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Stimulation of B-cell lymphopoiesis by interleukin-7 leads to aggravation of murine leishmaniasis

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SUMMARY

The effect of recombinant interleukin-7 (IL-7) on the clinical course of murine leishmaniasis and the development of the accompaning immune response was investigated. Previously, IL-7 has been shown to possess stimulatory capacity for different cell types of the immune and haematopoietic system critically involved in the defence against *Leishmania major* (*L. major*), such as macrophages which are activated for the elimination of the parasite by IL-7. In contrast to these *in vitro* data, the present study indicates that treatment of genetically susceptible BALB/c mice with IL-7 at the onset of the infection leads to enhanced lesion development and a significantly accelerated death of the animals. This was correlated with a 40-fold increased parasite burden in spleens and lymph nodes. While the specific antibody response against *L. major* was not altered and lymphocytes of IL-7-treated mice produced comparable amounts of the T-helper type-2 (Th2) cytokines IL-4 and IL-10, less interferon-γ (IFN-γ) was measurable after antigenic stimulation of lymph node and spleen cells *in vitro*. One of the major changes appearing by the first week after infection in IL-7-treated mice was the increase of the total cell number in spleen and lymph nodes draining the local infection. Analysis of the cellular composition revealed that the enhanced cellularity was predominantly due to a rise in the B-cell compartement. Since antigen presentation by B cells has been implicated in the development of Th2 cells, the disease-aggravating activity of IL-7 is thought to be primarily due to augmentation of B lymphopoiesis.

INTRODUCTION

Interleukin-7 (IL-7), a bone marrow stromal cell-derived cytokine, was identified and cloned as a soluble molecule with preB-cell growth factor activity.[1,2] In addition to multiple effects on B cells, IL-7 promotes the proliferation of thymocytes[3,4] and mature T cells,[5–7] the generation of alloreactive, anti-tumour, and antiviral cytotoxic T cells (CTL),[8–10] and the proliferation of natural killer (NK) cells.[11] Most important for the present study, IL-7 possesses stimulatory capacity for cells of the monocyte/macrophage compartment. IL-7 promotes the production and secretion of inflammatory cytokines by human peripheral blood monocytes,[12,13] and suppresses the synthesis of transforming growth factor-β (TGF-β), a macrophage deactivating cytokine, by murine macrophages.[14] These stimulatory effects of IL-7 are paralleled by an enhanced monocyte/macrophage tumoricidal activity.[12]

Macrophages are important host cells for intracellular parasites, such as leishmania, in the mammalian host. Recently, we have shown *in vitro* that IL-7 activates the leishmanicidal effector function of murine macrophages.[15] However, so far no studies are available concerning the effects of IL-7 during an anti-infectious immune response *in vivo*. Since several of the described functional activities of IL-7 could potentially have a beneficial influence on the immune response during a parasitic infection, we evaluated the clinical and immunomodulatory effects of recombinant IL-7 in the model of murine cutaneous leishmaniasis.

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MATERIALS AND METHODS

Reagents

Purified recombinant human IL-7 (lipopolysaccharide content: 39 pg/mg protein) was a gift of Sterling Drug Inc. (Malvern, PA) and had a specific bioactivity of $4{\cdot}7 \times 10^7$ U/mg, as determined by a bioassay using the IL-7-dependent I × N/2b preB-cell line. A single dose of IL-7 (5 μg/mouse) was injected subcutaneously into the right hind footpad simultaneously with the infection.

Mice and parasites

Female mice of the inbred strains BALB/c and C57BL/6 were obtained from Charles River Breeding Laboratories (Sulzfeld, Germany), and used at 6–12 weeks of age. *Leishmania major* (*L. major*) promastigotes were grown *in vitro* in blood agar cultures, as described elsewhere.[16] Stationary-phase promastigotes

were washed in phosphate-buffered saline (PBS) and $2{\cdot}5 \times 10^6$ organisms were injected in a volume of 50 μl intradermally into the right hind footpad.

