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Expression and characterization of recombinant interferon gamma (IFN-γ) from the nine-banded armadillo (*Dasypus novemcinctus*) and its effect on *Mycobacterium leprae*-infected macrophages

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Abstract

Armadillos (*Dasypus novemcinctus*) manifest the full histopathological spectrum of leprosy, and are hosts of choice for *in vivo* propagation of *Mycobacterium leprae.* Though potentially useful as a model of leprosy pathogenesis, few armadillo specific reagents exist. We have identified a region of high homology to the interferon gamma (IFN-γ) of other mammals within the recently published armadillo whole genomic sequence. cDNA was made from ConA-stimulated armadillo peripheral blood mononuclear cells (PBMC), amplified, and cloned into a pET expression vector for transformation and over-expression in *E. coli.* The recombinant protein (r*Dn*IFN-γ) was characterized by western blot and its biological function confirmed with biosassays including intracellular killing of *Toxoplasma gondii* and induction of indoleamine 2, 3-dioxygenase activity. In using rIFN-γ to activate macrophages from mice, humans or armadillos, similar to humans, rIFN-γ-activated armadillo MΦ did not produce nitrite and or inhibit the viability of *M. leprae in vitro*. Conversely, murine rIFN-γ-activated mouse MΦ produced high levels of nitrite and killed intracellular *M. leprae in vitro.* These data indicate that the response of armadillo MΦto r*Dn*IFN-γ is similar to that which occurs in humans, and demonstrates a potentially important value of the armadillo as a model in leprosy research.

Keywords

Dasypus novemcinctus; interferon; leprosy; Mycobacterium leprae; armadillo

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1. INTRODUCTION

Leprosy continues to be an important public health problem for the developing world and an estimated 2-3 million people currently live with deformity brought by their disease [1]. Though significant progress has been made in reducing new case presentations [2], improved diagnostic tests and therapeutic regimen are still needed. Two major obstacles impeding the progress of research in these areas have been our inability to cultivate *Mycobacterium leprae* (the etiological agent for leprosy) on artificial media in the laboratory, and the lack of a robust animal model for studying this infection [3].

Nine-banded armadillos (*Dasypus novemcinctus*) are the only immunologically intact animal species that exhibits high susceptibility to *M. leprae*. Like man, armadillos manifest leprosy over a broad clinical and histopathological spectrum that is classifiable from lepromatous to tuberculoid [4], and they have been developed as the hosts of choice for *in vivo* propagation of leprosy bacilli [5] [6]. Because of their unique natural susceptibility to infection with *M. leprae*, armadillos could be valuable models of leprosy pathogenesis and help advance development of new diagnostic tests, immunotherapies or vaccines [6]. Unfortunately, because of their exotic nature and scant commercial value, relatively few armadillo-specific immunological reagents have been generated, and consequently few translational benefits actually have been realized with this model to date.

Resistance to *M. leprae* is mediated through cellular immune processes and involves a complex interplay of cytokines and chemokines. Prominent among these is interferon gamma (IFN-γ), which stimulates macrophages (MΦ) to up-regulate antimicrobial, anti-tumour, and antigen processing and presentation pathways [7]. In rodent immune systems, activation of MΦ by IFN-γ results in effective growth restriction and clearance of mycobacteria with production of reactive nitrogen intermediates (RNI) as effector molecules [8;9]. However this potent antimicrobial mechanism varies from species to species. Human IFN-γ-activated peripheral blood MΦ demonstrate little or no production of nitric oxide (NO)[10;11] and are unable to kill several different mycobacterial species.

The IFN-γ genes of many other mammals have been cloned and over-expressed in *E. coli* [12;13]. Commercially available recombinant IFN-γ proteins and antibodies also are available for a variety of species, but they rarely exhibit functional cross reactivity between species and it has not been possible to monitor the production of IFN-γ among armadillos over the course of infection by *M. leprae*. However, because of the armadillo's evolutionary and medical significance, a low (2 X) coverage of the *D. novemcinctus* genome sequence (http://www.ncbi.nlm.nih.gov/BLAST) was recently published, and more extensive 6 X sequence coverage also is underway. Genomic sequence data is an invaluable resource for the identification and generation of specific immunological reagents [14] and exploitation of the armadillo sequence data can significantly benefit efforts to advance these animals as models for leprosy. We probed the available sequence data for an armadillo homolog to human IFN-γ, and report here the sequence, cloning, expression, biological activity and development of associated specific reagents of recombinant *D. novemcinctus* IFN-γ (r*Dn*IFN-γ). In addition, we also used these reagents to examine the functional character of armadillo IFN-γ-activated armadillo MΦ to live *M. leprae* and compared their function to both human and mouse activated macrophages.

2. MATERIALS AND METHODS

2.1. Identification of *Dn*IFN-γ

Bioinformatic tools were used to identify the putative coding sequence of *Dn*IFN-γ. The amino acid sequence of *Homo sapiens* IFN-γ (GI: 56786138) and tBLASTn

(http://www.ncbi.nlm.nih.gov/BLAST/) were used to search for homologous translated sequences in the *D. novemcinctus* whole genome sequence (WGS)[15]. The putative coding region of *Dn*IFN-γ (GI: DQ094083) was found in two *D. novemcinctus* genomic contigs (GI: 64640499 and GI: 64640497). The partial genomic sequence was used to derive a putative cDNA and a corresponding translation for the putative amino acid sequence was identified using ExPASy Transalate tool (http://us.expasy.org/tools/dna.html). The cDNA and the amino acid sequence were submitted to BLAST to compare homology to other IFN-γ molecules [15].

