Characterization of a novel salivary immunosuppressive protein from *Ixodes ricinus* ticks.

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Running title: *Ixodes ricinus* immunosuppressive protein (Iris).

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SUMMARY

In tick salivary glands, several genes are induced during the feeding process leading to the expression of new proteins. These proteins are typically secreted in tick saliva and are potentially involved in the modulation of the host immune and haemostatic responses. In a previous study, the construction and the analysis of a subtractive library led to the identification of *Ixodes ricinus* immunosuppressor (Iris), a novel protein, differentially expressed in *I. ricinus* salivary glands during the blood meal. In the present study, the data strongly suggest that this protein is secreted by tick salivary glands into the saliva. In addition, Iris is also found to modulate T lymphocyte and macrophage responsiveness by inducing a Th2 type response and by inhibiting the production of pro-inflammatory cytokines. In conclusion, these results suggest that Iris is an immunosuppressor, which might play an important role in the modulation of host immune response.
INTRODUCTION

In contrast to most hematophagous arthropods that feed to repletion rapidly, *Ixodid* female ticks feed for an extended period of over 2 weeks (1).

Completion of the blood meal is dependent on the relationships of ticks with hosts species (2). Resistance to tick infestation implicates both innate and acquired immunity, and is characterized by reduced feeding, molting, and mating capabilities that may lead to the death of the parasite. Acquired immunity of resistant hosts is mediated by a polarized Th1-type immune response, involving IFN-γ production and delayed type hypersensitivity reaction (3, 4).

Some hosts are unable to counteract the tick infestation (4). Indeed, during their blood meal, ticks circumvent host defenses via pharmacologically active components secreted in their saliva. These factors of major importance for tick survival can modulate innate and acquired immunity. In this way, the leukocyte responsiveness is modified during tick feeding (5). For example, cytokine production is modulated inducing a polarized Th2 immune response (4, 6). Therefore, the complex tick-host molecular interactions can be considered as a competition between host defenses raised against the parasite and the tick evasion strategies, facilitating feeding for an extended period.

Although, there is extensive information about the effects of tick bioactive factors on host immune response, little is known about the mechanisms of their actions. However, it has been observed that a wide range of new proteins are expressed during the blood meal (7). Several of them might be essential for the completion of the tick feeding process. At present time, only a few of them have been characterized (8, 9).

In order to identify cDNAs encoding proteins specifically expressed during the blood meal in the salivary glands of *I. ricinus* female ticks, a representational difference analysis (RDA) subtractive library was set up using mRNAs extracted from salivary glands of unfed and 5
day fed female *I. ricinus* ticks. One clone, formerly named *seq24* (accession number: AJ269658), was selected for further characterization of its recombinant protein (previously Seq24/MBP and Seq24/His), due to its similarity to the pig leukocyte elastase inhibitor (10). Elastase inhibitors have been identified in many mammalian species but also in arthropods like the leech *Hirudo nipponia* (11). The immunological properties of such inhibitors are not clearly established. However, these proteins are secreted by human macrophages, monocytes, and neutrophils (12). Recently, it has been also shown that the elastase inhibitor mRNA is abundantly expressed during delayed type hypersensibility responses (13). Based on the results, we have decided to call this tick protein “Iris”, for “*Ixodes ricinus* Immunosuppressor”.

In this study, we describe the characterization of Iris. We demonstrate that Iris is induced during the tick feeding process but is also secreted into the saliva. Moreover, the characterization of its properties shows that Iris has the unique capacity to modulate both the innate and the acquired immunity of the host.

