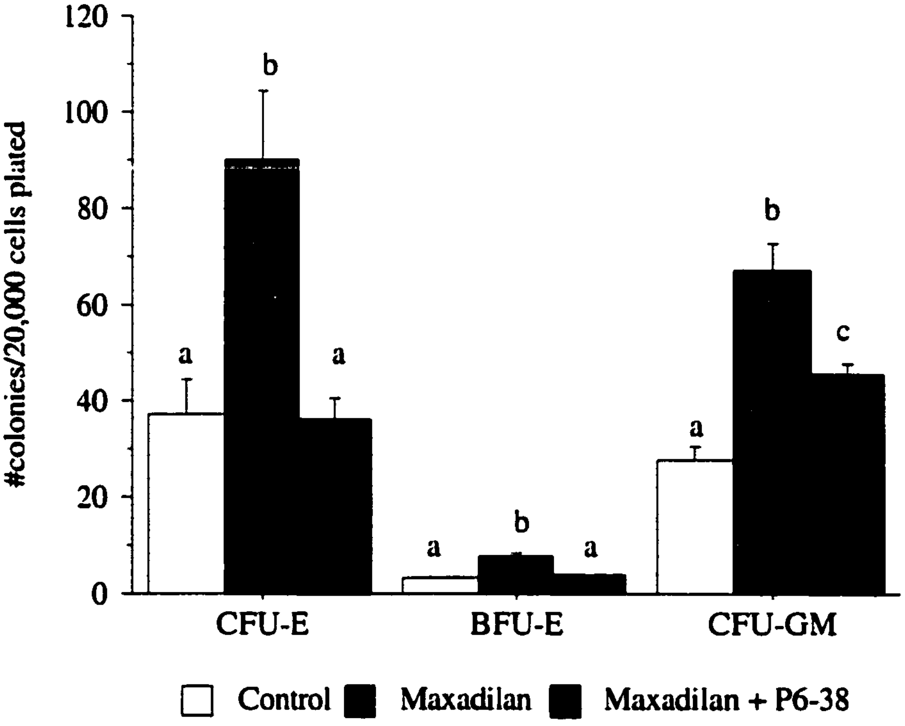

Figure 3.12: Effect of a PACAP receptor inhibitor (P6-38) on the bone marrow the response to maxadilan in BALB/c mice

Bone marrow cells were plated with or without P6-38 on irradiated bone marrow stromal cells that had been stimulated with or without maxadilan. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a, b and c) denote significant difference between treatment groups (p value < 0.05).

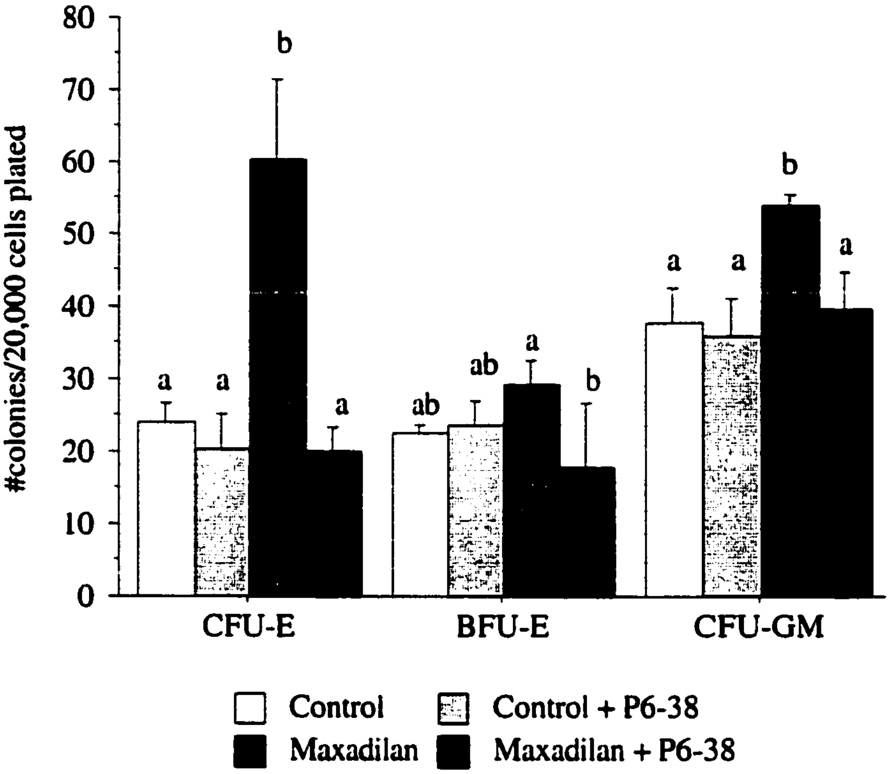

Figure 3.13: Effect of a PACAP receptor inhibitor (P6-38) on the bone marrow response to maxadilan in CBA mice

