

IL-4 and IL-10 inhibition of IFN- γ - and TNF- α -dependent nitric oxide production from bovine mononuclear phagocytes exposed to *Babesia bovis* merozoites

W.L. Goff^{a,*}, W.C. Johnson^a, S.M. Parish^b, G.M. Barrington^b,
T.H. Elsasser^c, W.C. Davis^d, R.A. Valdez^a

^aAnimal Disease Research Unit, USDA-ARS, Washington State University, 3003 ADBF,
P.O. Box 646630, Pullman, WA 99164-6630, USA

^bDepartment of Veterinary Clinical Sciences, Washington State University, Pullman, WA 99164, USA

^cGrowth Biology Laboratory, Animal and Nutrition Research Institute, Beltsville Agricultural Research Center,
USDA-ARS, Beltsville, MD 20705-2350, USA

^dDepartment of Veterinary Microbiology and Pathology, Washington State University,
Pullman, WA 99164, USA

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Abstract

The requirement for IFN- γ and/or TNF- α as co-stimulants with *Babesia bovis* merozoites for nitric oxide (NO) production was examined, as well as the regulatory role of IL-4 and IL-10. Purified *B. bovis* merozoites did not induce the production of NO in undifferentiated monocytes without addition of exogenous IFN- γ and TNF- α unless the monocytes taken ex vivo were producing TNF- α endogenously. Under the latter condition, the NO production resulting from merozoite stimulation remained IFN- γ -dependent. There was no evidence for endogenous synthesis of TNF- α in monocyte-derived macrophages (MDM), and merozoites alone were incapable of inducing TNF- α mRNA in MDM. However, while merozoites plus IFN- γ induced TNF- α mRNA expression in MDM, NO was not produced. Both IL-4 and IL-10 inhibited expression of iNOS and production of NO in merozoite-stimulated monocytes. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Bovine; Monocyte/macrophage; Cytokines; iNOS/nitric oxide; *Babesia bovis*

* Corresponding author. Tel.: +1-509-335-6003; fax: +1-509-335-8328.
E-mail address: wgoff@vetmed.wsu.edu (W.L. Goff).

1. Introduction

Monocytes and macrophages play a central role in immune responses as antigen presenting cells, immunoregulatory cells and as effector cells. While monocytes and macrophages share many characteristics, there are differences in response to cytokine signals and phagocytic stimuli: differences based upon the state of cell maturation and differentiation (Cao et al., 1989; Watanabe and Jacob, 1991; Van Hal et al., 1992; Rutherford et al., 1993; Jungi et al., 1996; Goff et al., 1996, 1998).

IFN- γ and TNF- α are co-stimulants for expression of iNOS in bovine monocytes (Jungi et al., 1996; Goff et al., 1996, 1998). Exposure of monocytes and macrophages to various microbial agents also induces nitric oxide (NO) production that may, or may not, be IFN- γ -dependent. Induction of NO in bovine monocytes and monocyte derived macrophages (MDM) after exposure to gram-negative bacteria like *Salmonella dublin* is an IFN- γ -independent event (Adler et al., 1995, Goff et al., 1996, 1998). Exposure to *Listeria monocytogenes*, a gram-positive bacterium, induces NO production that is greatly enhanced in the presence of IFN- γ (Jungi et al., 1997). In addition, *S. dublin* also upregulates TNF- α mRNA expression indicating that TNF- α may be required as an autocrine stimulant (Adler et al., 1995). Exposure to *Babesia bovis* merozoites or merozoite membranes also induces NO, but induction is entirely IFN- γ -dependent (Stich et al., 1998). The role of TNF- α in the response to *B. bovis* merozoite exposure has not been determined.

Recently, an in vivo analysis of the cytokine response to *B. bovis* infection in the spleen of innately immune calves revealed the importance of IL-12, IFN- γ and NO to protection (Goff et al., 2001), suggesting immunity is associated with development of a type-1 response involving NO that may be modulated by IL-4 and/or IL-10.

IL-10 has been shown to down-regulate NO production and inhibit macrophage microbicidal activity (Oswald et al., 1992; Gazzinelli et al., 1992). Jungi et al. (1997) have demonstrated the antagonistic effects of IL-4 and IFN- γ for expression of iNOS in bovine MDM following exposure to *S. dublin* and *L. monocytogenes*. The experiments we report here extend these observations to a protozoan parasite. We show that iNOS expression in bovine monocytes, in the presence of *B. bovis* merozoites, is dependent on both IFN- γ and TNF- α and that expression is inhibited by both IL-4 and IL-10.

2. Materials and methods

2.1. Source and concentrations of reagents

Recombinant bovine interferon gamma (rBo-IFN- γ) was a generous gift of Drs. Loren Babiuk and Dale Godson, Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada, and was used at 50 U/ml. Recombinant bovine tumor necrosis factor-alpha (rBo-TNF- α) was used at 250 pg/ml. Recombinant human IL-4 (rHu-IL-4) and recombinant human IL-10 (rHu-IL-10) (R&D Systems) were used at 10 ng/ml and 100 U/ml, respectively. To neutralize rBo-IFN- γ , 2 μ l of a specific antibody (Raggio et al., 2000)

was added to each well containing 10 U of IFN- γ . TNF- α produced endogenously by cultured cells was neutralized by incubating 200 μ l cell cultures with 5 μ l rabbit anti-bovine TNF- α (Kenison et al., 1990; Rewinski and Yang, 1994) for 2 h at 37 °C, 5% CO₂ prior to the addition of cytokine or microbial stimulants. For microbial stimulants, 200 μ g/ml heat killed *S. dublin* was used (generously provided by Dr. Tom Besser, Washington State University), or *B. bovis* merozoites isolated as previously described (Goff et al., 1988).