1 Abbreviations used in this paper: Iris, *Ixodes ricinus* immnosuppressor; NEG, negative control; rIris, recombinant Iris; SGE, salivary gland extracts.
**EXPERIMENTAL PROCEDURES**

**Biological materials.**

Unfed and five day fed tick salivary gland proteinic extracts were obtained from 300 and 70 *I. ricinus* salivary glands, respectively. These salivary glands were crushed for 10 min using a potter and a pestle in extraction buffer (PBS 1X, pH 7.4; EDTA 10 mM, AEBSF 1mM - Sigma-Aldrich, Bornem, Belgium). The samples were centrifuged at 10,000g for 8 min, and the supernatants were stored at −20°C.

Saliva was collected from ticks fed for 5 days on the ears of a rabbit. Engorging ticks were removed, washed, and a 0.2% Dopamine (Sigma-Aldrich, Bornem, Belgium) solution in PBS 1X pH 7.2 was injected in the tick body. A finely drawn capillary tube fitted over the mouthparts of each tick permitted to collect saliva.

**Production of recombinant Iris (rIris) protein in bacterial and mammalian expression systems.**

Recombinant Iris (rIris) protein was obtained as described by Leboulle *et al* (10). Briefly, *iris* cDNA was recovered by analyzing a RDA subtractive library. This library was constructed as described by Hubank and Schatz (14) by using mRNA populations extracted from unfed and 5 day engorged tick salivary glands. The complete *iris* cDNA sequence was recovered by performing the RACE methodology as described by Frohman (15). The recombinant rIris/MBP protein was expressed in *E. coli* by using the pMALC2-E vector (NEB, Hitchin, UK) containing MBP fusion partner. The rIris/His protein was expressed in CHO-K1 cells by using the pCDNA3.1/V5-His A vector containing the Epitag V5/6X His tag fusion partner (10).

The rIris/His protein was purified in batch by Ni-chelate chromatography (Ni-NTA superflow resin - Qiagen, Hilden, Germany), according to manufacturer’s instructions. Different buffers were used to purify the rIris/His protein: the lysis buffer (PBS 1X, NaCl 500mM, Zwittergent
the washing buffer (PBS 1X, NaCl 500 mM, Zwittergent 3.12 0.1%, imidazole 17.25 mM, pH 7.5) and the elution buffer (PBS 1X, NaCl 500 mM, Zwittergent 3.12 0.1%, imidazole 103 mM, pH 7.5). The eluate was dialyzed (in a 7.000 Da cut-off membrane) in PBS 1X, NaCl 500 mM, pH 7.5. The concentration of the protein was evaluated on a Comassie blue stained acrylamide gel, at ~10 ng/µl (250 nM), by comparison with known quantities of BSA. This result was confirmed by performing a microbicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL; data not shown).

Immunodetection of Iris.

To examine the expression of native proteins in salivary glands and to detect rIris/His, the same quantities of fed and unfed tick salivary glands, and a rIris/His sample were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with diluted sera directed against rIris/MBP (1:1,000) (10) and revealed with NBT-BCIP.

Confocal microscopy.

*I. ricinus* salivary glands were dissected from unfed, 3 day or 5 day fed ticks and, immobilized on silanated slides (Biorad, Nazareth EKE, Belgium). Salivary glands were fixed in a 4% paraformaldehyde solution. After a treatment with 0.5% Triton X-100, the samples were incubated in PBS 1X containing 5% FCS. The anti-rIris/MBP serum was used at a 1:10 dilution, and the secondary antibody, a FITC Anti-Mouse IgG (H+L) (ICN, Asse-Relegem, Belgium) at a 1:32 dilution. The slides were mounted in Vectashield mounting medium (Vector Lab, Peterborough, UK) and observed with a Leica confocal laser microscope using a Leica TCS 4D operating system (Leica, Wetzlar, Germany).

Preparation of rIris/His cellular extract and rIris negative control (NEG) extracts used in activity tests.