Bone marrow cells were plated with or without P6-38 on irradiated bone marrow stromal cells that had been stimulated with or without maxadilan. After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

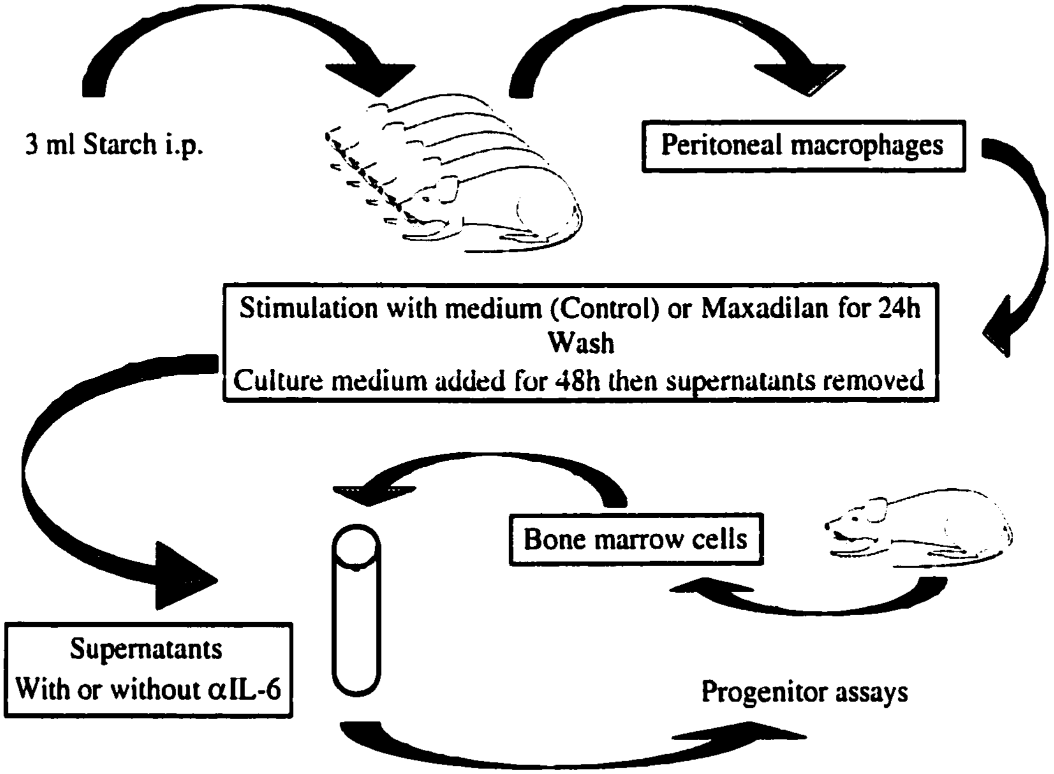

Figure 3.14: Effect of cell-free maxadilan-treated macrophage supernatants on bone marrow progenitors

Mice were injected i.p. with 3 ml of a sterile starch solution. Four days later, macrophages were harvested, washed and plated in 24-well plates at 3 x 10^6 cells/ml. Macrophages were to allowed to attach to the bottom of the wells for 24h. Non-adherent cells were removed and macrophages were treated for 24h with either maxadilan (4 ng/ml) or culture medium alone, the latter being used as control. Supernatants were removed, wells were washed, and fresh medium was added to macrophage cultures for an additional 48h. The supernatants were incubated for 2h on ice with bone marrow hematopoietic progenitor cells from four mice (each incubated and cultured separately) with or without addition of anti-IL-6 (αIL-6) (5 μg/ml). After incubation, colony progenitor assays were performed.