2.2. Generation of *D. novemcinctus* cDNA

Armadillo peripheral blood mononuclear cells (PBMC) were purified from 8 mL peripheral blood collected in BD Vacutainer® CPT Mononuclear Cell Preparation Tubes (BD Biosciences, San Jose, CA) and mononuclear cells were isolated after centrifugation (1600 × *g* for 45 mins, 25°C). The mononuclear cell layer was removed, washed 3 X with cold PBS, resuspended in culture medium (RPMI 1640 medium containing 2mM glutamine and HEPES) supplemented with 20% fetal bovine serum (FBS), and plated at 2×10^6 cells/mL in a T_{75} tissue culture flask. The cells were stimulated with ConA (Sigma-Aldrich, St. Louis, MO) at a final concentration of 5 μg/mL for 4 h at 37°C. Aliquots of the ConA-stimulated cells were washed 3 X in cold PBS, resuspended in 500 μL cold PBS, snap frozen in liquid nitrogen, and stored at −70°C for RNA purification. Total RNA was purified from these cells using the FASTRNA™ kit and the FastPrep® FP120 Instrument and manufacturer's recommendations (Q-Biogene, Carlsbad, CA). The cDNA was generated from 1 μg total RNA using the Advantage RT-for-PCR kit with random hexamers (BD Biosciences Clontech, Palo Alto, CA) in a final volume of 50 μL according to the manufacturer's recommendations.

2.3. *Dn*IFN-γ Amplification and Recombinant Plasmid Construction

Primers (DnIFNγ-F 5′-AGAAAAGATCAGCCAAGTCC-3′ and *Dn*IFNγ-R 5′ TTCAAATATTACAGGGAGGATG 3′) (BIOMEDD, Baton Rouge, LA) and armadillo cDNA from Con A-stimulated PBMCs were used with high fidelity polymerase, (*Pfu*, Strategene, La Jolla, CA), and PCR to generate a fragment encoding the entire *Dn*IFN-γ cDNA. This product was purified using QIAquick columns (QIAgen, Valencia, CA) and verified by automated DNA sequencing using an ABI prism 3310 DNA sequencer (Applied Biosystems, Foster City, CA) (BIOMMED). DNA encoding the mature peptide (the protein without the signal peptide) was amplified from the cDNA using primers containing the “topo” sequence (CACC) on the 5′ terminus (*Dn*IFN-γTOPOF: 5′CACCTGCTACTGCCAGGCCAC3′ and *Dn*IFNγTOPO-R: 5′CAAATATTACAGGGAGGATGACCA3′) and ligated into pET 200/DTOPO® vector (Invitrogen, Carlsbad, CA) using standard procedures. The recombinant plasmid was transformed into *E. coli* BL21star competent cells (Invitrogen) according to manufacturer's recommendations. Clones were identified by antibiotic selection and further characterized using *Dn*IFNγ PCR/direct sequencing.

2.4. *Dn*IFNγ Protein Expression and Characterization

r*Dn*IFNγ protein was produced from a positive clone using the following protocol. Recombinant bacteria were grown to mid-log phase (OD_{600}= 0.6) and induced with a final concentration of 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich). The expressed protein was purified using a Ni-NTA Purification System (Invitrogen) and dialyzed using the recommended, denaturing regimen (Invitrogen). The r*Dn*IFN-γ was refolded as described by Jeevan *et al*. [16] and concentrated using a Vivaspin 15 mL concentrator with a 10 kDa molecular weight cut-off (Vivascience, Hanover, Germany). The recombinant protein was separated by SDS-PAGE using a 4% to 20% gradient Novex TBE-urea polyacrylamide gel (Invitrogen), stained with Coomassie® Brilliant Blue (Bio-Rad, Hercules, CA), and

compared to Kaleidoscope pre-stained polypeptide standards (Bio-Rad). Western blot analysis using rabbit polyclonal antibody prepared with synthetic *Dn*IFN-γ peptide epitopes (below) was used to verify the size and presence of the recombinant product.

2.5. Generation of anti-r*Dn*IFN-γ Antibodies

Polyclonal antibodies (Abgent, Inc, San Diego, CA) were prepared in rabbits against selected synthetic *Dn*IFN-γ peptide epitopes without Freund's complete adjuvant. Based on the deduced amino acid sequence, two epitopes Anti-*Dn*IFN-γ #1 (LKNWKEESDKKIIQS) and Anti-*Dn*IFN-γ #2 (PKSNLRKRKRSQSTF) were selected using *in silico* predictions of antigenicity. The complexity, hydropathy (Hopp-Woods method) [17], ß-turns (Chou-Fasman method) [18], flexibility, and accessibility of epitopes in the deduced *Dn*IFN-γ amino acid sequence were assessed to generate the regions of the protein most likely to produce antibodies.

Monoclonal antibodies (Mabs) to *Dn*IFN-γ were produced by immunizing Balb/c mice (Harlan, Indianapolis, IN) intra-peritoneally (IP) with 50 μg of the purified recombinant protein in 1:1 TiterMax Gold (Titermax, Inc., Norcross, Ga.) twice in 3-week intervals followed by a final IP injection of 20 μg protein in PBS. Hybridomas were made by fusing primed mouse spleen cells and the myeloma B-cell line SP2/0 and cultured in hypoxanthine, aminopterin and thymidine (HAT) selection medium using a protocol previously described [19]. Hybridoma culture supernatants were screened in an ELISA with either r*Dn*IFN-γ or synthetic peptides. HRP-Rabbit anti-mouse IgG conjugate or HRP-Goat anti-Rabbit IgG (Zymed laboratories, San Francisco, CA) was used to detect the positive clones. Specific antibody reactive sites were determined by ELISA using synthetic 15mer peptides overlapping by 5 amino acids each (15 × 5) and extending over the entire length of the *Dn*IFN-γ (Mimotopes: PharmAus, Ltd., Nedlands, Aus.).