CHO-KI cells expressing rIris/His protein, obtained from a confluent culture in five 150 cm² flasks, were resuspended in 1 ml of RPMI-1640 complete medium. The sample was frozen.
and thaw 3 times before being centrifuged at 50,000 g for 1 hour at 4°C. The supernatant was recovered and used in the different activity tests. The negative control (NEG) was a proteinic extract of CHO-KI cells resistant to G418 that do not express the recombinant protein and prepared as the rIris/His extract. The concentration of rIris/His in the cellular extract was evaluated at ~4 ng/µl (~100 nM), by comparing rIris/His contained in cellular extracts and purified rIris/His, on Western blot revealed with anti-V5 antibody (Invitrogen, Groningen, The Netherlands). The toxicity of the different samples for human PBMCs was evaluated by using the 7-amino actinomycin D (7-AAD) viability dye (Immunotech, Marseille, France), according to manufacturer’s instructions.

**Cell Culture.**

*Naive Balb/c spleen cells*: A suspension of spleen cells (SC) was isolated from naive Balb/c mice. $10^6$ lymph node cells per well were cultivated with serial dilutions of rIris/His or NEG cellular extracts for 2 hours in 100 µl of complete culture medium containing RPMI-1640 (Gibco, Basel, Switzerland) supplemented with 10% fetal calf serum (v/v), 2mM L-glutamin, 1mM sodium pyruvate, 1mM non-essential amino acids (Sigma, St Louis, MO), 0,05 mM mercaptoethanol, 100 U/ml penicillin/streptomycin (Gibco, Basel, Switzerland) and 25 µg/ml Funigizone (Gibco, Basel, Switzerland). Cells were then stimulated with 1 µg/ml of ConA in a final volume of 200 µl for 15 hours. 1 µCi/well of $[^3]$H]thymidine (Amersham Int., Amersham, UK) was added 24 hours before harvesting the cells. Tritiated thymidine incorporation was determined by liquid scintillation counting. Results show the means (+/- S.D.) of duplicate rIris/His or NEG stimulated wells realized in 2 independent experiments. Means of ConA-unstimulated wells were previously subtracted (net $10^3$ c.p.m.).

*Naive human PBMCs*: Experiments were realized with PBMCs, obtained from 5 different donors, used separately. PBMCs were resuspended in RPMI-1640 medium supplemented with FCS 10% (v/v), L-glutamine 2 mM, penicilline-streptomycine (100U/ml)
and IL-2 (20 U/ml). 2.10^6 cells were pre-cultivated in 1 ml of culture medium. Cells were then diluted at different concentrations in 96 well plates (2.10^5 cells/100 µl for PPD stimulation and 5.10^4 cells/100 µl for LPS stimulation for the ELISPOT technique and 2.10^5 cells/100 µl for the ELISA technique). Finally, PBMCs were incubated, for 72 hours at 37°C, with different dilutions of rIris/His or NEG cellular extract or anti-rIris/MBP serum and the different activators : PHA (at a final concentration of 1 µg/ml), LPS (1 µg/ml), anti-CD3/CD28 mAb, (500 ng/ml), PMA/CD28 (PMA 25 ng/ml – CD28 500 ng/ml) and PPD (5 µg/ml).

Detection of cytokines.

ELISPOT assay : 96 well nitrocellulose bottom coated plates (Multiscreen-HA Mahan, Millipore, Brussels, Belgium) were coated with coating antibodies directed against IFN-γ (clone C1-D16 MAB 1-D1K, Nodia, Antwerp, Belgium) and IL-10 (Clone JES3-9D7, BD Pharmingen, San Diego, CA). Cells were stimulated with PHA or LPS and rIris/His or NEG cellular extracts for 72 hours at 37°C. Supernatants were recovered and stored at –20°C. The cytokines were detected, in the 96 well plates, with biotinylated anti-IFN-γ antibody (clone JES3-5A10 MAB 7-B6-1, Nodia, Antwerp, Belgium) and anti-IL10 antibody (clone JES3-12G8, BD Pharmingen, San Diego, CA) diluted in PBS Tween 0,25% (1µg/ml). Finally, the captured antibodies were revealed with extravidine peroxdyase and AEC substrate (Sigma-Aldrich, Bornem, Belgium). Results show the mean of triplicate wells (+/- S.D.). Means of unstimulated wells were previously subtracted. The rIris/His and NEG protein extracts do not affect the cell viability (data not shown).