Measurement of lesions and determination of parasite burden
The lesion development was monitored by measuring the thickness of the infected and non-infected contralateral footpad by means of a vernier caliper (Kroeplin, Schlüchtern, Germany) and calculated as described elsewhere (thickness infected foot/thickness control foot).[17] At the indicated time-points of infection, mice were bled and the inguinal lymph nodes and spleens removed. Single-cell suspensions of these organs were prepared and cultivated *in vitro* for 48 hr in the presence of *L. major* antigen. A limiting dilution (LD) *in vitro* culture assay was used to quantify the number of viable *L. major* parasites in spleens and lymph nodes, as described elsewhere.[17] Briefly, serial twofold dilutions of the cell suspensions were plated in 96-well flat-bottomed microtitre plates containing 50 μl blood agar and 100 μl complete medium (Click's RPMI-1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 10 mM HEPES, 100 μg/ml penicillin, 160 μg/ml gentamicin, 13 mM $NaHCO_3$ and 5×10^{-5} M 2-mercaptoethanol). After incubation at 28° for 2 weeks, the wells were assessed for growth of *L. major* promastigotes microscopically. The minimal estimates of the number of viable *L. major* promastigotes were analysed using an ELIDA program (C. Tasswell, Harvard University, Boston, MA) by applying Poisson statistics and the χ^2 minimization method.[18]

In vitro *restimulation of spleen and lymph node cells*
Unseparated spleen and lymph node cells were cultured *in vitro* at a density of 4×10^6/ml or 2×10^6/ml, respectively, in the presence or absence of *L. major* freeze-thawed promastigote antigen (LmAg) (5×10^6 cell equivalents/ml), prepared as described previously.[19] After 48 hr of culture the supernatants were analysed for the presence of cytokines.

Cytokine assays
The concentrations of IL-4, IL-5, IL-10 and interferon-γ (IFN-γ) in supernatants of antigen-stimulated lymph node, or spleen cloned T cells were measured by specific two-site enzyme-linked immunosorbent assays (ELISAs) with reference standard curves using known amounts of the respective murine recombinant cytokines. The antibody pairs for the detection of IL-4 (BVD4–1D11 and biotinylated BVD6-24G2), IL-5 (TRFK 5 and biotinylated TRFK 4) and IL-10 (JES5–2A5 and biotinylated SXC-1) were purchased from Dianova (Hamburg, Germany). IFN-γ was determined using microtitre plates coated with affinity-purified R4-6A2 monoclonal antibody (mAb) and a rabbit anti-mouse IFN-γ antiserum for detection.[20] The IFN-γ ELISA was further developed with swine anti-rabbit antiserum conjugated with peroxidase (Dianova).

Immunofluorescence
Staining of lymph node and spleen cells was performed using the following antibodies: rat anti-mouse CD4 [YTS191.1, fluorescein isothiocyanate (FITC)-conjugated], rat anti-mouse CD8 [YTS 169.4, phycoerythrin (PE)-conjugated] and rat anti-mouse B220 (RAQ3-6B2, FITC-conjugated), all purchased from Medac (Hamburg, Germany). Analysis of cell staining was performed in a FACScan® (Becton Dickinson, Heidelberg, Germany) using the Lysis II software® (Becton Dickinson).

Immunoglobulin determinations
For determination of IgG and IgM serum levels, microtitre plates were coated overnight with 10 μg/ml goat anti-mouse IgG + IgM antibodies (Dianova). After blocking with 5% fetal calf serum (FCS), plates were incubated with individual mouse sera for 2 hr. Peroxidase-labelled goat anti-mouse IgG or IgM antibodies (both from Dianova) were used for detection. ELISAs were developed with 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS; 20 μg/ml) as substrate. Specific anti-leishmanial antibody titres (IgM and IgG) were detected with an ELISA, as described elsewhere.[21]

RESULTS

Aggravation of the clinical course of *L. major* infection in BALB/c mice by IL-7 treatment

