EXPERIMENTAL LEISHMANIASIS: THE GLIBENCLAMIDE-TRIGGERED DECREASE IN PARASITE GROWTH CORRELATES WITH CHANGES IN MACROPHAGE FEATURES

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ABSTRACT

Previous studies from our laboratories revealed the susceptibility of *Leishmania sp.* to glibenclamide (GLIB), a potassium channel blocker which selectively interacts with ***a***denosine-***b***inding-***c***assette transporters. In the present work, we analyzed whether the drug sensitivity of intracellular amastigotes correlates with changes in macrophage features that are related to their function as antigen-presenting cells. We provide evidence that in BALB/c murine macrophages, GLIB induced a decrease in the interferon-gamma-stimulated expression of major histocompatibility complex class II molecules and the co-stimulatory molecule CD86 (B7-2). Furthermore, it caused a decrease in the interleukin-1 secretion by macrophages. The data indicate that the treatment with GLIB inhibits the Th2 development and polarizes macrophage functions towards the induction of a protective Th1 response.

INTRODUCTION

Leishmania are intracellular parasites that produce a spectrum of human disease manifestations. The control of these infections requires the activation of macrophages through a Th1-type immune response. Macrophages play a key role in the cellular immune response since they have the capacity to a) present antigens, in association with major histocompatibility complex (MHC) class I and II molecules to T cells, b) provide co-stimulatory signals, via expression of, e.g., CD80 (B7-1) and CD86 (B7-2) molecules for the activation of T cell effector functions and c) modulate the immune response by the production of various cytokines. There is evidence suggesting that differential expression of CD80 and CD86 may affect the development of a Th1 *vs.* a Th2 immune response. The up-regulation of CD86 has been shown to direct naive $CD4^+$ T cells preferentially towards a Th2 phenotype, while the expression of CD80 directs naive $CD4^+$ T cells towards a Th1 phenotype (9). On the other hand, cytokines released by macrophages, play a crucial role in *Leishmania* infections. For example, it has been shown that the levels of interleukin–1 (IL-1) produced by macrophages from *Leishmania*-susceptible BALB/c mice are consistently higher than those from non-infected controls stimulated with bacterial lipophosphosaccaride (LPS) (5, 15).

The parasite cell homeostasis must be maintained in the different physical environments of the *Leishmania* life cycle (extracellular in the vector; intracellular in the mammalian host). Regulatory mechanisms that are fundamental for the development of the promastigote, its transformation into the metacyclic form, its interaction with the host cell and its establishment inside the phagosome need to be activated by triggering intracellular signals. In an effort to further our knowledge of the physiology of this parasite and the mechanisms associated with the maintenance of cell homeostasis through its life cycle, we analyzed the sensitivity of *Leishmania* against the voltage-gated K^+ channel blocker 4-aminopyridine, the ***a***denine-***b***inding-***c***assette (ABC) transporter blocker glibenclamide (GLIB) and the Na^+ channel and Na^+/H^+ transport blocker amiloride. We demonstrated that these drugs induce a decrease in the percentage of macrophage infection with *Leishmania* and survival of intracellular parasites (7). As the ion transport systems blocked by these compounds are critical for cell homeostasis and survival, the sensitivity of *Leishmania* to these

drugs may be caused by their direct effect on the parasite. However, the observations that the macrophage ABC transporter ABC1, blocked by GLIB, is required for the phagocytosis of apoptotic cells (3), and for IL-1 secretion (2), and that the GLIB effect on the clearance of *Leishmania* is enhanced by interferon-gamma (IFN-γ) (8) suggest that the activity of GLIB may be based on a modification of the parasite-host cell interaction. In the present study, we evaluated whether the influence of GLIB on the growth of intracellular parasites correlates with changes in macrophage features and demonstrated that macrophage treatment with GLIB may counteract, at least partially, the strategies used by the parasite to evade the host´s protective immune response.

MATERIALS AND METHODS

Chemicals

GLIB was purchased from Research Biochemicals International (Natick, Mass, USA), and prepared and used as described (7). The monoclonal primary antibodies (mAb) against, I-A^{d}, CD80, CD86, IL-1–receptor type I and II (IL-1R I and IL-1R II) and isotype control antibodies IgG1a and IgG2a, purchased from Pharmingen (Heidelberg, Germany), as well as the streptavidin-fluorescein isothiocyanate (streptavidin-FITC) and phycoerythrin (PE)-conjugated donkey anti-rat conjugated antibodies, purchased from Dianova (Hamburg, Germany), were used for the fluorescence sorter cell (FACS) analysis according to the manufacturers instructions. IFN-γ was purchased from Gibco Chemical Co., (Hamburg, Germany) and used at 20 U ml^{-1}. The mAbs anti-IL-1β (MAB401) and detection antibody (BAF401) were purchased from R&D systems (Wiesbaden, Germany) and used in enzyme-linked immunosorbent (ELISA) assays at a concentration of 5 µg ml^{-1} and 1 µg ml^{-1}, respectively.