2.2. Source and preparation of leukocytes

Peripheral blood mononuclear cells (PBMC) and spleen mononuclear cells (SMC) were obtained from Holstein Friesian calves less than 8 months of age. The animals were maintained according to the American Association for Laboratory Animal Care procedures, and were provided an acceptable bovine ration, with water and mineral block ad libitum. Each animal underwent a surgical procedure at 3 months of age to marsupialize the spleen for ready access of SMC (Varma and Shatry, 1980; Goff et al., 1991). Initially, spleen architecture, cell phenotypes and function (including cytokine expression) were evaluated after multiple daily collections without evidence of significant changes due to the technique alone.

SMC were obtained from splenic aspirates aseptically collected in 60 ml syringes containing 15 ml acid-citrate-dextrose (ACD) pH 7.3 under local anesthesia. The splenic aspirate was processed into a single cell suspension using a tissue homogenizer, and the mixture diluted 1:1 with phosphate buffered saline (PBS) without Mg⁺² or Ca⁺². PBMC were obtained from 500 ml of blood. SMC and PBMC were prepared as previously described (Goff et al., 1996). Briefly, splenic cell suspensions or buffy coat cells from blood were layered onto 20 ml of Hypaque-Ficoll (1.086 g/l) (Accu-Paque, Accurate Chemicals, Westbury, NY) and centrifuged for 30 min at 1500g at 4 °C. SMC or PBMC from similar gradients were collected, pooled, and washed in 50 ml Dulbecco's modified eagle's medium (DMEM), pH 7.2, for 7 min at 1500g at 4 °C. The cells were suspended with DMEM and centrifuged twice for 7 min at 400g and 4 °C to remove platelets. Residual erythrocytes were lysed by suspending the pellet in three volumes of sterile, ambient temperature lysis buffer (AKC: 0.15 M NH₄Cl, 1.0 mM KHCO₃, 0.1 mM EDTA, pH 7.3). After 1 min, the SMC and PBMC were centrifuged for 5 min at 200g and 4 °C, washed in 50 ml DMEM, and suspended to 1 \times 10⁷ cells/ml in Iscove's medium (Gibco BRL, Gaithersburg, MD) containing 25 mM Hepes, 2 μ M glutamine, 10 μ g/ml gentamicin, 50 μ M mercaptoethanol, and 15% essentially endotoxin-free fetal bovine serum (FBS) (<0.06 EU/ml as assayed by limulus amoebocyte lysate gelation) (Hyclone, Logan, UT). Experiments involved total (monocyte-containing) cells, adherent cells, or differentiated macrophages from PBMC and SMC. Adherent cells were separated from total SMC and PBMC by incubating at 37°, 5% CO₂ for 2 h in FBS-treated polystyrene flasks. Non-adherent cells were removed by washing five times with warm DMEM. The flasks were further incubated overnight and washed again. Adherent cells were recovered by incubating in PBS without Mg⁺² or Ca⁺² containing 0.2% EDTA and 5% FBS, pH 7.35, on ice for 30 min. Dislodged cells were collected and washed once with DMEM and suspended in Iscove's medium. Adherent cells were either used immediately or allowed to differentiate

into tissue derived macrophages by transferring cells to a Teflon bag (AFC, Gaithersburg, MD) and incubating as before for 7 days. Total SMC and PBMC preparations were plated at a density of 1×10^7 cells/ml and adherent SMC, PBMC or differentiated macrophages at 1×10^6 cells/ml in Iscove's medium.

2.3. Culture conditions

For release of NO, SMC and PBMC were incubated in the presence of medium alone or under conditions described in each figure legend. Cultures were incubated for 3 days at 37 °C, 5% CO₂ before supernatants were assessed for evidence of NO production by detection of nitrite, the stable oxidized form of NO, by the Griess reaction. For wells harvested for RT-PCR, the SMC and PBMC were stimulated and incubated as described above for 8 h before the collection of cells and isolation of RNA by Trizol (BRL, Bethesda, MD).

2.4. Griess reaction

Accumulation of nitrite in culture supernatants was determined as previously described (Ding et al., 1988) by mixing 50 µl of supernatant with 200 µl of Greiss reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H₃PO₄). The assay was incubated at room temperature for 10 min and the absorbance at 540 nm determined with a microplate spectrophotometer and compared with a standard of 1.5–200 µM NaNO₂.