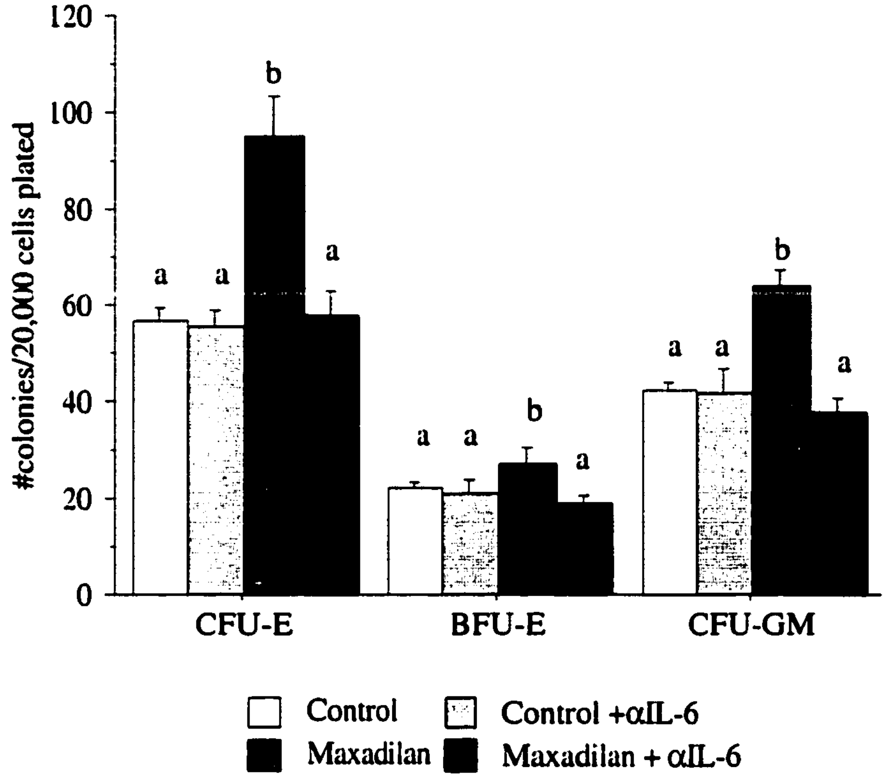

Figure 3.15: Effect of cell-free supernatants from macrophages stimulated with maxadilan in BALB/c mice

Cell-free supernatants from macrophages stimulated with or without maxadilan were incubated for 2h with bone marrow cells with or without anti-IL-6 antibody (αIL-6). After incubation, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

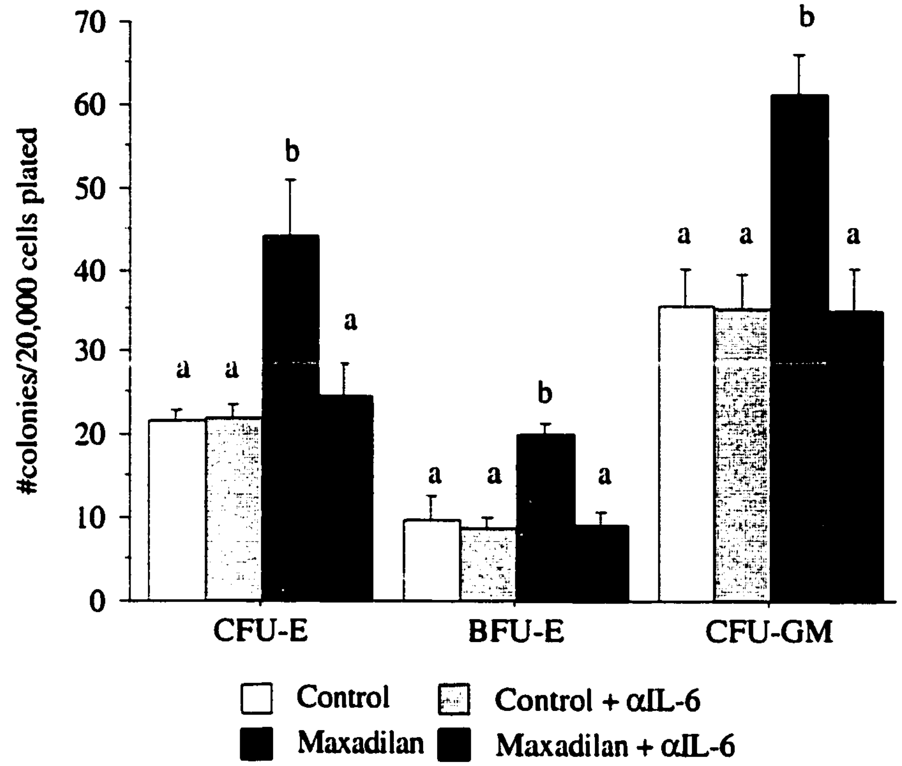

Figure 3.16: Effect of cell-free supernatant from macrophages stimulated with maxadilan in CBA mice

Cell-free supernatants from macrophages stimulated with or without maxadilan were incubated for 2h with bone marrow cells with or without anti-IL-6 antibody (αIL-6). After incubation, colony progenitor assays were performed as described in Material and Methods Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b)denote significant difference between treatment groups (p value < 0.05).