Experimental model

Peritoneal exudate cells (PEC) obtained from thioglycolate-treated BALB/c mice were used as the source of macrophages (7). *Leishmania* (*L.*) *major* (MHOM/IL/81/FE/BNI) was cultured as described (12). Freshly isolated BALB/c PEC (2 5 10^5 cells ml^{-1}) were infected for 4 h with stationary-phase *L. major* promastigotes at a parasite to macrophage ratio of 2 to 1, in a final volume of 0.5 ml of Click-RPMI 1640 culture medium (Gibco), supplemented with 2 x 10^{-5} M L-glutamine, 20 µg ml^{-1}gentamicin, 2 x 10^{-5} M 2-mercaptoethanol and 10 % fetal bovine serum. After removal of extracellular parasites by thorough rinsing with fresh Click-RPMI 1640 medium, the cells were incubated for further 40 h in the absence of drugs, or in the presence of 3 µM GLIB or 20 U ml^{-1} IFN-γ or 3 µM GLIB plus 20 U ml^{-1} IFN-γ. Intracellular parasites were quantified after different incubation times by staining with acridine orange and ethidium bromide (7) and were analyzed by fluorescence microscopy at 495 nm.

FACS analysis

Two color FACS analysis was performed after PEC surface staining with antibodies directed against CD80, CD86, IL-1-R I or IL-1-R II and simultaneously with I-A^d antibodies. Isotype-matched antibodies used as controls caused a mean fluorescence intensity (MFI) between 30 and 50 arbitrary units. Phenotypic analysis was done with cells incubated in the absence of drugs or in the presence of 3 µM GLIB, 20 U ml^{-1} IFN-γ or 3 µM GLIB and 20 U ml^{-1} IFN-γ for 40 or 72 h. In each case 10.000 events were accumulated and the analysis was carried out with the Cell Quest software in a FACScalibur (Beckton-Dickinson, Heidelberg, Germany).

ELISA assays

Levels of IL-1β in culture supernatants were quantified by sandwich ELISA using an enzyme linked immuno-absorbent kit (R&D systems) according to the manufacturer´s instructions. Briefly, flat-bottom high binding plates (Costar, Cambridge, Mass. USA) were coated by incubation overnight at 4°C with the anti-cytokine mAb diluted in binding solution (0.1 M $NaHCO_3$, pH 9.0), 50 µl per well. Sites for non-specific binding

of protein were blocked with 10 % bovine serum albumin (BSA) in Tris buffered saline (TBS) (Tris 100 mM, pH 7.0, 0.05% Tween 20), 100 µl per well, for 2 h at room temperature. The plates were then washed extensively with TBS for this and subsequent steps. Samples or medium blanks were added (100 µl per well) and a standard curve was constructed for each plate by using dilutions of recombinant cytokine, and plates were incubated again overnight at 4 °C prior to adding the detection anti-cytokine mAb (100 µl per well). After incubation for 1 h at room temperature, 100 µl of alkaline phosphatase (1:100) (DAKO, Hamburg, Germany) was added to each well and the plates were incubated at room temperature for 90 min. To detect the cytokines, the alkaline phosphatase substrate (Sigma Chemical Co., Heidelberg, Germany) was used. The developing color in the wells was read at 405/490 nm using an ELISA reader (Dynatech, Denkendorf, Germany).

Statistical analysis of the data

The data (number of parasites per hundred infected macrophages) are expressed as mean values ± SEM of three experiments in which at least 500 macrophages were analyzed. The levels of IL-1β are expressed as mean values ± SEM of three experiments where each culture supernatant was assayed in quadruplicate. Differences among treated and untreated macrophages were tested for statistical significance by the unpaired Student *t* test (10).