2.6. *Dn*IFN-γ RT-PCR assay

Semi-quantitative RT-PCR assays were developed for detection of *Dn*IFN-γ and *Dn*G_3PDH gene transcripts. The following PCR primers were designed from cDNA sequences of *Dn*IFN-γ (Assession # DQ094083) and the contig containing *Dn*G3PDH (Assession # 64811560) using Primer Express software: *Dn*IFNγ-F 5′GAATTACACGGGCTATCTCTTAGCTT3′, *Dn*IFNγ-R 5′ AAGGTCGGCCTGGCAGTAG3′, *Dn*G3PDH-F 5′ AATGGGCATCCCATCACTAT CT3′. Both gene fragments were amplified in PCR from the cDNA of PBMC both stimulated and unstimulated with ConA for 8 hr and specific parameters specified by the Primer Express software. The resultant amplicons were separated on a Novex® 10% TBE mini-gel (Invitrogen) and visualized after ethidium bromide staining using GelDoc2000 Instrument (Bio-Rad). The relative quantity of *Dn*IFN-γ transcripts was estimated by comparing the intensity of the amplicon bands of ConA-stimulated to that of its non-stimulated control for both assays. Amplicons were purified and the DNA sequence of each amplicon was obtained to verify the gene fragments.

2.7. Preparation of MΦ monolayers

Armadillo PBMC were isolated with BD Vacutainer® CPT Mononuclear Cell Preparation Tubes (BD Biosciences) as described above and the mononuclear cells were resuspended in culture medium containing 20% FBS. Cell viability was determined using trypan blue exclusion. Human PBMC were separated over Ficoll-Hypaque (Pharmacia) and resuspended in culture medium supplemented with 10% autologous serum. Cell suspensions were seeded individually into 24 well culture plates (0.5 mL/ well) containing 13 mm round LUX cover slips at a concentration of 4×10^6 cells/mL. Resident murine (Balb/c) peritoneal cells were obtained by lavage, and adherent peritoneal MΦ were cultured on coverslips in culture medium supplemented with 10% FBS. All cultures were incubated at 37°C with 5% CO_2 for 7 days to

allow differentiation of monocytes to MΦ. Media was changed at least once for all cultures before performing the bioassays.

2.8. MΦ activation

MΦ were activated by replacing the medium in each well with culture medium and appropriate serum supplement along with either r*Dn*IFN-γ (125.0 ng), rHuman IFN-γ (R&D Systems, Minneapolis, MN) (500 U/ml) or rMurine IFN-γ (Genzyme, Cambridge, MA) (500 U/ml). LPS (Sigma-Aldrich) at 5 ng/mL was used as the second signal and plates were incubated overnight at 37°C. Controls with LPS alone showed no activation.

2.9. *Toxoplasma* killing assay

Bioassays for the IFN-γ-mediated killing of intracellular *Toxoplasma gondii* were performed as described before [20;21]. Briefly, *T. gondii* strain RH was maintained by serial 2-day i.p. passage in Swiss-Webster mice (Harlan), harvested from the ascites fluid, and resuspended at 1-1.5 × 10^6 *T. gondii*/mL in culture medium containing the appropriate serum. Normal and activated MΦ monolayers were washed with PBS and 0.5 mL of the *T. gondii* suspension was added to each well. The plates were incubated for 1 h at 37°C and the coverslips were washed and transferred to a new plate with fresh medium. Some cover slips from both controls and activated cells were fixed and stained (Dif-Quick; Dade Behring, Inc., Newark, DE). The rest of the plates were incubated for 20 h at 37°C to accommodate the growth of the *T. gondii*. At this time the remaining cover slips were fixed, stained, and the individual tachyzoites in rosettes were enumerated.

2.10. Determination of *M. leprae* viability

Viable *M. leprae* were harvested from the footpads of athymic nu/nu mice and determined to be free of other microbial contaminants as described previously [22]. The viability of *M. leprae* was assessed by the oxidation of [^{14}C] palmitate in radiorespirometry using a procedure described before [23].

2.11. Determination of nitrite production

The MΦ culture supernatants were collected in 2 ml centrifuge tubes and stored at −70°C until the nitrite assay was performed. Nitrite production was determined using the Griess Reagent System (Promega, Madison, WI) according to the manufacturer's recommendations. Briefly, 50 μl of the culture supernatants were added in duplicate to a 96-well plate, 50μl of 1% sulfanilamide solution in 5% phosphoric acid was added and the plate was incubated for 10 min 25°C, protected from light. After incubation, 50μl of 0.1% N-1-napthylethylenediamine dihydrochloride solution was added, the plate was incubated for additional 10 min, and absorbance was measured with a plate reader (Bio-Rad) at 520-550 nm.