In a first series of experiments, IL-7 was given subcutaneously in a single dose of either 1, 5, 10 or 15 μg to BALB/c mice simultaneously with the infection into the right hind footpad. Mice were infected with $2{\cdot}5 \times 10^6$ living *L. major* promastigotes. The footpad swelling following the infection was scored twice a week. Since the effects observed were optimal with a dosage of 5 μg IL-7 and were not enhanced further by applying higher amounts of IL-7 or repeated subcutaneous injection of the cytokine (day 0, 2, 4, and 6 after infection; data not shown), the following experiments were performed with a single dose of 5 μg IL-7. In all of the experiments the increase in footpad thickness of mice receiving IL-7 significantly exceeded that of controls after the first 2–3 weeks of infection and later on (Fig. 1), which was not the case in similarily treated and infected C57BL/6 mice, which are genetically resistant towards *L. major* (data not shown). The accelerated development of local lesions correlated with a reduced survival time of the IL-7-treated BALB/c mice (Fig. 2). Thirteen weeks after infection only one out of eight mice was alive in the IL-7-treated group, while five of the control mice had survived the infection.

Effects of IL-7 treatment on organ cellularity and parasite content of lymph nodes and spleens

The unexpected aggravation of the clinical course of murine leishmaniasis by single-dose IL-7 treatment prompted us to analyse the influence on the parasite load in the organs of the mice. The cell number per organ significantly increased in IL-7-treated and infected mice compared to infected control animals. The increase became measurable on day 6 after infection in lymph nodes and spleens, and was still present on day 70 after infection in the spleens (Table 1). The observed reduction of cell number in lymph nodes of IL-7-treated mice compared to control mice on day 70 of infection was caused by massive necrotic changes in two out of five lymph nodes, reflecting the enhanced progression of the disease after IL-7 treatment. However, while the cell number per organ had maximally doubled in IL-7-treated versus control mice, the parasite burden per organ increased more than 40-fold in spleens and lymph nodes of IL-7-treated mice, as determined 10 weeks after infection (Table 2). These data demonstrate that the massive

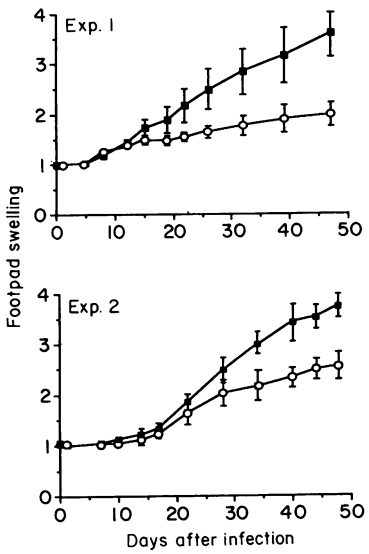

Figure 1. Influence of IL-7 on the clinical course of murine cutaneous leishmaniasis. Female BALB/c mice were infected with $2{\cdot}5 \times 10^6$ *L. major* promastigotes in 50 μl PBS in the right hind footpad, and were treated with a single dose of 5 μg recombinant IL-7 (■) or PBS (○) subcutaneously at the day of infection. Data shown represent the mean values of footpad swelling of six mice per group, calculated as (thickness of infected footpad/thickness of control footpad) (SD of measurements < 0·25, $P = 0{\cdot}07$), of two independent experiments (1 and 2). Later than 7 weeks after infection the lesions of the mice of both groups became necrotic so that no useful measurements could be performed.

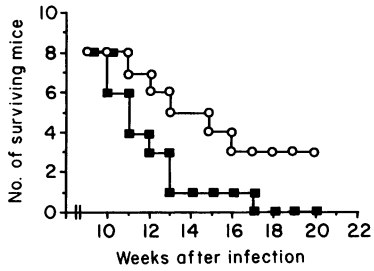

Figure 2. Effect of a single injection of 5 μg IL-7 (■) on survival of BALB/c mice after infection with *L. major*. Mice (eight per group) were infected with *L. major* promastigotes as described in the legend to Fig. 1. The number of surviving animals was assessed daily. Control mice received PBS (○).

parasite load caused by IL-7 was not simply due to the enlargement of lymphoid organs but reflected an impaired ability of host cells to eliminate the parasites.