RESULTS

Replication of intracellular parasites

In order to determine whether GLIB inhibits the replication of intracellular parasites, macrophage cultures were infected with stationary-phase *L. major* promastigotes. After removal of the excess of parasites, GLIB or IFN-γ alone or GLIB plus IFN-γ were added to the macrophage-parasite cultures, which were further incubated for 12, 18 or 40 h. Figure 1

shows that the number of parasites per hundred infected macrophages incubated without drugs increased significantly within 40 h from 100 parasites per hundred infected macrophages to values higher than 400 parasites per hundred infected macrophages. On the contrary, the parasite levels found at the end of the 40 h (Fig. 1) or 72 h incubation period (8, data not shown) in cultures treated with GLIB, IFN-γ or GLIB plus IFN-γ increased only slightly to 200 parasites per hundred infected macrophages. This result suggests that *Leishmania* replication in macrophages may be affected by GLIB.

Macrophage surface molecules

To evaluate whether the effect of GLIB on the proliferation of intracellular *Leishmania* parasites correlates with a modulation of the phenotype of macrophages, freshly isolated PEC were incubated with the drugs for 40 h and analyzed for the expression of cell surface markers. Treatment with GLIB alone did not affect the basal expression of MHC class II and CD86 (Fig. 2a) but the IFN-γ–stimulated expression of MHC class II and the level of CD86 were decreased after simultaneous incubation with GLIB (Fig. 2b). On the other hand, GLIB did not significantly modify the expression of CD80, IL-1 RI and IL-1 RII (data not shown).

To determine the kinetics of this GLIB effect, we evaluated the macrophage phenotype after 72 h of the incubation with the drug. Table 1 shows that this prolonged treatment with GLIB inhibited not only the IFN-γ–stimulated expression of MHC class II and CD86 molecules, but also the basal expression of MHC class II molecules. Altogether, these results suggest that macrophage exposure to GLIB is able to modulate the expression of macrophage surface molecules that are involved in antigen presentation to T cells and their co-stimulation.

Levels of IL-1β

The function of macrophages in the stimulation of a T cell-mediated immune response involves not only the MHC-restricted presentation of antigen and the delivery of

co-stimulatory signals, but also the secretion of cytokines such as IL-1β. To evaluate whether the effect of drug treatment on *Leishmania* replication in macrophages correlates with changes in their IL-1β activity, culture supernatants of cells treated with GLIB were assayed for the presence of this cytokine. Macrophages incubated in the abscence of drugs produced IL-1β levels of 150 pg ml^{-1}, which were significantly decreased (36 %) in the presence of IFN-γ or GLIB plus IFN-γ (Fig. 3). These levels dropped dramatically when macrophages were incubated in the presence of GLIB alone. These results suggest that a decrease in IL-1β secretion may be involved in the effect of GLIB on parasite replication in macrophages.

TABLE 1

Modulation of CD86 and MHC class II by prolonged exposure to GLIB

	Mean fluorescence intensity	
	MHC class II	CD86
no drugs	29.74 ± 5.92[a]	26.08 ± 10.56
GLIB	14.86 ± 3.47[a]	25.58 ± 6.76
IFN-γ	194.95 ± 56.75[b]	54.53 ± 4.86[c]
GLIB + IFN-γ	91.12 ± 26.81[b]	30.93 ± 1.73[c]

Macrophages from BALB/c mice were treated with 3 µM GLIB, 20 U ml^{-1} IFN-γ or 3 µM GLIB + 20 U ml^{-1} IFN-γ for 72 h and assayed for surface marker expression. [a,b,c] $p<0.001$ for comparison between values identified with the same letter.

DISCUSSION

The capacity to survive and replicate within mammalian host cells is a characteristic of pathogenic intracellular parasites such as *Leishmania.* Drug action against these parasites should be directed to one or both of these physiological phenomena. In the experimental model of *L. major*-susceptible BALB/c mice, the parasite-host cell interaction

results in an impaired capacity of macrophages to control intracellular parasite growth. In a previous study (7) we demonstrated that *Leishmania* parasites are sensitive to GLIB, a drug which has been described as a selective K^+ATP channel blocker and as a potent inhibitor of the anion exchange activity of murine ABC transporters (3, 11). In the present study we investigated whether the GLIB effect on the replication of *L. major* in macrophages correlates with changes in macrophage features that are related to antigen presentation and T cell stimulation.