2.12. Indoleamine 2, 3-dioxygenase activity

r*Dn*IFN-γ bioactivity also was assessed by measuring indoleamine 2, 3-dioxygenase (IDO) activity. Armadillo MΦ monolayers were stimulated with serial dilutions of r*Dn*IFN-γ at 33° C for 72 h. For blocking experiments, monolayers were stimulated with 7.0 μg/mL r*Dn*IFN-γ in the presence of serial dilutions of the polyclonal antibody, anti-*Dn*IFN-γ #1 (500 μL/well) and L-tryptophan (500 μM/well). Supernatants were harvested, combined with 250 μL of 30% trichloroacetic acid, vortexed and heated to 50° C for 30 min to hydrolyze N-formylkynurenine to L-kynurenine. Samples were centrifuged at 10,000 rpm for 5 min and a 125 μl aliquot of the supernatant was added to 125μl of Ehrlich reagent (100 mg of *p*-dimethylbenzaldehyde + 5 ml of glacial acetic acid), loaded in a 96-well plate and absorbance read at 470 nm.

2.13. Protein Quantification

Protein concentrations were determined spectrophotometrically using BCA Protein Assay Kit (Peirce, Rockford, IL) according to the manufacturer's recommendation.

2.14. Statistical Analyses

The means and standard deviations of groups and replicate samples were compared in ANOVA and Tukey-Kramer *t*-tests using InStat statistical software (GraphPad Software, Inc, San Diego, CA).

3. RESULTS

3.1. *Dn*IFN-γ Sequence analyses

The 510 bp cDNA sequence encoding *Dn*IFN-γ was identified in the *D. novemcinctus* WGS, verified by direct PCR/DNA sequencing of armadillo cDNA and translated *in silico* into a 166 aa protein (Fig. 1). ClustalW multiple alignment analysis of the putative 166 aa *Dn*IFN-γ and other mammalian IFN-γs demonstrated that the *Dn*IFN-γ protein had the greatest homology to *Equus caballus* (horse) (E-value: 1e-70, 89% match), and a lesser homology to *Camelus bactrianus* (bactrian camel) (E-value: 9e-69, 87% match), *Lama glama* (llama) (E-value: 3e-67, 86% match), and *Canis familiaris* (dog) (E-value: 6e-67, 86% match) (Fig.2). Commercially available IFN-γs from porcine (E-value: 4e-55, 66% match), human (E-value: 2e-49, 62% match) and mouse (E-value: 1e-28, 43% match) were examined for potential homology to *Dn*IFN-γ. Porcine (*Sus scrofa*) IFN-γ was seen to be the most similar (Fig. 2) and subsequent studies showed porcine IFN-γ also to be effective for activating armadillo MΦ (data not shown).

3.2. Protein Purification

When expressed as recombinant protein, the r*Dn*IFN-γ was found in the insoluble fraction of the *E. coli* lysate (data not shown). The size of the purified r*Dn*IFN-γ protein (21.2 kDa) was determined *in silico* and by analyzing the size of the recombinant protein in Coomassie Blue-stained SDS PAGE gels (Fig. 3A) and in Western blot analysis (Fig. 3C) using rabbit polyclonal antibodies (antibodies Anti-*Dn*IFN-γ #1 (Fig. 3B) and Anti-*Dn*IFN-γ #2 (Fig. 3D). The r*Dn*IFN-γ protein appeared larger than the other calculated, mature, native IFN-γ's produced without glycosylation: *Sus scrofa* 17.1 kDa [24], *Homo sapiens* 17.5 kDa [25], and *Mus musculus* 15.8 kDa [26]. This increase in molecular weight likely was due to the inclusion of the 6 X His-tag and enterokinase recognition site (M R G S H H H H H H G M A S M T G G Q Q M G R D L Y D D D D K D H P F T) added to the mature polypeptide sequence and starting at residue C-21 in the recombinant product (Fig. 1).

3.3. r*Dn*IFN-γ Antibodies

Although both rabbit polyclonal *Dn*IFN-γ antibodies reacted specifically with their synthetic peptides and were useful in identifying the recombinant product by western blot analysis (Fig. 3 B, D), unfortunately, they failed to show sufficient reactivity when used in combination in capture ELISA for quantifying protein production. The hybridoma production resulted in only one monoclonal antibody that was reactive with armadillo r*Dn*IFN-γ. While it reacted specifically with its synthetic 15mer peptide LKVQRKAVNELFKVM, it also failed to work in combination with the polyclonal antibodies in capture ELISA (data not shown).

3.4. *Dn*IFN-γ gene transcript analysis

Stimulation of armadillo PBMC with ConA resulted in the induction of increased levels of *Dn*IFN-γ gene transcription as indicated by the increase in the *Dn*IFN-γ amplicons over that of the non-stimulated control using the *Dn*IFN-γ RT-PCR assay (Fig 4).

3.5. Effect of r*Dn*IFN-γ on IDO activity

In order to determine if *Dn*IFN-γ was biologically active we investigated its ability to induce IDO activity in armadillo MΦ. Results demonstrated that armadillo MΦ stimulated with r*Dn*IFN-γ induced IDO activity whereas MΦ cultured in medium alone produced no L-kynurenine (Table 1). The peak production (104.95 μg/mL L-kynurenine) was induced in armadillo MΦ with 7.0 μg/mL r*Dn*IFN-γ. Polyclonal antibody, anti-*Dn*IFN-γ #1, at 385 μg/mL, successfully blocked IDO activity after stimulation with these maximal levels of r*Dn*IFN-γ, with the resulting 11.2 μg/mL L-kynurenine indicating effective inhibition of the r*Dn*IFN-γ biological activity. Titered results showed 50% and 25% inhibition with 200ug and 50ug pAb concentrations respectively.