Predominant increase of B cells in spleens and lymph nodes of *L. major*-infected BALB/c mice after IL-7 treatment

IL-7 was originally described as a growth factor for immature B cells.[1,2] Since there is evidence that the kind of antigen-presenting cell, e.g. B cells versus macrophages or cells of the monocytic lineage, might influence the development of either T-helper type-1 (Th1) or Th2 cells,[22,23,24] it was of interest to determine the influence of IL-7-treatment on the cellular composition in lymphoid organs of *L. major*-infected mice. Cells of spleens and lymph nodes were analysed by immunocytofluorometry for the percentage of B cells, $CD4^+$ and $CD8^+$ T cells. As depicted in Fig. 3, the increase in cellularity of spleens in IL-7-treated mice was predominantly due to the increase of B220-positive B cells, which was similarily found in the lymph nodes (data not shown). The relative decrease of $CD4^+$ and $CD8^+$ T cells during the course of infection was comparable in IL-7-treated and control mice and was most prominent in the spleens. While approximately 25% of the spleen cells were T cells at day 6, only 10–12% belonged to either the $CD4^+$ or $CD8^+$ subset 10 weeks after infection in both groups of mice.

As a marker for the function of B cells *in vivo*, we measured the *L. major*-specific antibody titres as well as the total concentrations of IgG and IgM in the sera of the respective mice. As summarized in Table 3, 6 days after infection there was no significant difference between sera of IL-7-treated and control mice. In sera of both groups of mice there was a marked increase of IgM as well as IgG concentrations when measured 70 days after infection, reflecting the previously documented polyclonal B-cell activation occuring during progression of leishmaniasis.[25,26] The enhanced IgG content in sera of IL-7-treated mice correlated with the increased number of B cells (Fig. 3).

Table 1. Influence of IL-7 treatment on cell number per organ*

Time after infection	Cells/spleen‡	% increase§	Cells/lymph node‡	% increase§
Day 6				
Control	$8{\cdot}0 \times 10^7 \pm 0{\cdot}5 \times 10^7$	—	$2{\cdot}11 \times 10^6 \pm 0{\cdot}2 \times 10^6$	—
IL-7†	$10{\cdot}7 \times 10^7 \pm 0{\cdot}8 \times 10^7$	33·8	$2{\cdot}7 \times 10^6 \pm 0{\cdot}3 \times 10^6$	28·0
Day 70				
Control	$21{\cdot}1 \times 10^7 \pm 2{\cdot}4 \times 10^7$	—	$12{\cdot}6 \times 10^6 \pm 1{\cdot}2 \times 10^6$	—
IL-7†	$46{\cdot}3 \times 10^7 \pm 4{\cdot}7 \times 10^7$	119·4	$10{\cdot}8 \times 10^6 \pm 3{\cdot}6 \times 10^6$	<

* Mice were infected with $2{\cdot}5 \times 10^6$ *L. major* promastigotes into the right hind footpad. Lymph nodes and spleens were harvested at the times indicated.

† Mice received 5 μg IL-7 subcutaneously simultaneously with the infection.

‡ Each value represents the mean of five mice ± standard deviation.

§ Percent increase of total cell numbers per organ in IL-7-treated versus control mice.

Table 2. Influence of IL-7 treatment on parasite load in organs of *L. major* infected BALB/c mice*

	Control	IL-7 treated†
Spleen		
Frequency‡	1/217	1/11·5
Total no. of parasites§	$9{\cdot}9 \times 10^5 \pm 3{\cdot}4 \times 10^5$	$403{\cdot}2 \times 10^5 \pm 36 \times 10^5$
Fold increase¶	—	41·7
Lymph node		
Frequency‡	1/119	1/2
Total no. of parasites§	$1{\cdot}06 \times 10^5 \pm 0{\cdot}5 \times 10^5$	$49{\cdot}7 \times 10^5 \pm 9{\cdot}8 \times 10^5$
Fold increase¶	—	46·8

* Mice were infected as described in the legend to Table 1. Lymph nodes and spleens were analysed at 10 weeks after infection. A limiting dilution (LD) *in vitro* culture assay was used to quantify the number of viable *L. major* parasites in organs of mice.