ELISA technique : Cytokine-specific ELISAs were realized using the Flexia-human kit (Biosource, Nivelles, Belgium) for the detection of IFN-γ, IL-10, TNF-α, IL-6, IL-1β and IL-8, and the IL-5 kit (Endogene, Woburn, Massachusetts) for the detection of IL-5. The assays were carried out using manufacturer’s instructions and were revealed using TMB substrate.
The concentration of the different cytokines in the experimental samples (pg/ml) was calculated against a standard curve generated with recombinant cytokines. Results show the mean of rIris/His or NEG cellular extracts stimulated wells realized in 5 independent experiments (+/- S.D.). Means of unstimulated wells were previously subtracted.

**Statistical Analysis.**

The significance of the differences obtained between NEG and rIris/His-treated groups was evaluated by Student \( t \)-test using the Pooled or Satterhwaite method.
RESULTS

Detection of Iris in *I. ricinus* salivary glands and saliva.

Immune sera recovered from mice immunized with rIris/MBP (formerly Seq24/MBP) were used to detect the native Iris protein in the salivary glands and saliva from unfed or engorged female *I. ricinus* ticks. Iris was revealed by Western blot in tick saliva at a molecular weight of 43 kDa (data not shown). By using confocal microscopy, it was found in 3 day and, more abundantly, in 5 day fed tick salivary glands on the external surface of salivary acini, within the cells and also in the acini’s light; but was not detected in unfed tick salivary glands (Figure 1). These results suggest that the expression of Iris is induced in the salivary glands during the tick feeding process and that Iris is secreted in tick saliva.

Fig. 1 inserted here

Characterization of the immunomodulatory properties of Iris.

Based on its homology to a leukocyte elastase inhibitor, the immunomodulatory properties of Iris were examined by using different activity tests that were mainly performed with soluble proteinic extracts of CHO-KI cells expressing rIris/His (formerly named Seq24/His) at a concentration of ~4 ng/µl (100 nM). Proteinic extracts of CHO-KI cells, which do not express rIris/His, were used as a negative control (NEG).

*In vitro proliferation of Balb/c naive spleen cells.* The proliferation of naive Balb/c spleen cells (SC) was analyzed *in vitro* by pre-incubating them with various dilution (1 :6.25 to 1 :50) of rIris/His cellular extracts, followed by stimulation with concanavalin-A (ConA). As shown on figure 2, the proliferative response of SC was strongly diminished (81% of inhibition at a 1 :6.25 dilution) in a dose dependent manner. The negative control had no significant effect on ConA-stimulated SC (average inhibition of 15%); even if it inhibited by 25% the SC proliferation at a 1 :6.25 dilution.
In vitro stimulation of human peripheral blood mononuclear cells (PBMCs). The effect of rIris/His on cytokine production was studied on PBMCs stimulated with different activators. The number of PBMCs secreting IFN-γ and IL-10, after stimulation either with LPS or phytohaemagglutinin-A (PHA), was assayed by the ELISPOT technique (Figure 3). After PHA stimulation, in presence of rIris/His cellular extract, a reduced number of PBMCs (more than 80%) secreted IFN-γ while the number of cells producing IL-10 remained unchanged. Furthermore, the NEG protein extract had no effect on the production of both cytokines by PHA-stimulated PBMCs. In contrast, after LPS stimulation, no difference in the number of cells producing IFN-γ was observed between PBMCs incubated with rIris/His and the NEG cellular extract. On the other hand, rIris/His extract enhanced by 400% the number of PBMCs expressing IL-10, while stimulation with NEG cellular extract did not enhance the number of cells producing IL-10.