3.6. Effect of *Dn*IFN-γ on intracellular growth of *T. gondii.*

A *T. gondii* killing assay was used to determine the ability of r*Dn*IFN-γ to activate armadillo MΦ antimicrobial activity. Results demonstrated that mouse MΦ supported growth of intracellular *T. gondii* while IFN-γ activated MΦ significantly limited parasite growth (P = 0.0067) (Fig. 5). Human MN-derived MΦ from eight different donors supported growth of intracellular *T. gondii* while IFNγ-activated MΦ significantly limited parasite growth (P = 0.001) (Fig. 5). Armadillo MΦ from four animals were permissive for the intracellular growth of *T. gondii*; in contrast, armadillo MΦ activated with r*Dn*IFN-γ markedly limited the growth of the intracellular protozoan over a 20 h period (P = 0.0034). Controls with LPS alone did not activate armadillo MΦ to kill *T. gondii* (data not shown)

3.7. Effect of *Dn*IFN-γ on *M. leprae* viability

To further evaluate the ability of *Dn*IFN-γ to activate armadillo MΦ, we also examined their antimycobacterial properties, comparing these to activated mouse and human MΦ. The metabolism of *M. leprae* was significantly reduced (p=0.023) in mouse MΦ activated with rIFN-γ 24 hours prior to *M. leprae* challenge compared to normal mouse MΦ (Fig. 5). In contrast, no inhibition of *M. leprae* metabolic activity (P = 0.31) was seen in bacilli recovered from activated human MΦ (Fig. 5) or activated armadillo MΦ (P = 0.59) (Fig. 5).

3.8. Effect of r*Dn*IFN-γ on nitrite production

Mouse MΦ activated with murine rIFN-γ and LPS generated copious amounts of nitrites compared to normal MΦ (P = 0.0001) (Fig.5). In contrast, no significant increase in nitrite production (P = 0.866) was seen in activated human MΦ (Fig. 5) or armadillo MΦ after activation with r*Dn*IFN-γ and LPS (Fig. 5).

4. DISCUSSION

IFN-γ is a pleiotropic molecule produced primarily by T lymphocytes that plays an important role in resistance to intracellular pathogens via its ability to activate MΦ for antimicrobial activity. Therefore, IFN-γ is one of the key cytokines necessary for the study of immunologic processes in the armadillo leprosy model. The data presented here demonstrate that the mature *Dn*IFN-γ has been successfully produced, purified and that it possesses immunological activity.

The *Dn*IFN-γ shows high homology to other mammalian IFN-γ as evidenced by their low Expect (E) values. However, glycosylation prediction analysis using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) demonstrated a lower N-glycosylation level in *Dn*IFN-γ compared to that of other IFN-γ such as *H. sapiens* and *Mus musculus* (data not shown). It has been shown that glycosylation of the other mammalian IFN-γ may increase bioactivity [24] [25], but it apparently was not necessary for *Dn*IFN-γ function as evidenced by the appropriate biological properties we observed.

One such biological property studied was the induction of IDO activity. Human MΦ activated with IFN-γ produce IDO which rapidly depletes intracellular tryptophan and has been associated with an inhibitory effect on the growth of intracellular pathogens [27;28]. In the IDO pathway, tryptophan is decyclized to L-Kynurenine which can be measured spectrophotometrically and used as an indicator of IFN-γ activity [29]. In the present study *Dn*IFN-γ appropriately up-regulated IDO activity in armadillo MΦ, and polyclonal antibodies (anti-*Dn*IFN-γ #1), made against a synthetic peptide epitope of r*Dn*IFN-γ, effectively blocked IDO activity.

We also examined the production of RNI by MΦ following IFN-γ activation. MΦ activated with IFN-γ and a microbe-derived trigger, such as LPS, generate inducible nitric oxide synthase (iNOS) which catalyzes the formation of nitric oxide radical (NO) from L-arginine [30]. NO induces cytotoxic effects via its ability to inhibit iron and iron/sulfur containing enzymes involved in cellular respiration and DNA synthesis [31]. The end products of iNOS activity are nitrites, nitrates, and citrulline that can be measured in culture supernatants. Activated mouse peritoneal MΦ produce large quantities of nitric oxide [30;32], and we and others have previously shown that such MΦ inhibit both *T. gondii* [20] and *M. leprae* in an RNI dependent manner [8;33]. In contrast, peripheral blood derived human MΦ produce little nitric oxide after activation with IFN [10;11]. In this study we showed that armadillo MΦ, like their human counterparts, did not generate nitrites after activation with *Dn*IFN-γ. Interestingly, RNI, as measured by nitrotyrosine staining, have been demonstrated in human tissues at sites of infection, including leprosy lesions [34;35] and *M. tuberculosis* infected lungs [36]. Whether or not armadillos express RNI in leprosy lesions is currently under investigation.

One of the most important functions of the activated MΦ is the killing of intracellular pathogens. This complex process is a culmination of a series of events that ultimately affords protection to the host. Previous reports have demonstrated a role for the enhanced microbicidal activity of the IFN-γ-activated mouse MΦ against a variety of mycobacterial pathogens, including *M. tuberculosis* [37] and *M. leprae*[38]. However, unlike the murine system, IFN-γ does not appear to activate human MΦ to kill or inhibit mycobacteria such as: *M. tuberculosis* [37], *M. avium*[39] and *M. phlei* [40]. In the well-controlled studies from Crowle's group [41], MdMΦactivated by IFN-γ efficiently killed the intracellular protozoan Leishmania but not *M. tuberculosis*. In the present study we explored killing of another intracellular protozoan, *T. gondii* in parallel with *M. leprae* by IFN-γ-activated MΦ from human, mice and armadillos. Our results confirm the finding that IFN-γ-activated mouse and human MΦcan kill intracellular protozoa [42;43] and we also showed that activated armadillo MΦ are microbicidal for *T. gondii*. In contrast however, while activated mouse MΦreadily cope with *M. leprae*, neither IFN-γ-activated human nor *Dn*IFN-γ-activated armadillo MΦ killed or inhibited the leprosy bacillus.