† Mice received 5 μg IL-7 subcutaneously simultaneously with the infection.

‡ Each value represents the mean of five mice ± standard deviation.

§ Total number of parasites per organ was calculated as (number of cells per organ) × frequency.

¶ Fold increase of parasite numbers per organ in IL-7-treated versus control mice.

Influence of IL-7 treatment on the *L. major*-induced synthesis of Th1 and Th2 cytokines

The production of either Th1 or Th2 cytokines appears to be decisive for the clinical outcome of murine cutaneous leishmaniasis. The expression of the Th2 cytokines IL-4 and IL-10 has been shown to be associated with disease progression, while IFN-γ is important for cure and elimination of the parasite *L. major*.[25,26] To determine the expression of IL-4, IL-5 and IL-10 of parasite-induced Th2 cells in relation to that of IFN-γ, a characteristic lymphokine of Th1 cells, lymph node and spleen cells were stimulated with the *L. major* antigen (LmAg) *in vitro*. As depicted in Fig. 4, early after infection (day 6) there was no difference in cytokine synthesis between IL-7-treated and control mice. In contrast, 10 weeks after infection the amounts of IFN-γ produced were significantly lower in supernatants of cells from IL-7-treated BALB/c mice. Thus, the reduced expression of the Th1 lymphokine IFN-γ correlated with the aggravated clinical course and the enhanced parasite load as a consequence of IL-7 treatment. The exact mechanism(s) causing the reduced concentrations of cytokines in supernatants of stimulated cells on day 70 compared to those of day 6 after infection are not known. Since we are not aware of any study analysing the cytokine synthesis in BALB/c mice as late as 10 weeks after infection with *L. major*, these findings cannot be directly compared with previous reports. Possible explanations for our observations might be the reduced percentage of $CD4^+$ T cells in the bulk cultures during restimulation (Fig. 3) and/or the exhaustion or suppression of cytokine-producing cells in the state of full-blown disease.

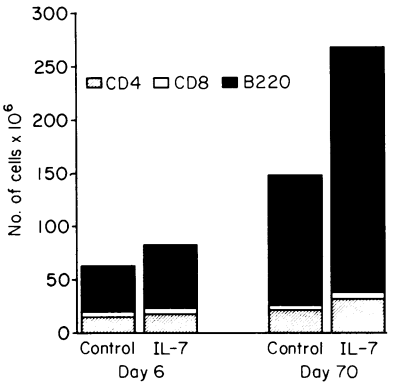

Figure 3. Predominant expansion of B cells in spleens of BALB/c mice after IL-7 treatment. Mice were infected and treated with IL-7 as described in the legend to Fig. 1. The data are the average phenotypic composition of spleens of IL-7-treated and control mice (means of five mice/group, SD < 2×10^6 for $CD4^+$ and $CD8^+$ cells and SD < 5×10^6 for $B220^+$ cells). Cell numbers were calculated on the basis of total cell number per organ and the percentage of marker positive cells as determined by cytofluorometric analysis.

Table 3. Influence of IL-7 treatment of immunoglobulin synthesis

Time after infection*	Σ IgM†	Σ IgG†	Specific IgM‡	Specific IgG‡
day 6				
Control	1 ± 0·13	1·7 ± 0·53	1:300	1:900
IL-7 treated	1 ± 0·17	1·8 ± 0·72	1:300	1:900
day 70				
Control	4·6 ± 0·53	8·3 ± 0·2	1:300	1:5400
IL-7 treated	3·1 ± 0·6	11·0 ± 0·25	1:300	1:5400

* Mice were infected and treated with IL-7 as described in the legend to Fig. 1.