The effect of rIris/His cellular extract (used at a 1:5 dilution) on the production of cytokines (IFN-γ, IL-8, IL-10, IL-6, TNF-α, and IL-1β) by PBMCs stimulated with a set of activators (PHA, CD3/CD28, LPS and protein purified derivative - PPD) was also evaluated by ELISA (Table 1).

The results indicated that the production of almost all tested cytokines (IFN-γ, IL-6, TNF-α, and IL-8) was inhibited by the rIris/His cellular extract, except for the IL-1β production that was unaffected after LPS stimulation (Table 1). rIris/His cellular extract inhibited IL-10 production by PMBCs after PHA stimulation, had no effect on PMBCs stimulated with CD3/CD28, and LPS. The NEG cellular extract had no significant effect on the cytokine production except after LPS stimulation that inhibited IL-10 production and enhanced IL-6.
production. The dose-dependent effect of rIris/His was examined by analyzing the IFN-\(\gamma\), IL-5 and IL-10 production under CD3/CD28 and PPD stimulation (Figure 4). In these cases, the maximum inhibition of IFN-\(\gamma\) production by rIris/His was of \(\sim 65\%\) (the difference between rIris/His and NEG cellular extracts were statistically significant: \(P<0.01\)) and \(\sim 75\%\) (\(P<0.05\)) after CD3/CD28 and PPD stimulation, respectively. This inhibition was still effective at a 1:12.5 dilution. In contrast, no difference in the production of IL-5 and IL-10 was observed between PBMCs incubated with rIris/His and NEG cellular extracts.

To confirm the role of Iris in the modulation of the production of some cytokines, PBMCs stimulated by CD3/CD28 activator were incubated with various dilutions (from 1:250 to 1:4,000) of anti-rIris/MBP serum (Figure 5A). It was observed that PBMCs treated with rIris/His cellular extract (at dilution 1:12.5) in the presence of anti-rIris/MBP serum restored the IFN-\(\gamma\) production in a dose-dependent manner (Figure 5A); while this antiserum itself had no immuno-stimulating effect on cytokine production by PBMCs (data not shown). At dilution 1:250, the antiserum re-established the IFN-\(\gamma\) production to a level similar to that obtained by CD3/CD28-stimulated PBMCs without rIris/His. To assert the specificity of the neutralizing activity of the anti-rIris/MBP serum, its effect was measured on the activity of cyclosporine-A (CsA) (Figure 5B), an immunosuppressive drug. The effect of a serum specific to an unrelated MBP fusion protein was also measured on rIris/His cellular extracts activity (Figure 5A). The anti-rIris/MBP serum (at a 1:250 dilution) did not affect the activity of 400 nM CsA, and the unrelated antiserum had no effect on the rIris/His immunomodulatory activity.

Finally, a small amount of rIris/His was purified from the rIris/His CHO-K1 cellular extract. This purified rIris/His protein, at a \(\sim 25\) nM concentration, completely inhibited the IFN-\(\gamma\)
production by CD3/CD28-stimulated PBMCs (Figure 5C), which was partially restored (50 % of the IFN-γ production by CD3/CD28-stimulated PBMCs) by using anti-rIris/MBP serum (at 1:250 dilution). Interestingly, it was found that the level of inhibition of ~25 nM of purified rIris/His was comparable to that of 400 nM CsA. The incubation of CD3/CD28-stimulated PBMCs with either purified NEG and anti-rIris/MBP serum (at 1:250 dilution) or purified NEG alone had no influence on the IFN-γ production.
DISCUSSION

