Modulation of innate immunity by African Trypanosomes

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SUMMARY

The experimental studies of *Brucei* group trypanosomes presented here demonstrate that the balance of host and parasite factors, especially IFN-γ GPI-sVSG respectively, and the timing of cellular exposure to them, dictate the predominant MP and DC activation profiles present at any given time during infection and within specific tissues. The timing of changes in innate immune cell functions following infection consistently support the conclusion that the key events controlling host resistance occur within a short time following initial exposure to the parasite GPI substituents. Once the changes in MP and DC activities are initiated, there appears little that the host can do to reverse these changes and alter the final outcome of these regulatory events. Instead, despite the availability of multiple innate and adaptive immune mechanisms that can control parasites, there is an inability to control trypanosome numbers sufficiently to prevent the emergence and establishment of virulent trypanosomes that eventually kill the host. Overall it appears that trypanosomes have carefully orchestrated the host innate and adaptive immune response so that parasite survival and transmission, and alterations of host immunity, are to its ultimate benefit.

Key words: African trypanosomes, innate immunity, immunoregulation, GPI.

INTRODUCTION

The evolution of immune evasion by microbes has occurred in step with evolution of the vertebrate immune system, and is therefore intimately associated with all aspects of innate and adaptive immunity. Like other pathogenic microorganisms, the African trypanosomes have evolved the means to manipulate host immune cell recognition, activation and regulation by employing a wide range of discrete mechanisms. For these parasites the necessity of (indeed the dependence on) immunological evasion mechanisms is a direct consequence of the fact that these organisms must live for prolonged periods within host tissues during their life cycle; at a minimum this period must be sufficient for trypanosomes to replicate and develop into the life cycle stages and numbers of organisms necessary for successful transmission by the tsetse fly in nature. This review paper examines one of the central mechanisms by which African trypanosomes sequentially activate and then modulate cells of the innate immune system to successfully evade host immunity.

In general, there are three critical stages in the expression and subsequent down-regulation of host immunity to the *Brucei* group African trypanosomes. The first stage is innate immune system recognition of trypanosome PAMPs (pathogen associated molecular patterns), which for trypanosomes are glycosylphosphatidylinositol (GPI) residues associated with shed and membrane-bound variant surface glycoprotein (VSG) molecules. GPI substituents trigger early pro-inflammatory responses in macrophages (MPs) and dendritic cells (DCs) as well as subsequent counter-inflammatory and counter-protective responses in the infected host. The second is activation of the VSG-specific adaptive immune response. This includes the B cell response to exposed VSG coat determinants that destroys parasites in the bloodstream, as well as the protective VSG-specific Th cell response that destroys parasites within extravascular tissues. The highly polarized VSG-specific Th cell response produces Type 1 cytokines that provide protection via IFN-γ activation of MPs for release of trypanocidal factors; these responses are highly regulated at multiple levels and to the parasite's advantage. The third stage is progressive outgrowth of highly virulent trypanosomes from less virulent infecting populations, which deliver a 'death blow' to all infected hosts regardless of their immune status and genetically determined levels of resistance. Trypanosome virulence expression impacts on both innate and adaptive immunity.

Each of these critical stages of host resistance is interlaced with the others and should be considered in any evaluation of host resistance to African trypanosomes. Therefore this review paper focuses on events linked to GPI activation of the innate immune system and the subsequent downstream immunomodulatory effects of such activation,

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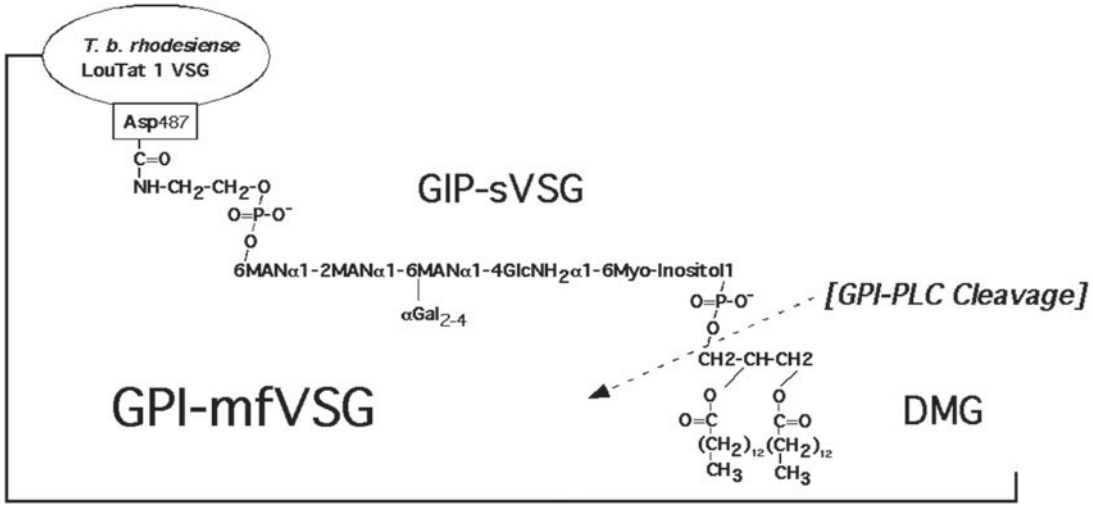