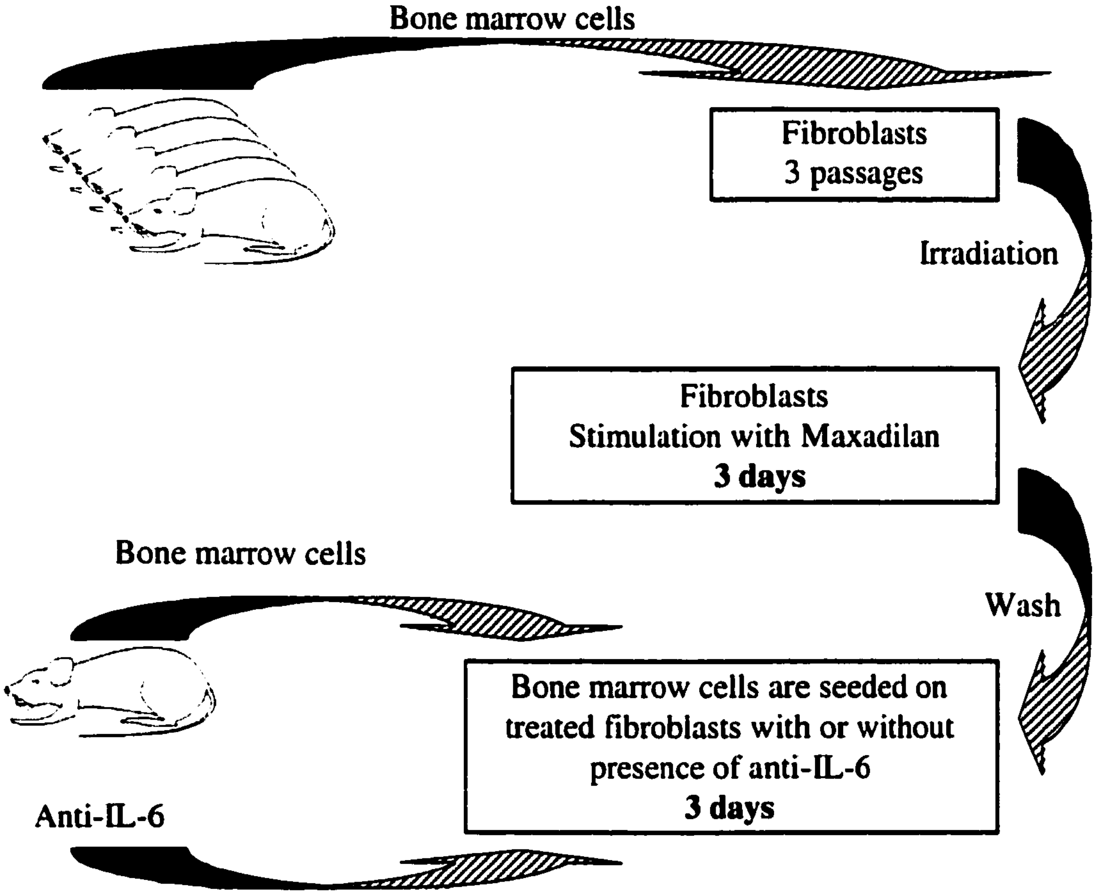

Figure 3.17: Effect of maxadilan on hematopoiesis supported by bone marrow-derived fibroblasts

BM-derived fibroblast cultures were initiated from bone marrow cells isolated from five mice. Cells were pooled first. Cultures were fed by medium replacement every other day and underwent three passages before being used. BM-derived fibroblasts were and plated in 48-well plates. After 24h incubation, BM-derived fibroblasts were stimulated with either maxadilan at a concentration of 4 ng/ml in Myelocult® or Myelocult® only for three days at 37°C. Supernatants were removed and wells were rinsed once with MEM-α. Bone marrow cells then were seeded on the feeder layer at 2 x 10^6 cells/well. In some cases, anti-IL-6 was added to the cultures.