It is now well established that the modulation of host immunity by tick saliva is of major importance both in the successful accomplishment of the blood meal and in the transmission of tick-borne pathogens such as *Borrelia burgdorferi*, the causal agent of Lyme disease (16). Although extensive information is available on the effects of tick feeding on host immune defenses, little is known about the nature of the immunomodulatory molecules expressed by tick salivary glands. Tick salivary gland extracts (SGE) modulates host immune response by modifying both lymphocyte and monocyte activities. For example, T lymphocyte proliferation in response to mitogens (17) and the production of Th1 cytokines as IFN-γ and IL-2 are inhibited by SGE (18). Moreover, Th2 type cytokine production such as IL-10, IL-5 and IL-4 is in general slightly enhanced (4, 5). Tick SGE also inhibits the production of several cytokines (IFN-γ, IL-8, IL-6, TNF-α, …) by human macrophages (19). Studies indicated that some of those immunomodulating phenomena are induced by proteins (20), (21).

In this work, we have characterized the properties of a protein induced during the tick feeding process, which is called Iris for “*I. ricinus* immunosuppressor”, due to its exceptional immune properties on leukocytes.

The corresponding mRNA sequence was first recovered by analyzing a RDA subtractive library, and by using the RACE method (10). In this report, we show by Western blot and confocal microscopy using a specific antiserum that Iris is not only specifically expressed during the blood meal in female *I. ricinus* salivary glands but is also present in saliva with an increasing expression from day 3 to day 5 of engorgement. In order to determine the immunomodulatory properties of Iris, we studied the effect of the corresponding recombinant protein (rIris/His) on naive Balb/c spleen cells proliferation, and on human PBMCs cytokine production, using specific T-lymphocytes (PHA, ConA, CD3/CD28 and PMA/CD28), macrophages (LPS) and APC (PPD) activators. The results indicated that rIris/His cellular...
extracts inhibited on a dose dependent manner the proliferation of murine lymphocytes. ELISA and ELISPOT assays showed that the rIris/His cellular extracts suppressed the production of IFN-γ by human T cell and APC, while IL-5 and IL-10 levels remained unchanged or were inhibited. Indeed, rIris/His cellular extract inhibited IL-10 production by T-lymphocytes stimulated with PHA, but had no effect on CD3/CD28 stimulated cells. In contrast, rIris/His extract enhanced the number of macrophages producing IL-10 (see Figure 3) and had no effect on the expression of this cytokine by macrophages (Table 1). This difference could be explained by the low level of IL-10 detected by ELISA, the high sensitivity (up to 200 times) of the ELISPOT technique and the variety in shape and size of the spots observed with ELISPOT. Nevertheless, these results suggest Iris does not inhibit the IL-10 production by macrophages. It was also shown that the expression of the pro-inflammatory cytokine IL-6 was inhibited by macrophages, T cell, and APC. In addition, the production of TNF-α by macrophages was inhibited while IL-1β expression remained unaffected. Furthermore, by neutralizing completely rIris/His cellular extract activity with a specific anti-rIris serum, and by showing that purified rIris/His protein inhibited IFN-γ production by T cell, we clearly established that the recombinant protein was responsible for the immunomodulation. Importantly, the inhibitory effect of ~25 nM rIris/His on IFN-γ production (inhibition of 94%) is comparable to 400 nM CsA activity (inhibition of 99%). This inhibition of IFN-γ production suggests that the T cell proliferation is also inhibited. This hypothesis was confirmed by showing that rIris/His protein inhibited murine T cell proliferation. These observations indicate that Iris is a novel immunosuppressor induced in the salivary glands of engorged ticks during the blood meal. It suppresses T lymphocytes proliferation, and induces a Th2 type immune response that is characterized by the inhibition of IFNγ production, an unaffected expression of IL-5 and IL-10 by T lymphocytes and APC. Moreover, IL-10 production by macrophages is not inhibited by Iris, confirming the pro-Th2
activity of Iris. In addition, Iris modulates the mechanisms of innate immunity by inhibiting the production of pro-inflammatory cytokines (IL-6 and TNF-α).