2.5. RT-PCR

Samples of isolated RNA were processed for RT-PCR amplification of IFN- γ , iNOS, TNF- α , IL-1, IL-10, IL-12 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as previously described (Goff et al., 1998). Briefly, RNA samples reverse transcribed by Superscript II Reverse Transcriptase (BRL) and oligo-(dT)₁₂ primer were compared to RNA samples without reverse transcription for specific amplification of mRNA. Taq DNA polymerase (BRL) amplified products were resolved on 4% NuSieve 3:1 (FMC BioProducts, Rockland, ME) agarose gels containing ethidium bromide. The level of mRNA expression in each sample was determined by densitometric image analysis and standardized against the GAPDH measurement (IS-1000 Digital Imaging System and Alpha-EASE 3.21 software, San Leandro, CA). Progressive GAPDH cDNA dilutions were evaluated and optimized by densitometry to confirm the reliability of the method as a semi-quantitative measure of mRNA expression (correlation coefficient of dilutions versus densitometric values: $r = 0.966$; slope of the dilution curve: densitometric value $Y = a + bx$, where $a = 3800$, $b = -65.53$, therefore, $Y = 3800 - 65.53x$). Each mRNA expression level is presented as relative units after normalization to the observed GAPDH expression level. In addition, a change of 40% in relative units from the basal medium control expression was arbitrarily established as a requirement for the expression to be considered significant up or down regulation.

2.6. Statistical analysis

Student's *t*-test was used to compare NO production between animal groups and treatments within groups. A *P* value of <0.05 was considered significant.

3. Results

3.1. *Ex vivo* demonstration of IFN- γ and TNF- α effects on nitric oxide production from monocytes

Bovine monocytes can respond to IFN- γ plus TNF- α as soluble signals for the production of NO for a short time in culture and early in the differentiation from monocytes to macrophages (Goff et al., 1998). In previous experiments (Goff et al., 1996), we determined that there was no difference in NO production in response to stimulation between total PBMC that contain monocytes and monocytes used immediately after purification by DH59B MoAb selection (data not shown). This allowed PBMC to be used saving time and cell manipulation. These monocytes are able to respond to exogenous TNF- α alone if the cells are either simultaneously, or have recently been exposed to IFN- γ (Goff et al., 1998). They typically respond to the addition of IFN- γ alone suggesting that TNF- α is operative in an autocrine fashion. In order to investigate this further, it was first necessary to establish that NO release from monocytes stimulated with IFN- γ alone is a measure of the presence of endogenous TNF- α . This was demonstrated as illustrated in Fig. 1, where NO levels produced as a result of stimulation with IFN- γ alone was inhibited up to 75% in a dose response fashion with increasing concentrations of a TNF- α -neutralizing antibody. Thus, the presence of TNF- α is necessary for the production of NO and indicates that NO release from monocytes stimulated with IFN- γ is a suitable

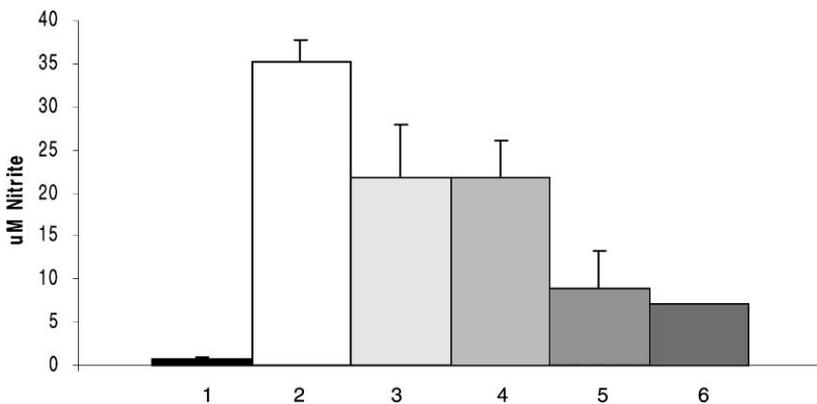


Fig. 1. The effect of neutralizing TNF- α on nitric oxide production from bovine monocytes responsive to stimulation with IFN- γ alone. Each lane represents a different stimulation condition: (1) medium control; (2) IFN- γ plus TNF- α control; (3) IFN- γ alone; (4) IFN- γ plus 1 μ l anti-TNF- α ; (5) IFN- γ plus 2 μ l anti-TNF- α ; (6) IFN- γ plus 5 μ l anti-TNF- α .

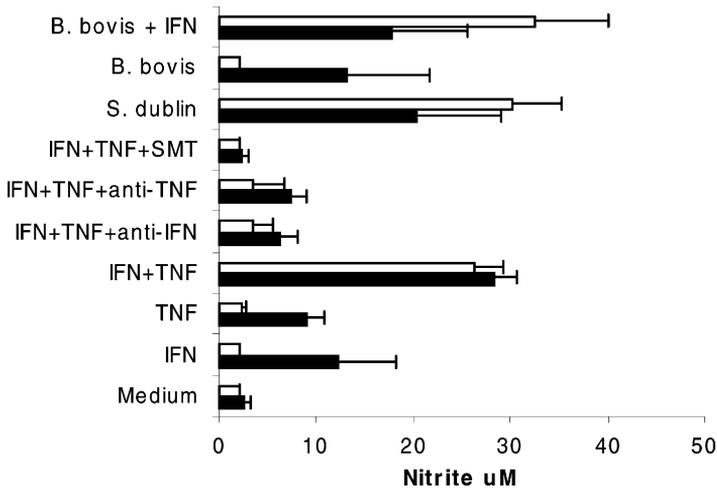


Fig. 2. Nitric oxide production from bovine monocytes in response to soluble signal and microbial stimulation. Black bars: monocytes from four calves that responded to IFN- γ and TNF- α alone. White bars: monocytes from five calves that were unresponsive to IFN- γ and TNF- α alone. Antibodies specific for IFN- γ and TNF- α were neutralizing antibodies as described in M&M. SMT: S-methylisothiourea, an iNOS inhibitor. Where indicated in Section 3, significance was at the level of $P < 0.05$.