Armadillos, like humans, manifest leprosy over a broad histopathological spectrum, and are considered potentially valuable models for studying leprosy pathogenesis, investigating susceptibility and resistance to *M. leprae*, monitoring the evolution of the immune response to this pathogen, and developing new diagnostic assays and vaccines. The *in vitro* response of MΦ to IFN-γ activation shown in this study highlights interesting similarities between humans and armadillos in their cell mediated immune response and affirms an important value of this animal as a model for the study of human leprosy.

Besides man, nine-banded armadillos are the only immunologically intact animal hosts that exhibit natural susceptibility to high levels of infection with *M. leprae*. Like man, armadillos also manifest their leprosy over a broad spectrum of clinical and histopathological responses. The factors which underlie their unique shared susceptibility to *M. leprae*, or that trigger the immune response of individual animals to manifest the disease over such a diverse clinical

spectrum remain mysteries. Access to the armadillo whole genomic sequence will greatly benefit our ability to develop additional armadillo immunological reagents. Availability of r*Dn*IFN-γand other cytokines, probes and cell markers that are now in development will help advance these animals as important models in leprosy research.

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  1      M   N   Y   T   G   Y   L   L   A   F   Q   L   C   I   I   15
  1    ATG AAT TAC ACG GGC TAT CTC TTA GCT TTT CAG CTT TGC ATC ATT  45
 16      L   G   S   S   S   C   Y   C   Q   A   T   F   L   R   E   30
 46    TTG GGT TCT TCT AGC TGC TAC TGC CAG GCC ACT TTT TTG AGA GAA   90
 31      I   Q   T   L   K   E   Y   F   N   A   S   D   S   D   V   45
 91    ATA CAA ACT CTA AAG GAA TAT TTT AAT GCA AGT GAT TCA GAT GTA 135
 46      A   D   G   G   P   L   F   L   D   I   L   K   N   W   K   60
136    GCA GAT GGT GGA CCT CTT TTC TTA GAT ATT TTG AAG AAC TGG AAA 180
 61      E   E   S   D   K   K   I   I   Q   S   Q   I   V   S   V   75
181    GAG GAG AGT GAC AAA AAA ATA ATT CAG AGC CAG ATC GTC TCT GTT 225
 76      Y   F   K   I   F   D   N   L   K   D   N   Q   I   I   Q   90
226    TAC TTC AAA ATC TTT GAT AAT TTA AAA GAC AAC CAG ATC ATC CAA 270
 91      K   S   M   A   T   I   K   E   D   L   I   A   K   F   F  105
271    AAG AGC ATG GCT ACC ATC AAG GAA GAC CTA ATT GCC AAG TTC TTC 315
106     N   S   S   S   S   K   L   N   D   F   L   K   L   I   R  120
316    AAT AGC AGT TCC AGC AAG CTG AAT GAC TTC CTG AAG CTG ATT CGA 360
121     T   P   V   N   D   L   K   V   Q   R   K   A   V   N   E  135
361    ACT CCG GTA AAT GAC CTG AAG GTC CAG CGC AAA GCA GTA AAT GAA 405
136     L   F   K   V   M   N   D   L   S   P   K   S   N   L   R  150
406    CTC TTC AAA GTG ATG AAT GAT CTG TCA CCC AAA TCT AAC CTA AGG 450
151     K   R   K   R   S   Q   S   T   F   Q   G   R   R   A   S  165
451    AAG CGA AAA AGG AGT CAG AGT ACG TTT CAA GGC AGG AGA GCA TCA 495
166     I stop 166
496    AAA TAA  501
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Figure 1.
Putative *Dn*IFN-γ cDNA (GI: DQ094083) and predicted protein. Italicized characters indicate the putative signal peptide.