Activated macrophages play an essential role in inflammatory processes and contribute to the adaptive immune response by acting as antigen-presenting cells. Antigen peptides associated with MHC molecules and the expression of co-stimulatory molecules on the macrophage surface constitute the two main signals that trigger this immune response. In *L. major* infection, Th2 subset differentiation and susceptibility to disease is critically dependent on interaction with the CD86 co-stimulatory molecule (4) IFN-γ is known to induce the expression of MHC class II as well as co-stimulatory molecules on the surface of macrophages, thereby enhancing the antigen presentation capacity (13), and to inhibit the efficacy of IL-1 to generate a Th2 cell biased response (15). In the present study, we showed that GLIB has minimal effects on the expression of co-stimulatory molecules by unstimulated macrophages. In contrast, the IFN-γ −stimulated expression of MHC class II and CD86 molecules is decreased on cells treated simultaneously with GLIB. As there is evidence that *L. major*-infected BALB/c macrophages enhance the proliferation of Th2 cells (6) and that the increased expression of CD86 augments Th2 subset activation (4), our results suggest that the specific down-regulation of CD86 after incubation of macrophages with GLIB might selectively contribute to a decrease in the development of a disease-exacerbating Th2 response. Furthermore, the GLIB effect on IL-1 secretion may be relevant for the development of T cells subsets. Our finding that GLIB treatment of murine PEC results in a dramatic inhibition of Il-1β activity is in line with the finding that treatment with GLIB inhibits both IL-1β secretion and accumulation in human monocytes (2). Noteworthy, low levels of IL-1 are associated with a mouse phenotype resistant to *Leishmania* major (5). the treatment of *L. major*-infected mice with anti-IL-1

receptor antibodies inhibits the development of cutaneous lesions in genetically susceptible and resistant mice (14) and recombinant IL-1 exacerbates leishmanial lesions in *L. major* infected BALB/c mice (1). Taken together, these results demonstrate a direct correlation between a decrease in parasite growth and a decrease in MHC class II and CD86 expression as well as IL-1β secretion by macrophages treated with GLIB and suggest that the GLIB effect on macrophages may diminish the differentiation of Th2 cells and thus promote the development of a protective immune response.

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FOOTNOTES

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[2] adenine-binding-cassette transporter, ABC-transporter; bacterial lipopolysaccharide, LPS; bovine serum albumin, BSA; enzyme-linked immunosorbent assay, ELISA; Fluorescence sorter cell analysis, FACS; fluorescein isothiocyanate, FITC; glibenclamide, GLIB; interferon-gamma, IFN-γ; Interleukin 1, IL-1; IL-1–receptor, IL-1-R; *Leishmania major*, *L. major*; major histocompatibility complex, MHC; mean intensity fluorescence, MIF; monoclonal antibodies, mAb; Peritoneal exudate cells, PEC; phycoerythrin, PE; Tris buffered saline TBS.

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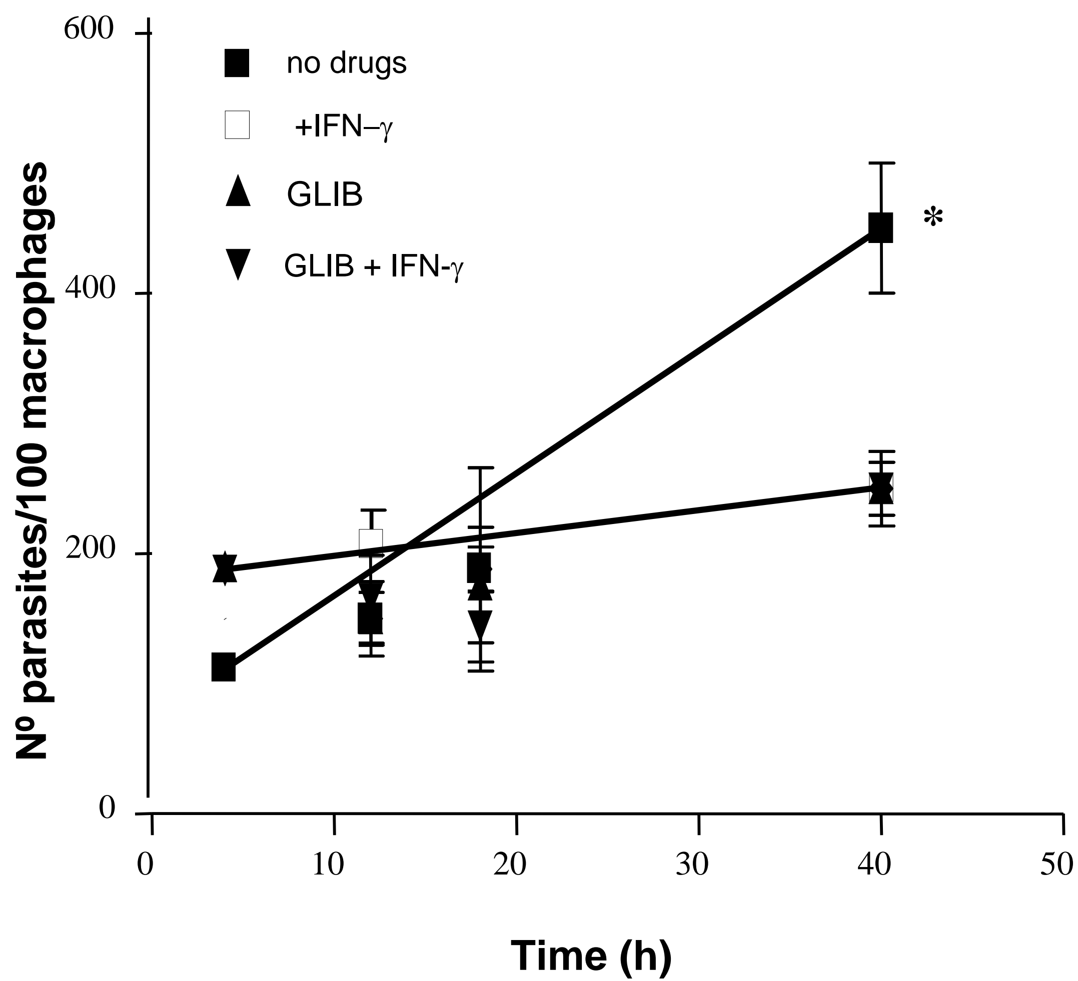
FIGURE LEGENDS