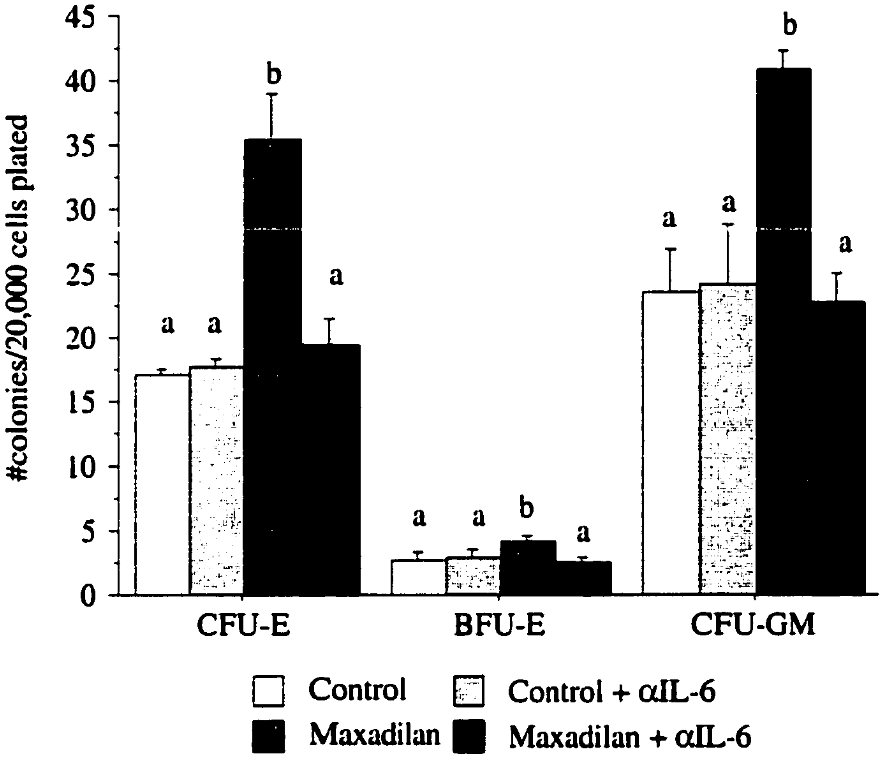

Figure 3.18: Effect of maxadilan on hematopoiesis supported by bone marrow-derived fibroblasts in BALB/c mice

Irradiated bone marrow-derived fibroblasts were stimulated for three days with maxadilan and then seeded with bone marrow cells with or without anti-IL-6 antibody (αIL-6). After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

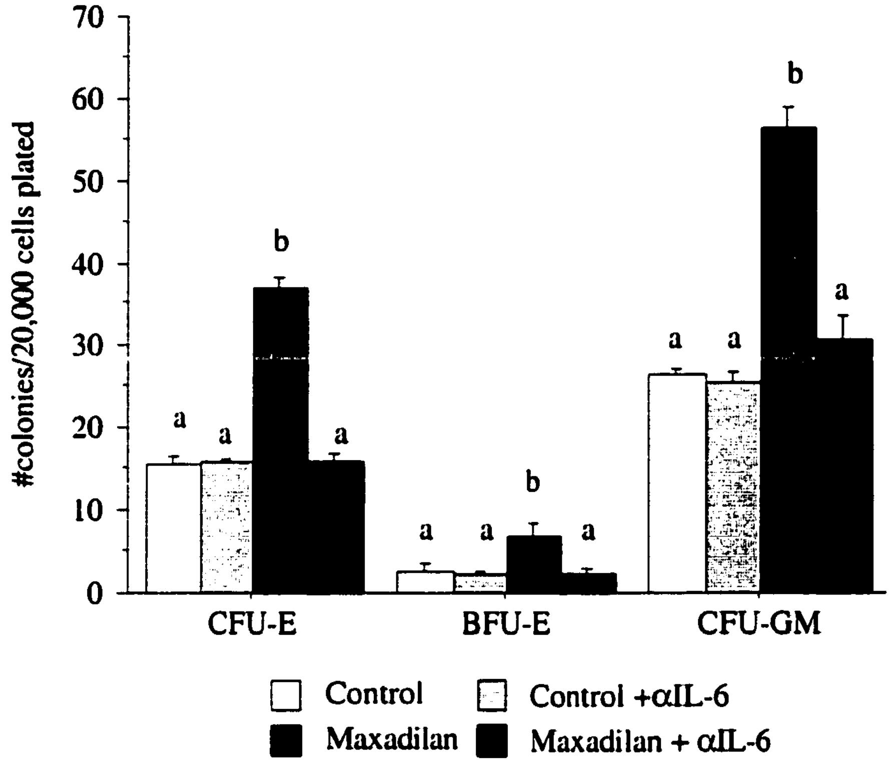

Figure 3.19: Effect of maxadilan on hematopoiesis supported by bone marrow-derived fibroblasts in CBA mice

Irradiated bone marrow-derived fibroblasts were stimulated for three days with maxadilan and then seeded with bone marrow cells with or without anti-IL-6 antibody (αIL-6). After three days, colony progenitor assays were performed as described in Material and Methods. Results are expressed as numbers of colonies per 20,000 cells plated. Values represent means ± SD (bar). Different letters (a and b) denote significant difference between treatment groups (p value < 0.05).