biological assay for the presence of TNF- α . The necessity for both IFN- γ and TNF- α in a NO burst was confirmed in cultures obtained from nine cattle (Fig. 2), where monocytes from four of the nine animals produced a significant ($P < 0.05$) amount of NO in response to stimulation with IFN- γ alone and TNF- α alone suggesting that both molecules were, or had recently been present and had interacted with these cells in vivo.

Bovine macrophages exposed to *S. dublin* has previously been demonstrated to up-regulate the production of TNF- α and to stimulate NO production in the absence of IFN- γ (Adler et al., 1995), while NO release from bovine macrophages exposed to *B. bovis* merozoites is IFN- γ -dependent (Stich et al., 1998). Fig. 2 also demonstrates bovine monocytes respond similarly. Monocytes from animals responding to TNF- α alone, responded to both *S. dublin* and *B. bovis* without exogenous addition of IFN- γ , while monocytes from animals that failed to respond to TNF- α responded to *S. dublin* but not to *B. bovis* unless exogenous IFN- γ was provided. The difference between groups was significant ($P < 0.05$).

3.2. TNF- α -dependent nitric oxide release from monocytes and IL-4 and IL-10 regulation

Monocytes from the same nine cattle used for the data in Fig. 2 were stimulated for NO production in the presence or absence of IL-4 and/or IL-10 (Fig. 3). Again, there was significant ex vivo evidence for either or both IFN- γ and TNF- α activity in four of the cattle. IL-4 and IL-10 significantly down-regulated the NO production resulting from stimulation of monocytes from these four animals with either IFN- γ or TNF- α . However, IL-10 was considerably less effective when both stimulants were used together (Fig. 3a).