Figure 1. Time course of the number of *Leishmania* parasites per 100 infected macrophages. Macrophages were infected 4h and further incubated for various time periods either in the absence of drugs or in the presence of GLIB (3 µM), GLIB (3µM) plus IFN-γ (20 U ml^{-1}) or IFN-γ (20 U ml^{-1}) * $p<0.005$.

Figure 2. Phenotype analysis of macrophages. Expression of MHC class II and CD86 was detected by FACS analysis after treatment with GLIB (3 µM) , GLIB (3 µM) plus IFN-γ (20 U ml^{-1}) or IFN-γ 20 U ml^{-1} for 40 h. Filled histograms represent the isotype controls, Grey lines are without treatment (a) and with IFN-γ treatment (b) and black lines after GLIB treatment (a) and IFN-γ plus GLIB treatment (b).

Figure 3. IL-1β levels in supernatants of macrophage cultures treated for 40 h with 3 µM GLIB, 3µM GLIB plus 20 U ml^{-1}IFN-γ, or 20 U ml^{-1} IFN-γ. *$p< 0.005$, ** $p<0.0001$.


600
400
200
0
Nº parasites/100 macrophages
no drugs
+IFN–γ
GLIB
GLIB + IFN-γ
*
0
10
20
30
40
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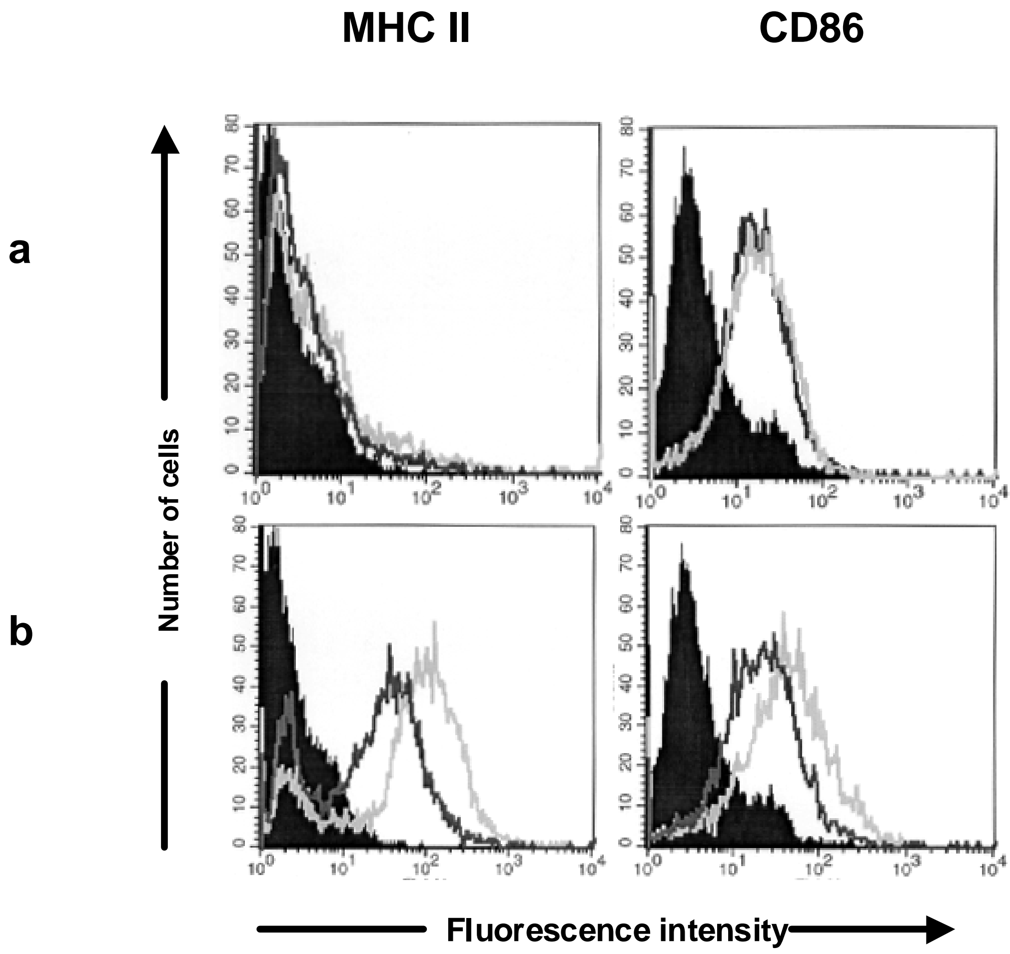
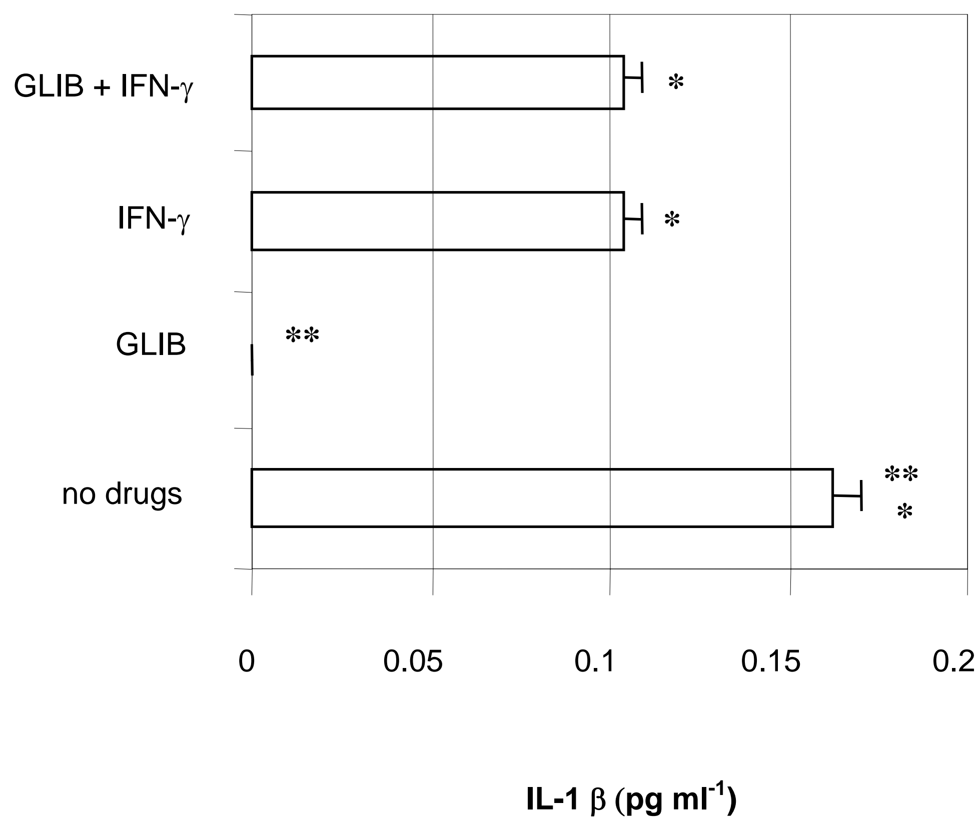
Time (h)


MHC II
CD86
a
b
Number of cells
Fluorescence intensity


GLIB + IFN-γ
IFN-γ
GLIB
no drugs
*
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0
0.05
0.1
0.15
0.2
IL-1 β (pg ml-1)