† Values are given in mg/ml serum, mean of five mice ± standard deviation.

‡ Titres specific for total *L. major* antigen measured by ELISA.

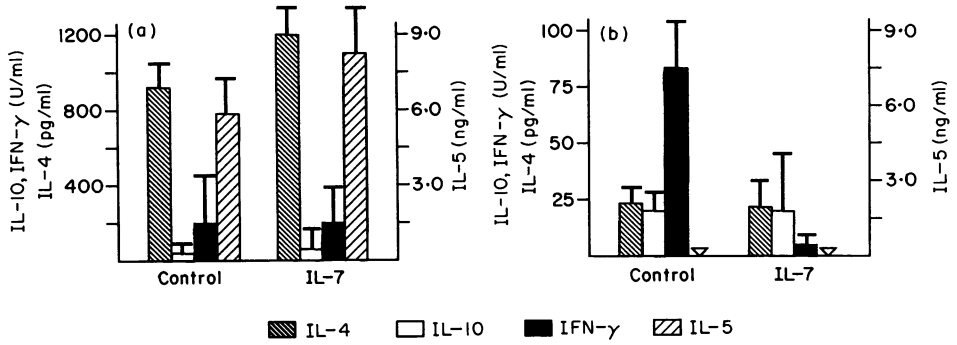

Figure 4. Effect of IL-7 treatment on LmAg-specific cytokine synthesis in BALB/c mice 6 days and 10 weeks after infection. BALB/c mice were infected as described in the legend to Fig. 1 and treated once with recombinant IL-7 or PBS (control). At days 6 and 70 after infection spleen cells were cultivated for 48 hr in the presence of LmAg (5×10^6 cell equivalents/ml). Concentrations of IL-4, IL-5, IL-10 and IFN-γ in the supernatants were measured by ELISA. Each value represents the mean of five mice. No cytokines could be detected if the cells were cultivated in the absence of LmAg in medium alone.

DISCUSSION

The potential role of IL-7 as an immunoregulatory cytokine during an anti-parasitic immune response *in vivo* was investigated in this study. The rationale for this approach was the idea that several activities of IL-7, previously demonstrated *in vitro*, might promote protective immune mechanisms *in vivo*. For example, IL-7 has been shown to activate murine macrophages for the destruction of *L. major* parasites, a process in which reactive nitrogen intermediates are critically involved.[15] In lipopolysaccharide (LPS)-stimulated murine macrophages, IL-7 suppresses the synthesis of TGF-β, a factor involved in the escape of leishmania parasites from destruction by the immune system.[27] In addition, IL-7 acts as growth factor for NK cells,[11] which participate in the early resistance against *L. major* by the production of IFN-γ.[28,29] Treatment with recombinant IL-7 during the early phase of infection could therefore be of potential benefit to the host organism, and thus of therapeutic value.

In contrast to the expectation, this study demonstrates that IL-7 treatment *in vivo* aggravated the clinical course of murine cutaneous leishmaniasis in BALB/c mice, paralleled by a massive enhanced parasite load in spleens and lymph nodes. Since IL-7 has no influence on the growth of promastigotes of *L. major*, which has been extensively tested *in vitro*, the possibility that IL-7 directly enhances parasite growth appears to be very unlikely. Furthermore, when mice were infected subcutaneously on the dorsum near the base of the tail, IL-7 also led to enhanced lesion development even when injected into a different site of the dorsum (data not shown). Despite a similar effect of IL-7 treatment on the B-cell compartment in *L. major*-resistant C57BL/6 mice (personal observations).[30] these mice displayed no reversal of their clinical phenotype. Thus, for so far unknown reasons, IL-7 alone does not interfere with the establishment of protective immunity in resistant mice but markedly enhances pathology in susceptible mice.