Discussion

We were interested in studying how maxadilan was able to stimulate hematopoiesis. Given its action on IL-6 production and the fact that PACAP receptors have been identified on stromal bone marrow cells of rats, it was reasonable to assume that maxadilan will act to stimulate hematopoiesis through stimulation of IL-6 secretion and that bone marrow stromal cells will be its primary targets.

We used long-term bone marrow cultures (LTBMC) to assess the effect of IL-6 on maxadilan-treated cultures. LTBMC is an *in vitro* system used to study hematopoiesis and it most closely resembles the *in vivo* environment. Hematopoiesis in this system is dependent upon formation of a confluent stromal layer that will provide cell to cell interactions and adequate growth factors to sustain renewal, proliferation and differentiation of early progenitors (18). Four weeks are needed for the system to be established. At that time, the stromal layer, composed mainly of fibroblasts, macrophages and adipocytes, reaches confluence (19) and the committed progenitors that were present at the initiation of the culture have completed their differentiation. Newly formed committed progenitors originate from early progenitors that are sustained by the stromal layer. This system is more efficient at sustaining the formation of myeloid progenitors (14, 20). Erythroid progenitors do not differentiate beyond BFU-E due to the lack of erythropoietin (EPO) production by the stromal layer and the strict requirement for EPO of erythroid progenitors after the BFU-E stage (21, 22). After stimulation with maxadilan, both early erythroid and myeloid progenitors were increased, which was consistent with the effect of maxadilan seen *in vivo*. When anti-IL-6 was added to maxadilan-treated LTBMC, the stimulatory effect of maxadilan on hematopoiesis was eliminated, suggesting a major role of IL-6 in mediating maxadilan action on hematopoiesis. Increased levels of IL-6 in maxadilan-treated culture further supported these conclusions.

LTBMC are useful model to study hematopoiesis, however, it is difficult to assess which cell population, whether bone marrow stromal cells or hematopoietic progenitors, is targeted by maxadilan action. To remedy this problem and study the effect of maxadilan on stromal bone marrow cells, we used a feeder layer-derived assay. In this *in vitro* system, irradiated bone marrow stromal layers, which are composed of bone marrow-derived fibroblasts, adipocytes, macrophages and a few endothelial cells, provide the essential interaction to sustain adequate hematopoeisis. These cells also maintain their ability to produce cytokines but have lost heir ability to divide (23, 24). Thus, only the bone marrow progenitors that are seeded on this stromal layers are evaluated during clonogenic progenitor assays. Feeder layers were treated for three days with maxadilan. When cells from whole bone marrow (mainly hematopoietic cells with accessory cells mixed within are added to these stromal layers, clusters of hematopoietic progenitors can be seen after a few days. By incubating bone marrow cells for three days on feeder layers, we evaluated mainly lineage-committed progenitors (25). Maxadilan-treated bone marrow-derived stromal cells were able to stimulate hematopoeisis, indicating that maxadilan was acting on bone marrow stromal cells to induce up-regulation of hematopoiesis. This stimulation of hematopoiesis seems to be mainly mediated through stimulation of IL-6 production by maxadilan-treated stromal cells, since when anti IL-6 was added with bone marrow progenitors, hematopoiesis in maxadilan-treated feeder layers was similar to control cultures. Co-treatment of feeder layers with P6-38, a potent PACAP antagonist (26), and maxadilan abolished the stimulatory effect of maxadilan treatment on hematopoiesis, suggesting that binding to PACAP receptors was necessary for maxadilan to exert its effect on IL-6 production and stimulation of hematopoiesis. We were not able, however, to detect IL-

6 by ELISA in these feeder layers treated with or without maxadilan and/or P6-38. Similar experiments using rat bone marrow-derived stromal layers, showed that PACAP itself was able to stimulate IL-6 production by bone marrow stromal cells. In that study, IL-6 levels were determined by using bioassays. Those authors also identified PACAP receptor synthesis in bone marrow, suggesting a regulatory role of PACAP on hematopoiesis (13). In our studies, P6-38 addition to maxadilan-treated culture had a partial effect on CFU-GM but only in BALB/c mice; stromal bone marrow layers treated with maxadilan were still able to slightly stimulate myeloid progenitors. It is possible that P6-38 was not enough in excess to block all PACAP receptors present. Moreover, it is also possible that maxadilan modulates cytokine production other than IL-6 or adhesion molecule expression by stromal cells. Maxadilan has been shown to down-regulate TNF-α production by macrophages (9). TNF-α is a potent inhibitor of hematopoiesis inducing apoptosis of hematopoietic progenitors produced by endothelial cells and macrophages, both elements of the bone marrow stroma (27, 19). Thus, down-regulation of TNF-α may account for the partial effect of anti-IL-6 on CFU-GM in maxadilan-treated feeder layer in BALB/c; however, CFU-E and BFU-E should be affected as well if not even more. Technical errors due to variation in counting of CFU-GM or variation of the feeder layer in its ability to support hematopoiesis ("plate effect") cannot be ruled out.