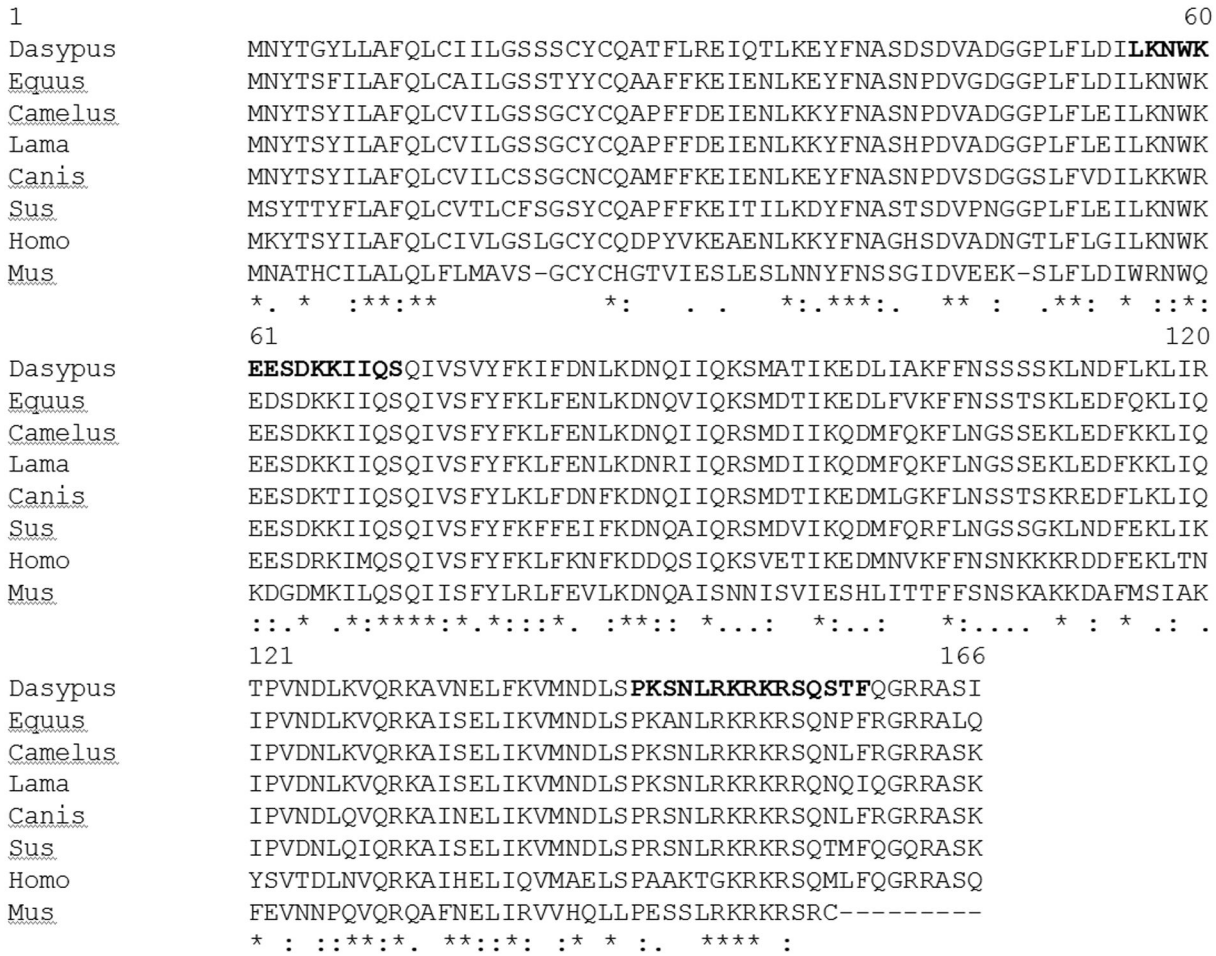

Figure 2.

Clustal W alignment of *Dn*IFN-γ-IFN-γ Dasypus (*Dasypus novemcinctus*) with Equus (*Equus caballus*), Camelus (*Camelus bactrianus*), Lama (*Lama glama*), Canis (*Canis familiaris*), Sus (*Sus scrofa*), Homo (*Homo sapiens*), and Mus (*Mus musculus*) IFN-γs. Epitopes used to generate polyclonal antibodies =Anti-*Dn*IFN-γ #1 and Anti-*Dn*IFN-γ #2 are indicated by bold text. The signal peptide is indicated in italics. "*" indicates positions which have a single, fully conserved residue. ":" indicates that one of the following "strong" groups is fully conserved: STA NEQK NHQK NDEQ QHRK MILV MILF HY FYW. "." indicates that one of the following "weaker" groups is fully conserved: CSA ATV SAG STNK STPA SGND SNDEQK NDEQHK NEQHRK FVLIH FYM.

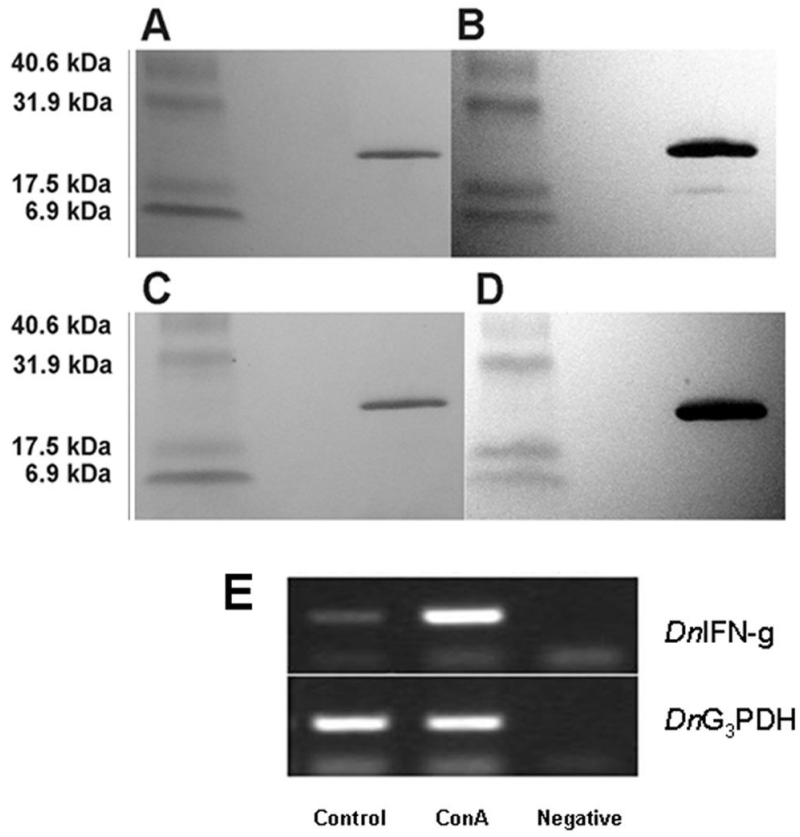

Figure 3.