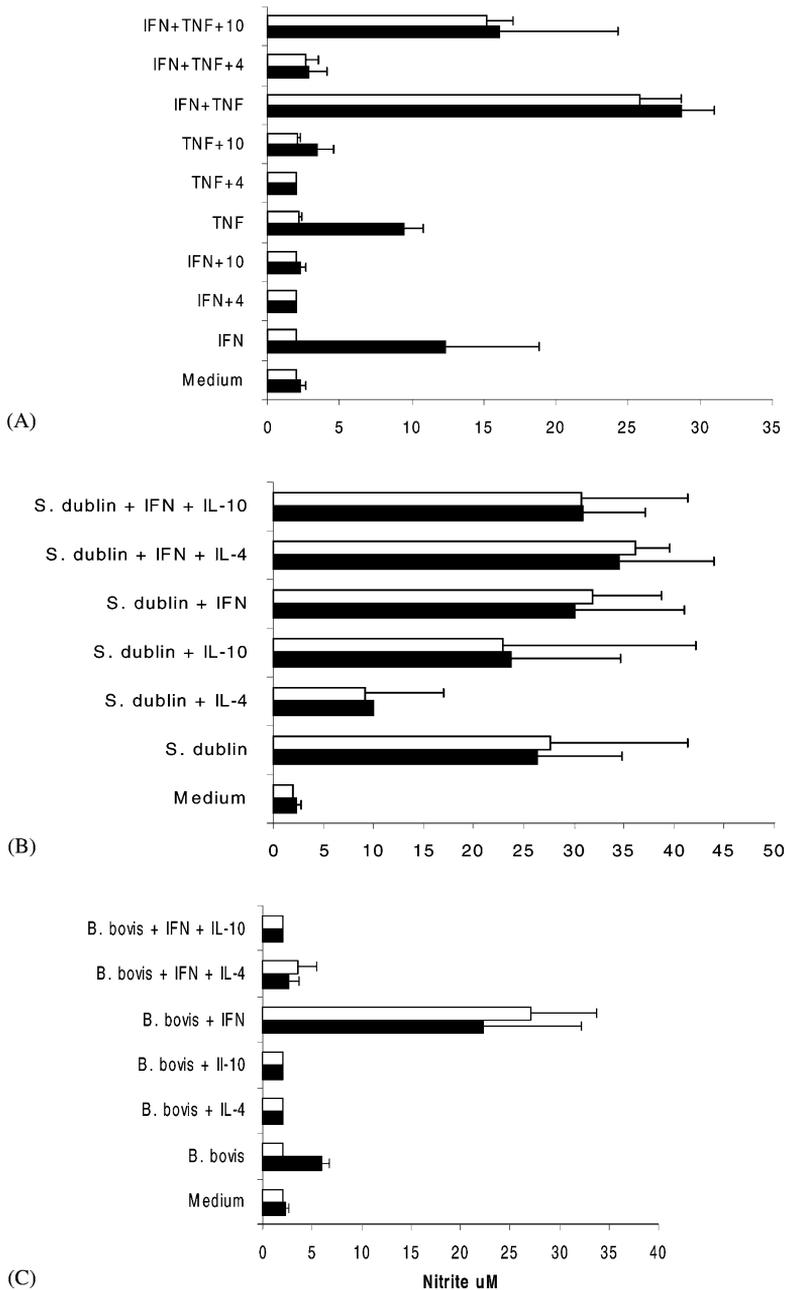


Fig. 3. IL-4 and IL-10 regulation of nitric oxide production from bovine monocytes in response to stimulation with IFN- γ and/or TNF- α (A), *S. dublin* (B), or *B. bovis* merozoites (C). Black bars: monocytes from four calves that responded to IFN- γ and TNF- α alone. White bars: monocytes from five calves that were unresponsive to IFN- γ and TNF- α alone. 4 = IL-4, 10 = IL-10. Where indicated in Section 3, significance was at the level of $P < 0.05$.

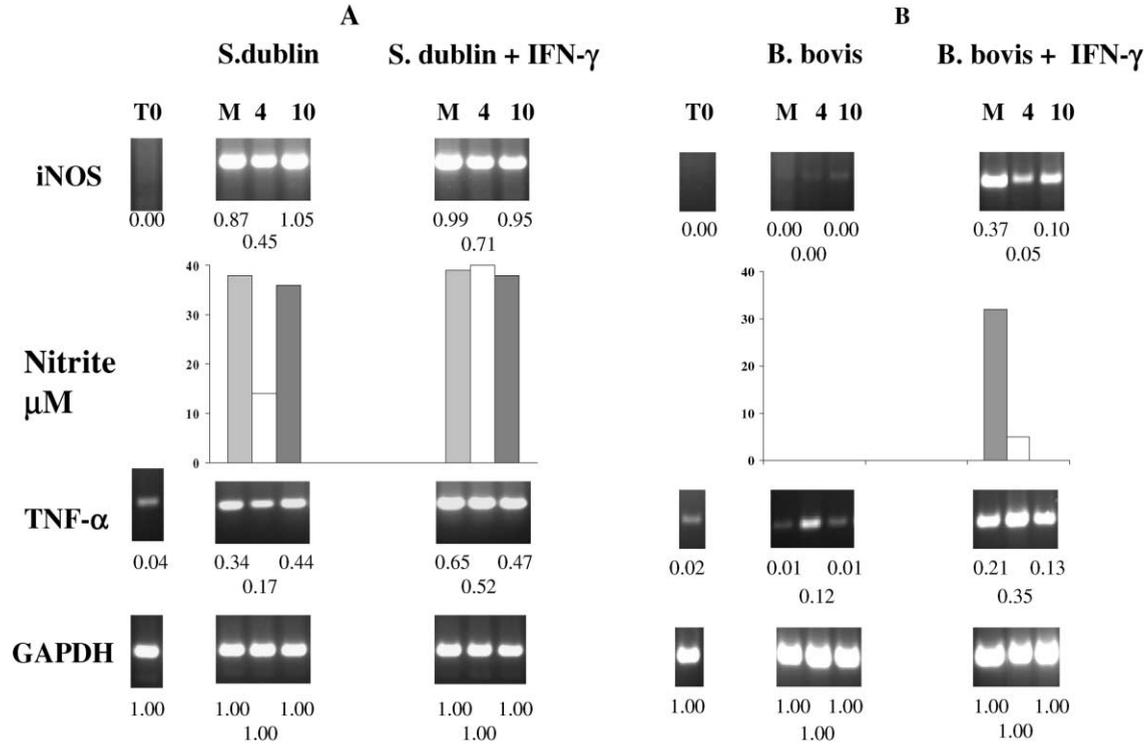


Fig. 4. iNOS and TNF- α mRNA expression and nitric oxide production from bovine monocytes exposed to *S. dublin* (A) or *B. bovis* merozoites (B) either alone or in the presence of IFN- γ , and under the influence of either IL-4 or IL-10. Data is representative of several experiments. T_0 : time zero cells before stimulation; M: media control; 4: IL-4; 10: IL-10. Numerical values beneath each lane of mRNA expression was determined by densitometric image analysis and standardized against the GAPDH measurement. Each mRNA expression level is presented as relative units after normalization to the observed GAPDH expression level. Nitrite levels, as a measure of NO production, were determined from culture supernatants corresponding to the cellular mRNA expression lanes above and below.

Monocytes from all nine animals responded to *S. dublin* exposure, and IL-4 significantly down-regulated the response in contrast to IL-10 (Fig. 3b). As previously demonstrated (Jungi et al., 1997), the addition of IFN- γ did little to enhance the response to *S. dublin*, but reversed the regulatory effect of IL-4 (Fig. 3b).

The only monocytes to produce significant amounts of NO in response to *B. bovis* merozoites alone were from the animals where there was evidence for in vivo activity of both IFN- γ and TNF- α , and both IL-4 and IL-10 significantly down-regulated this response (Fig. 3c). Unlike with *S. dublin*, IL-10 inhibited as well as IL-4 and IFN- γ failed to reverse either IL-4 or IL-10 inhibition when added exogenously.