It is known that several immunomodulatory factors are secreted in saliva at various times of the feeding process. Indeed, SGE, prepared daily from engorging *Dermacentor andersoni*, suppressed IL-1 secretion by macrophages only from day 0 to day 5 of engorgement while TNF-α production was suppressed during the entire blood meal by these cells (18). Except for the fact that IL-1 expression is not inhibited, the modulating effect of Iris on T cell and macrophage responsiveness seems to mimic the SGE properties, suggesting that Iris is an important salivary factor in the modulation of the local host immune response.

Finally, it is known that IL-4 produced following *I. ricinus* tick bites strongly influences the immune response against *B. burgdorferi* (22). Moreover, Th1 cytokines are down regulated during the initial spirochete transmission period in mice infested with *Ixodes scapularis* infected ticks (23). IFN-γ, TNF-α and IL-2 given at the time of tick feeding suppress spirochetes transmission by *I. scapularis* (16). In addition, it has been recently shown that this Th2 type immune response makes possible the transmission of the human granulocytic ehrlichiosis agent (24). Consequently, based on its immune properties, Iris could be a key factor facilitating the transmission of a large number of tick borne pathogens.

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REFERENCES


*Ixodes ricinus* immunosuppressive protein (Iris)


*Ixodes ricinus* immunosuppressive protein (Iris)
LEGENDS TO FIGURES

**Figure 1**  *Confocal microscopy of female I. ricinus salivary glands.* Negative control corresponding to 5 day fed tick salivary glands incubated only with the secondary antibody (A). Unfed (B), 3 day (C) and 5 day (D) fed tick salivary glands incubated with anti-Iris/MBP serum.

**Figure 2**  *Balb/c spleen cells stimulated with ConA.* SC were incubated only with ConA (N.S.) or with different dilutions of rIris/His and NEG cellular extracts. Tritiated thymidine incorporation was determined by liquid scintillation counting (10³ c.p.m. +/- S.D.).

**Figure 3**  *IFN-γ and IL-10 ELISPOT of human PBMCs.* The number of activated cells producing the cytokines upon treatment with PHA or LPS was evaluated (spots/10⁶ cells +/- S.D.). Activated cells were counted after treatment with rIris/His or NEG cellular extracts. A positive control was realised by stimulating the cells only with the activator (PHA or LPS).

**Figure 4**  *IFN-γ and IL-5 production by human PBMCs.* Cells were incubated only with the activator (N.S.) or with various dilutions of rIris/His and NEG cellular extracts. The production of the cytokines (Conc. - pg/ml +/- S.D.) was evaluated by ELISA after treatment with CD3/CD28 or PPD.

**Figure 5**  *IFN-γ production by human PBMCs stimulated with CD3/CD28.* The cells were stimulated with NEG and rIris/His cellular extracts at a 1:12.5 dilution. All the assays were realised by stimulating the cells: only with CD3/CD28 (P.C.), with CD3/CD28 in the presence of NEG cellular extract (NEG), or with CD3/CD28 in the presence of rIris/His cellular extract (rIris/His). A. CD3/CD28 stimulated cells were incubated with rIris/His cellular extract in the
presence of various dilutions of either anti-rIris/MBP serum (Anti-rIris/MBP+rIris/His) or a non-specific serum (ns Ab+rIris/His). **B.** CD3/CD28-stimulated cells were incubated with 400 nM CsA (CsA), with 400 nM CsA in the presence of anti-rIris/MBP serum (CsA + Anti-rIris/MBP), or with 400 nM CsA and the non-specific serum (CsA + NS AB); both antisera were used at a 1:250 dilution. **C.** CD3/CD28 stimulated PBMCs were also incubated with purified NEG (pNEG) or rIris/His (pIris/His) protein, and with purified NEG or rIris/His proteins in the presence of anti-rIris/MBP serum at a 1:250 dilution (pNEG + Anti-rIris/MBP and pIris/His + Anti-rIris/MBP).
TABLE 1: Cytokine production by PBMC treated with rIris/His cellular extract.