(**A** and **C)**. Purified r*Dn*IFN-γ (21.22 kDa) separated on a 4% to 20% gradient polyacrylamide gel and stained with Coomassie Blue Brilliant; (**B** and **D**) Western blots with polyclonal antibodies Anti-*Dn*IFN-g #1 and Anti-*Dn*IFN-g #2, respectively. **E.** Semi-quantitative RT-PCR analysis of *Dn*IFN-γ gene expression in armadillo PBMC: Control, non-stimulated armadillo PBMC cDNA; ConA, ConA-stimulated armadillo PBMC cDNA; Negative, buffer control; *Dn*IFN-γ, *Dn*IFN-γ PCR products; *Dn*G_3PDH, *Dn*G_3PDH housekeeping gene PCR products.

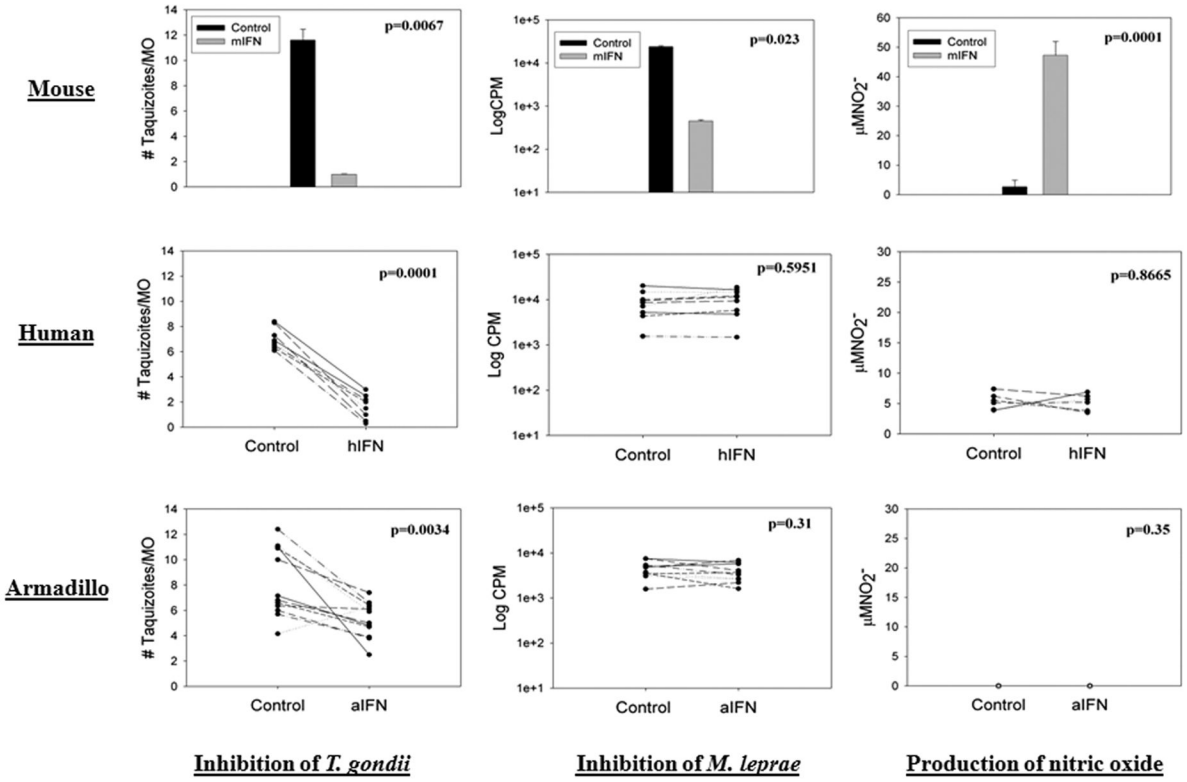

Figure 4.

Inhibition of intracellular growth of *T. gondii*, viability of *M. leprae*, and Nitrite production in mouse, human and armadillo ṂΦ. MΦ were unstimulated (Control) or activated for 24 h with the appropriate rIFNγ + 5 ng/ml LPS (ACT) prior to challenge with *T. gondii or M. leprae*. Growth of *T. gondii* indexed by enumerated intracellular rosettes, and viability of *M. leprae* indexed with radiorespirometry of ^{14}C-palmitate. Nitrites were assessed on supernatants using the Griess reagent system. Results are representative of three independent experiments in mouse, 8 different human donors, and 4 different armadillos.

Table 1

Indoleamine 2,3-dioxygenase (IDO) production by armadillo MΦ

Concentration of r*Dn*IFN-γ (μg/mL)	**L-Kynurenine production (± SD) in armadillo MΦ (μg/mL)[a]**
7.0	103.6 ± 43.7
1.6	31.3 ± 28.8
0.36	4.7 ± 1.7
0.078	3.7 ± 1.2
0.0156	3.6 ± 1.1
0.0	3.7 ± 0.9
7.0 + anti-IFN-γ	11.2 ± 2.6

IDO production by armadillo MΦ showed a dosal response to stimulation with r*Dn*IFN-g, and was significantly inhibited with anti-IFN-γ antibody.

[a] IDO activity was measured by L-Kynurenine production.