As shown in Fig. 4, NO regulation is at the level of iNOS expression, with iNOS significantly downregulated by IL-4 in both *S. dublin* (Fig. 4a) and *B. bovis* merozoite plus IFN- γ (Fig. 4b) stimulated monocytes. IL-10 also significantly downregulated iNOS and NO production from monocytes stimulated with *B. bovis* merozoites plus IFN- γ . Interestingly, in the presence of IL-4, TNF- α message was significantly reduced in *S. dublin*-stimulated monocytes, while significantly increased in *B. bovis* merozoite-stimulated monocytes. TNF- α mRNA expression levels were unaffected with the additional presence of IFN- γ .

Table 1
Nitric oxide production from monocytes in blood and spleen versus fully differentiated macrophages

Stimulant	Source			
	PBMC		SMC	
	Total ^a	Differentiated ^b	Total	Differentiated
Medium	<2 ^c	<2	<2	<2
IFN- γ	12.90 (0.74) ^d	<2	10.90 (0.23)	<2
TNF- α	3.29 (0.33)	<2	5.67 (0.17)	<2
IFN- γ + TNF- α	24.41 (0.20)	<2	22.52 (1.03)	<2
IFN- γ + TNF- α + SMT ^e	<2	<2	<2	<2
<i>S. dublin</i>	22.70 (1.99)	26.61 (1.17)	32.32 (1.10)	12.48 (0.51)
<i>S. dublin</i> + IL-4	10.49 (0.20)	<2	ND	2.26 (0.14)
<i>S. dublin</i> + IL-10	20.82 (0.54)	26.70 (2.21)	ND	12.37 (0.82)
<i>S. dublin</i> + IFN- γ	25.14 (2.68)	65.15 (1.46)	23.48 (1.41)	38.69 (1.51)
<i>S. dublin</i> + IFN- γ + IL-4	25.84 (0.19)	61.25 (3.22)	ND	38.81 (1.07)
<i>S. dublin</i> + IFN- γ + IL-10	26.35 (0.22)	69.18 (3.22)	ND	41.54 (1.90)
<i>B. bovis</i>	<2	<2	<2	<2
<i>B. bovis</i> + IL-4	<2	<2	ND	<2
<i>B. bovis</i> + IL-10	<2	<2	ND	<2
<i>B. bovis</i> + IFN- γ	24.04 (1.13)	<2	17.70 (2.10)	<2
<i>B. bovis</i> + IFN- γ + IL-4	3.75 (0.25)	<2	ND	<2
<i>B. bovis</i> + IFN- γ + IL-10	<2	<2	ND	<2

^a Total cells containing monocytes before adherence and culture for 7 days in Teflon bags.

^b Adherent cells cultured for 7 days in Teflon bags.

^c μ M nitrite in culture supernatant determined by the Griess reaction where 2 μ M is the lower limit of detection.

^d SEM in parentheses.

^e Nitric oxide synthase inhibitor.

3.3. Downregulation of TNF- α during differentiation from monocytes to macrophages

Monocytes and monocyte-derived macrophages (MDM) from both blood and spleen were compared for NO production when stimulated with IFN- γ and/or TNF- α and *S. dublin* and *B. bovis* merozoites with and without IL-4 and IL-10. Table 1 shows the results from an experiment where the animal's monocytes produced NO in response to either IFN- γ or TNF- α alone. There was no difference due to the source of monocytes, whether circulating in blood (PBMC) or through the spleen (SMC). However, MDM were not responsive to IFN- γ or TNF- α alone, nor did they produce NO when stimulated by the combination of IFN- γ and TNF- α . MDM and monocytes produced NO following *S. dublin* exposure and the production was inhibited by IL-4. This inhibition was reversed in the presence of IFN- γ . As before, IL-10 had no regulatory effect. The only difference was that unlike monocytes where IFN- γ has typically not enhanced NO production in response to *S. dublin* exposure, MDM produced considerably more NO when stimulated by both the bacterium and IFN- γ .

Neither monocytes nor MDM produced NO following exposure to *B. bovis* merozoites alone (Table 1). Monocytes produced low levels of NO in response to TNF- α alone, suggestive of little or very recent exposure to IFN- γ , a conclusion supported by the fact that the monocytes required exogenous IFN- γ with merozoites for a NO burst. MDM did not produce NO in response to merozoites plus IFN- γ . The failure of MDM to produce NO under any condition except when exposed to *S. dublin*, where TNF- α is known to be up-regulated, indicated that fully differentiated but unstimulated MDM may not produce TNF- α . This was directly demonstrated at the mRNA level where there was no evidence of TNF- α mRNA expression with unstimulated MDM (Fig. 5). Moreover, unstimulated

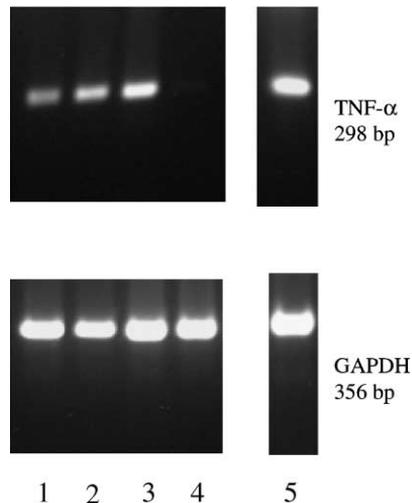


Fig. 5. TNF- α mRNA expression from unstimulated total PBMC containing 8% monocytes as defined by flow cytometry and the monocyte-specific monoclonal antibody DH59B, lane 1; >95% unstimulated plastic adherent monocytes, lane 2; unstimulated non-adherent cells containing approximately 2% DH59B⁺ cells, lane 3; unstimulated monocyte-derived macrophages after 7 days of differentiation in Teflon bags, lane 4; TNF- α and GAPDH gene sequences cloned into pCR2.1 as positive controls, lane 5.