<table>
<thead>
<tr>
<th>Cell Stimulation</th>
<th>PHA</th>
<th>CD3/CD28</th>
<th>PPD</th>
<th>LPS</th>
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<tbody>
<tr>
<td>IFN-γ</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>36%</td>
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<td>-</td>
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<tr>
<td></td>
<td>22%</td>
<td>12%</td>
<td>13%</td>
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<tr>
<td>IL-10</td>
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<td>/</td>
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<tr>
<td></td>
<td>27%</td>
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<td>16%</td>
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<td>10%</td>
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<tr>
<td>TNF-α</td>
<td>-</td>
<td></td>
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<tr>
<td></td>
<td>11%</td>
<td></td>
<td></td>
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<tr>
<td>IL-1β</td>
<td>/</td>
<td></td>
<td></td>
<td>97%</td>
</tr>
</tbody>
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2 Expression is: inhibited (-) or unchanged (/). Values represent the % of cytokine production calculated in comparison with cells stimulated only with the activator.
FIGURE 2

Spleen Cells Prolif. - ConA

Tritium Uptake (10^-3 c.p.m. +/- S.D.)

Dilutions

- N.S.
- 50 X
- 25 X
- 12,5 X
- 6,25 X

Legend:
- rIris/His
- NEG
**FIGURE 3**

**PHA stimulation of PBMCs**

**IFN-γ production**

<table>
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<tr>
<th>Condition</th>
<th>Spots/10^6 cells +/- S.D.</th>
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<td>PHA</td>
<td>3000</td>
</tr>
<tr>
<td>PHA + NEG</td>
<td>2500</td>
</tr>
<tr>
<td>PHA + rIris/His</td>
<td>1500</td>
</tr>
</tbody>
</table>

**IL-10 production**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spots/10^6 cells +/- S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>1000</td>
</tr>
<tr>
<td>PHA + NEG</td>
<td>600</td>
</tr>
<tr>
<td>PHA + rIris/His</td>
<td>400</td>
</tr>
</tbody>
</table>

**LPS stimulation of PBMCs**

**IFN-γ production**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spots/10^6 cells +/- S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPS</td>
<td>500</td>
</tr>
<tr>
<td>LPS + NEG</td>
<td>1000</td>
</tr>
<tr>
<td>LPS + rIris/His</td>
<td>1500</td>
</tr>
</tbody>
</table>

**IL-10 production**

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<tbody>
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</tr>
<tr>
<td>LPS + NEG</td>
<td>500</td>
</tr>
<tr>
<td>LPS + rIris/His</td>
<td>1500</td>
</tr>
</tbody>
</table>
FIGURE 4

PBMCs stimulation

CD3/CD28

IFN-γ production

Conc. (pg/ml)

NEG

rIris/His

Dilutions

N.S. 66 X 40 X 12.5 X 5 X

IL-5 production

Conc. (pg/ml)

NEG

rIris/His

Dilutions

N.S. 12.5 X 5 X

IL-10 production

Conc. (pg/ml)

NEG

rIris/His

Dilutions

NS 66 X 12.5 X 5 X

PPD

IFN-γ production

Conc. (pg/ml)

NEG

rIris/His

Dilutions

N.S. 66 X 40 X 12.5 X 5 X

IL-5 production

Conc. (pg/ml)

NEG

rIris/His

Dilutions

N.S. 12.5 X 5 X

IL-10 production

Conc. (pg/ml)

NEG

rIris/His

Dilutions

NS 66 X 12.5 X 5 X

PBMCs stimulation

CD3/CD28

IFN-γ production

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Dilutions

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IL-5 production

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rIris/His
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Gérard Leboulle, Mara Crippa, Yves Decrem, Naceur Mejri, Michel Brossard, Alex Bollen and Edmond Godfroid

J. Biol. Chem. published online January 15, 2002

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