Macrophages and fibroblasts are major components of the bone marrow stroma, both *in vivo* and *in vitro* (28), and both are potential targets for maxadilan. Supernatants from maxadilan-treated macrophages were able to stimulate CFU-E, BFU-E and CFU-GM formation after incubation with bone marrow progenitors, implying a role for macrophages in stimulating hematopoiesis. This stimulation was dependent upon IL-6 production since anti-IL-6 eliminated the stimulatory effect of maxadilan-treated macrophage supernatants. Bone marrow-derived fibroblasts were able to support hematopoiesis, and pre-treatment with maxadilan lead to an increase in colony formation of both erythroid and myeloid lineage. As with macrophages, IL-6 appeared to have a major role in mediating the effect of maxadilan.

These latter results may shed some light on the effect of maxadilan incubation with bone marrow cells we observed in Chapter 2. Maxadilan may have interacted with resident macrophages and fibroblasts present in the cell suspension rather than directly with hematopoietic progenitors. A direct action of maxadilan on progenitors cannot be ruled out, however, and further evaluation is warranted. From our results, it appears that maxadilan is able to act on the bone marrow microenvironment, more precisely at least on fibroblasts and macrophages, through binding to PACAP receptors and stimulation of IL-6 production. A possible effect of maxadilan on other cytokines and possible adhesion molecules as well as other components of the bone marrow stroma requires further study. It is interesting to note that maxadilan increases hematopoiesis, which has been associated with susceptibility to the disease. It appears that maxadilan favors infection by providing the necessary cells that will host the parasite.

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Discussion

Numerous cell types are involved in the response to *L. major* infection: macrophages where the parasite is going to reside, dendritic and natural killer (NK) cells in the early stages of infection, and T cells and B cells as effectors of the acquired immunity (1). These cells communicate with each other through secretion of molecules such as cytokines and chemokines, and up- and down-regulation of surface markers (2). A local phenomenon quickly becomes a more systemic one with the recruitment of lymphocytes and white blood cells, and the production of cytokines with systemic effects such as TNF-α or IL-6 (3). Most of the cytokines produced during an immune response will act on the hematopoietic system. More than 50 cytokines have been shown to affect hematopoiesis (4). Hematopoietic homeostasis relies on complex interactions between inhibitory and stimulatory signals. Some of these signals are given by cytokines that can act directly on hematopoietic progenitors to stimulate their proliferation and differentiation, others target bone marrow stromal cells (5). The bone marrow stromal microenvironment is essential in providing the cell to cell interactions necessary to sustain renewal and differentiation of early progenitors as well as to induce maturation of the lineage-committed progenitors and the release of mature cells (6). Stromal bone marrow cells control hematopoiesis not only through the adhesion molecules and surface markers they express, but also through the synthesis of the extracellular matrix, cytokines, and neuropeptides (5, 7). Disruption of one element of this equilibrium will affect hematopoiesis toward proliferation or inhibition.

Myeloproliferative disorders and myelodysplastic syndromes provide numerous examples of the complexity of hematopoiesis (8-11). Moreover, cytokines have different effects on hematopoiesis depending not only on their levels, but also on the other cytokines present at that time within the microenvironment. TNF-α is the perfect example of this type of cytokine. At low levels, it will act in synergy with stimulatory molecules such as GM-CSF, IL-3, or IL-6 to stimulate hematopoiesis (12). At higher levels, it will either act in synergy with other inhibitory molecules such as IFN-γ (13) or block either the inhibitory effect of others such as TGF-β (14) or the stimulatory effect of cytokines such as G-CSF or erythropoietin (12).