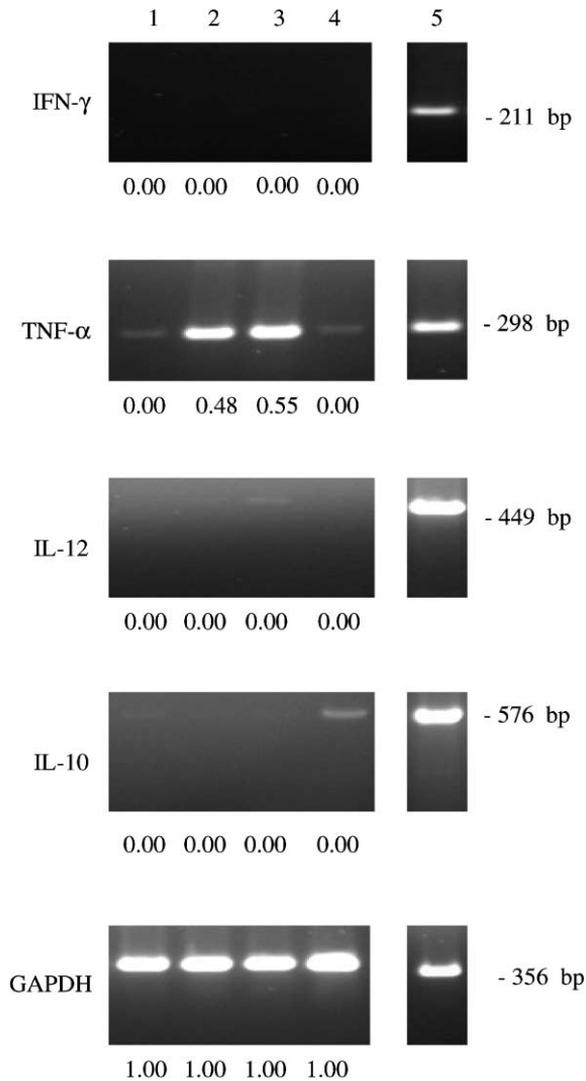


Fig. 6. Cytokine mRNA expression from unstimulated monocyte-derived macrophages after 7 days in Teflon bags, lane 1; or stimulated for 8 h with IFN- γ , lane 2; with *B. bovis* merozoites plus IFN- γ , lane 3; with *B. bovis* merozoites alone, lane 4. Lane 5 represents amplification of each gene sequence cloned into pCR2.1 as a positive control.

MDM appeared to be quiescent with no evidence of IL-10 or IL-12 mRNA expression (Fig. 6). There was no IFN- γ mRNA expression in any of the conditions indicating that either there were no contaminating lymphocytes in these cultures or if present, they were not expressing IFN- γ message. When IFN- γ alone or with merozoites was used to stimulate MDM, TNF- α message was expressed (Fig. 6). Thus, IFN- γ appears to be sufficient to

up-regulate TNF- α mRNA expression. In contrast, merozoites alone or with IFN- γ failed to induce IL-12 mRNA expression.

4. Discussion

Monocytes present in PBMC will produce NO when stimulated with TNF- α alone if there has been recent exposure to IFN- γ (Goff et al., 1998). The reciprocal also occurs, where typically these cells produce NO when stimulated with IFN- γ alone. NO production from monocytes stimulated with IFN- γ can therefore serve as a biological assay for recent or concurrent exposure to TNF- α . This was verified by showing that production of NO is inhibited in cultures stimulated with IFN- γ in the presence of a neutralizing antibody to bovine TNF- α . This bioassay was used to assess the in vivo activation state of monocytes with respect to NO production, and to determine whether IFN- γ and/or TNF- α are required as co-stimulants for NO production following exposure to *B. bovis* merozoites.

Unlike the response following exposure to *S. dublin*, induction of iNOS by *B. bovis* merozoites is IFN- γ -dependent (Stich et al., 1998). Our results demonstrate that TNF- α is also required. The only time that merozoites alone stimulated NO production *ex vivo*, was when there was evidence of recent or ongoing exposure to both IFN- γ and TNF- α . The most common condition was the need for addition of exogenous IFN- γ along with merozoites for induction of NO synthesis, suggesting that with monocytes, TNF- α is operating in an autocrine fashion, and is so readily induced that it has the appearance of constitutive expression. If, for some reason, TNF- α is not active in monocytes, IFN- γ alone is insufficient for up-regulation of NO (Goff et al., 1998). MDM grown in Teflon bags were quiescent with respect to most monokines and TNF- α message was never apparent in unstimulated cells. However, IFN- γ was capable of inducing TNF- α mRNA expression in MDM. Unlike with *S. dublin*, extracellular *B. bovis* merozoites were incapable of inducing TNF- α in MDM without IFN- γ , and also incapable of inducing IL-12, even in the presence of IFN- γ . Intact *B. bovis*-infected erythrocytes, as opposed to free merozoites, may provide better induction of cytokines. IL-12 and, variably, TNF- α were induced in MDM grown on plastic in response to stimulation with intact infected erythrocytes (Shoda et al., 2000). We did not determine whether other unstimulated tissue macrophages also fail to express TNF- α , but the data with MDM suggest that the monocyte is an effector cell involved in production of NO. Monocytes producing TNF- α traffic through the spleen and, together with local IFN- γ , could trigger production of NO following uptake of the parasite.