Previous studies with other cytokines have shown that treatment of BALB/c mice with IFN-γ, tumour necrosis factor-α (TNF-α), and IL-12 led to amelioration of leishmaniasis or induction of protection, while application of granulocyte–macrophage colony-stimulating factor (GM-CSF) resulted in exacerbation of disease (reviewed by refs 25 and 26). In each of these studies the *in vivo* effect of the cytokine correlated well or could be explained by known activities of the respective cytokine *in vitro*. Due to the pleiotropic activities of IL-7 on monocytes and macrophages, as well as on NK cells and T- and B-cell lymphopoiesis, the influence of IL-7 on the immune response was less predictable.

IL-7 was given as a single dose at the day of infection. Therefore, the altered clinical course observed during the following weeks is most likely a consequence of processes initiated during the onset of infection and not caused by the presence of recombinant IL-7 itself. According to the accepted current view, the development of either protective or disease-promoting immunity in murine cutaneous leishmaniasis is determined within the first few days after infection. This view is based on the observation that all known treatments inducing protection in BALB/c mice had to be performed prior to or during the first week after *L. major* infection.[25,26] While several types of cells of the immune system, such as $CD4^+$ T cells, NK cells and Langerhans' cells, have been shown to be critically involved in this induction phase of the anti-parasitic immune response,[25] the role of B cells is not yet well established.

Cell-transfer studies, in which spleen cells from BALB/c mice were injected into *L. major*-infected C.B-17 scid mice, have shown no critical involvement of B cells in the transferred resistance of the recipient mice. Transfer of as few as 10^4 T cells was sufficient to protect the host, while neither enrichment nor depletion of B cells from the transferred T cells had a measurable effect.[31] In contrast to this, B-cell depletion by treatment of newborn BALB/c mice with polyclonal anti IgM has been demonstrated to protect mice against systemic leishmaniasis.[32] In line with the latter studies, BALB.XID mice, which lack B1 cells and display reduced numbers of B2 cells, are more resistant towards *Trypanosoma cruzi*[33] as well as to *L. major* infection[34] when compared to their normal counterparts, also arguing for a disease-promoting role of B cells in murine cutaneous leishmaniasis.

What mechanism(s) could cause aggravation of leishmaniasis by B cells? One possible mechanism resides in the interaction of B cells with $CD4^+$ T cells. In this context it is of interest that antigen presentation by B cells was shown to lead to activation and differentiation of predominant Th2 cells, while other antigen-presenting cells such as macrophages and

dendritic cells promoted the expansion of Th1 cells.[22–24] We therefore analysed the influence of IL-7 on the B-cell compartment in lymphoid organs of *L. major*-infected mice. A significant increase in the total number of B cells (30–40%) was found in lymph nodes as well as in spleens of mice on day 6 after *L. major* infection and IL-7 treatment. This effect was independent of the infection with *L. major*, since it also occurred in uninfected but IL-7-treated BALB/c mice (data not shown). These data are concordant with previous studies showing IL-7 as a cytokine enhancing murine lymphopoiesis[30,35] *in vivo*. Application of IL-7 to normal mice led to a transient twofold increase of $B220^+$ preB and B cells in the spleen and lymph nodes.[30] In these studies, the B-cell numbers declined to normal values within 1 week after the cessation of IL-7 administration. Thus, the early increase in cellularity in *L. major*-infected and IL-7-treated mice is most likely caused directly by IL-7, whereas the effects as late as 10 weeks after infection must be the consequence of altered regulatory processes during the onset of immunity. The early changes induced by IL-7 obviously provoke a subsequent augmentation of the polyclonal B-cell proliferation, which also occurs to a lesser extent during the physiological course of progressive leishmaniasis.[25,26] It remains to be elucidated whether this is simply due to the enlargement of the initial pool of B cells responsive to the proliferative stimuli also present during the infection in the absence of IL-7 or if additional regulatory mechanisms are involved. Considering the well-known B lymphopoietic activity of IL-7 and the results of our study, we propose that the number of B cells confronted with leishmania parasites during the onset of the immune response is critically involved in the determination of immunity and thus the clinical course of disease.

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