Increased hematopoiesis and decreased macrophage maturation have been associated with susceptibility to *L. major* (15, 16). As others shown (15, 17), we found that in susceptible mice hematopoiesis was increased in response to infection. Resistant mice, however, had the complete opposite response with the inhibition of hematopoiesis. The underlying mechanism appears to reside within the cytokines produced during the immune response to parasite infection. In the early stages of infection, macrophages and NK cells are essential in sending the signals that will decide the outcome of infection. Upon infection, macrophages will produce cytokines such as TNF-α or TGF-β, and interact with NK cells that will produce IFN-γ. TGF-β will in turn activate CD4$^+$ T cell production of IL-4. A cascade of events is started and depending on the host genetic background, type and levels of cytokines produced, and surface molecules expressed, infection will resolve or progress (2). Early in the infection, the cytokines produced in BALB/c mice are favorable to bone marrow stimulation. We showed that IL-4 was mainly responsible for the stimulation of hematopoiesis observed in response to *L. major* infection in BALB/c mice. IL-4 is able to act both directly on hematopoietic progenitors and on bone marrow stromal cells, as was the case in our system. Indeed, IL-4 has been shown to stimulate IL-6 production by stromal bone marrow cells (18-20). IL-6 has a potent stimulatory effect on hematopoiesis and is essential for the development of myeloid and erythroid progenitors (21). On the other hand, CBA mice produce IFN-γ, TNF-α, and nitric oxide early on in response to *L. major* infection, and all of these molecules are potent inhibitors of hematopoiesis (13, 22, 23). We found that TNF-α was involved in the down-regulation of hematopoiesis that we observed in response to parasite infection of CBA mice.

One of the elements of *L. major* infection, whose effect is not negligible at least in the early stages of infection, is the role of the sand fly vector saliva. Numerous studies have shown its ability to enhance infection and saliva effect on macrophages (27, 30). We were particularly interested in maxadilan, a vasodilatory peptide isolated from saliva of the sand fly *Lutzomyia longipalpis* (31). This peptide acts on pituitary adenylate cyclase-activating peptide (PACAP) receptors present on macrophages and bone marrow stromal cells as well as on numerous other cell types. Maxadilan is able to stimulate the production of IL-6, an important molecule in hematopoiesis (21, 32, 33). Indeed, we found that maxadilan was able to stimulate hematopoiesis in mice through its action on PACAP receptors, targeting bone marrow stromal cells, macrophages, and bone marrow-derived fibroblasts, and potentially hematopoietic progenitors themselves. Maxadilan mimics somehow the effect of PACAP itself. PACAP has a dual role in controlling the immune response. PACAP inhibits proliferation and mobility of T cells (34, 35), whereas it will activate resting macrophages or macrophages receiving a weak activation signal and increase their mobility (36). It increases IL-6 production and as well as phagocytosis in resting macrophages and on the other hand increases and inhibits IL-10 and TNF-α production in activated macrophages respectively, thus down-regulating macrophage functions (37, 38). Maxadilan has been shown to induce IL-6 and IL-10 production and inhibit TNF-α synthesis in macrophages. Thus an action of this peptide on macrophage and lymphocyte mobility is not unlikely (39, 40).

Maxadilan may act at different levels to enhance *Leishmania* infection. Limited numbers of parasites are inoculated during the sand fly blood meal, which create a stimulatory signal of low amplitude on the immune system. Without the presence of saliva, parasites are cleared and disease does not progress even in susceptible animals such as BALB/c mice (28, 41). Maxadilan might be the catalyst necessary to allow parasites to establish infection. By enhancing phagocytosis, parasite exposure to the toxic factors of complement is limited, thus favoring survival of the parasites. Then, by activating IL-6 production, it may stimulate hematopoiesis and production of new targets for the parasite. Moreover, by possibly acting on macrophage mobility, maxadilan might favor arrival and accumulation of macrophages while delaying T cell arrival and the instigation of acquired immune mechanisms which are necessary to clear the parasite. Subsequently, when macrophage activation and proliferation can become detrimental to parasite survival and the outcome of the infection, maxadilan may down-regulate macrophage functions by stimulating IL-10 production and inhibiting TNF-α production. Macrophages, therefore, will not be as efficient in killing the parasites that infect them and infection progresses farther than it might without the presence of this salivary component. This scenario is not unlikely given the effect of PACAP on down-regulation of IL-12 and NO production (42, 43). These neuropeptides also affect the expression of B7 molecules and appear to support development of a Th2 immune response (44, 45). The enhancing role of maxadilan in *L. major* might be more extensive than expected.

This scenario involves a molecule that mimics the functions of an endogenous neuropeptide in the control of immune response and hematopoiesis. More work is needed to elucidate the complex interactions between neuroendocrine, immune and hematopoietic systems, which seem to function as whole rather than distinct and independent systems.

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