Even though TNF- α mRNA was induced in MDM in the presence of exogenous IFN- γ plus merozoites, NO was not produced. A number of reasons were considered. First, it is possible that with MDM, some undefined post-transcriptional regulation of protein expression occurred under these experimental conditions. Second, TNF- α message may simply represent expression preceding the production of the protein. This was ruled out due to the fact that nitrite detection was assayed 72 h after stimulation, sufficient time for TNF- α to have been produced, interact with its receptor and exert co-stimulatory activity for induction of iNOS and NO production. Third, intact *B. bovis*-infected erythrocytes may be a better stimulant than free merozoites for induction of iNOS as well as TNF- α . TNF- α and IFN- γ induction in naïve murine $\gamma\delta$ T-cells and TNF- α in CD14⁺ (LPS receptor) murine

MP was greater in response to intact *Plasmodium falciparum*-infected erythrocytes than free parasites (Hensmann and Kwiatkowski, 2001) and it follows that iNOS induction would mimic this. Finally, the TNF- α receptor may not be expressed in MDM. Monocytes produce NO in response to stimulation with IFN- γ plus TNF- α for only a brief period after being placed in culture, suggesting that one or both receptors are repressed during differentiation in culture (Goff et al., 1998). Moreover, exogenous IFN- γ plus TNF- α did not stimulate NO production in MDM. MDM did produce NO in response to *S. dublin*, but it has been demonstrated that mice with the TNF receptor gene disrupted respond to LPS and induce both TNF- α and iNOS mRNA expression and produce NO (Clemons-Miller et al., 2000). The failure of IFN- γ plus merozoites to induce IL-12 in our MDM is further evidence for repression of the TNF- α receptor. Mice lacking TNF- α receptors are deficient in IL-12 production (Zhan and Cheers, 1998).

At the present time, it is unclear how IL-4 modulates iNOS in bovine monocytes. It does not seem to modulate iNOS through regulation of TNF- α . In the absence of IFN- γ , IL-4 downregulated TNF- α mRNA expression during exposure to *S. dublin* but up-regulated TNF- α message during exposure to merozoites. In the presence of IFN- γ , IL-4 had no effect on TNF- α mRNA expression after exposure to *S. dublin* or merozoites. These results are somewhat at odds with a previous study involving murine macrophages, where TNF- α mRNA expression was unaffected by IL-4 in response to LPS (Gautam et al., 1992), and in another study showing that IL-4 suppressed NO production from mouse macrophages without affecting the level of TNF- α (Bogdan et al., 1994).

iNOS induction in murine macrophages has been shown to require binding of interferon regulatory factor-1 (IRF-1) to IRF response elements within the iNOS promoter (Kamijo et al., 1994; Martin et al., 1994). Recently, Paludan et al. (1999) reported that IL-4 suppresses iNOS transcription by increasing IRF-2 levels that, by competition for the same IRF response element, prevents the binding of IRF-1. We are investigating whether similar factors are involved in the induction and IL-4 modulation of iNOS from bovine monocytes.

Recently, the antagonistic effects of IL-4 and IFN- γ for iNOS expression in bovine monocyte/macrophages following exposure to either *S. dublin* or *L. monocytogenes* was demonstrated, with the expression dependent on the balance between IFN- γ and IL-4 (Jungi et al., 1997). IL-10 was not considered in those studies. We previously demonstrated that IL-10 down-regulates TNF- α mRNA expression in unstimulated bovine monocytes (Goff et al., 1998). In the current study, NO production resulting from merozoite plus IFN- γ stimulation was profoundly inhibited in the presence of IL-10, unlike with *S. dublin*. This suggests different regulatory pathways are being used following exposure to bacteria and protozoa. Our results are in agreement with those of Oswald et al. (1992) demonstrating that IL-10 is effective at regulating production of endogenous TNF- α , and bypassing the autocrine co-stimulant requirement for NO production. IL-10 also down-regulates basal levels of IFN- γ mRNA expression (Goff et al., 1998) as well as antigen stimulated IFN- γ production and T-cell proliferation (Brown et al., 1994) even in the presence of IL-12 (Collins et al., 1999). These results are also in agreement with our recent study characterizing the immune response of calves and adult cattle to an initial infection with *B. bovis*. IL-10 was the primary cytokine regulating synthesis of IFN- γ , TNF- α and NO in vivo during infection with *B. bovis*, as evidenced by expression of IL-10 mRNA in the spleen where expression of IL-4 was not detected (Goff et al., 2001).

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