Fig. 1. Membrane-associated GPI-PLC cleaves the GPI anchor to release soluble VSG with GIP substituents linked to the C-terminal residue of the molecule adapted from Magez *et al*. (2002) and Paulnock and Coller (2001).

alterations in protective parasite-specific Th1 cell responses as the result of altered antigen processing cell (APC) function, and evidence that trypanosome virulence overrides both innate and adaptive immunity. We present and discuss both published and unpublished work derived from our experimental studies of *Trypanosoma brucei rhodesiense* infections, as well as the work of others studying experimental *Brucei* group trypanosome infections.

Interaction of the innate immune system with GPI residues of the VSG molecule

African trypanosomes have evolved a structured monomolecular surface coat that covers the entire plasma membrane of the parasite, including the flagellum (Vickerman and Luckins, 1969; Cross, 1975). This surface coat consists of a highly ordered and densely packed array of ~10^7 identical GPI-anchored VSG homodimers that both determine the antigenic phenotype of the parasite and prevent Ab and innate immune elements such as complement from binding to subsurface invariant epitopes of the coat or to membrane determinants. Despite extensive primary sequence variation among VSGs that comprise different surface coats (see papers on antigenic variation by Schwede and Carrington and Black *et al*. in this special issue), secondary and tertiary structural features of these molecules (and the amino acids residues that determine such features) are highly conserved within the ordered coat structure (Metcalf *et al*. 1987; Freymann *et al*. 1990; Carrington *et al*. 1991; Reinitz *et al*. 1992; Blum *et al*. 1993; Chattopadhyay *et al*. 2005).

During infection, GPI anchors are cleaved by activation of an endogenous GPI-phospholipase C that appears to be localized to an external linear array along the parasite flagellum (Mensa Wilmot *et al*. 1990; Field *et al*. 1991; Carrington *et al*. 1998; Hanrahan *et al*. 2009). When GPI-anchored VSG flows across the membrane into this site, the activated enzyme cleaves the anchor and releases VSG from the membrane with residual glycosylinositolphosphate (GIP) residues attached to the shed VSG (GIP-sVSG) (see Fig. 1) (Webb *et al*. 1994,1997; Hanrahan *et al*. 2009). The release of GIP-sVSG seems to occur during episodes of high parasite burden in the presence or absence of functional adaptive immunity, and is readily detectable in host tissues (see Fig. 2A). It is the GIP residues that primarily activate cells of the innate immune system during infection and, as discussed in more detail below, there appear to be two critical points of modulation of host immunity: regulation of the MP and DC secreted cytokine profiles and alteration of DC and MP antigen-presenting cell functions (Tachado *et al*. 1997; Magez *et al*. 1998, 2002; Paulnock and Coller, 2001; Mansfield and Paulnock, 2005; Dagenais *et al*. 2009*b*).

GIP-sVSG-induced activation events

Studies in our laboratory and elsewhere have demonstrated that during early stages of infection MPs and DCs display multiple hallmarks of a 'classical' or pro-inflammatory, activation profile; this includes production of trypanocidal molecules (ROI, RNI, and TNF-α), pro-inflammatory cytokines (including IL-6, IL-12 and IFN-γ), and changes in functional capacity (including increased expression of MHC class II and co-stimulatory molecules for APC activity) (Magez *et al*. 1998, 2002; Paulnock and Coller, 2001; Coller *et al*. 2003; Harris *et al*. 2006; Barkhuizen *et al*. 2007; Leppert *et al*. 2007; Lopez *et al*. 2008; Dagenais *et al*. 2009*b*).

The initial event triggering this activation profile is binding of GIP-sVSG to Type A scavenger receptors (SR-A) on the MP and DC membrane. Binding

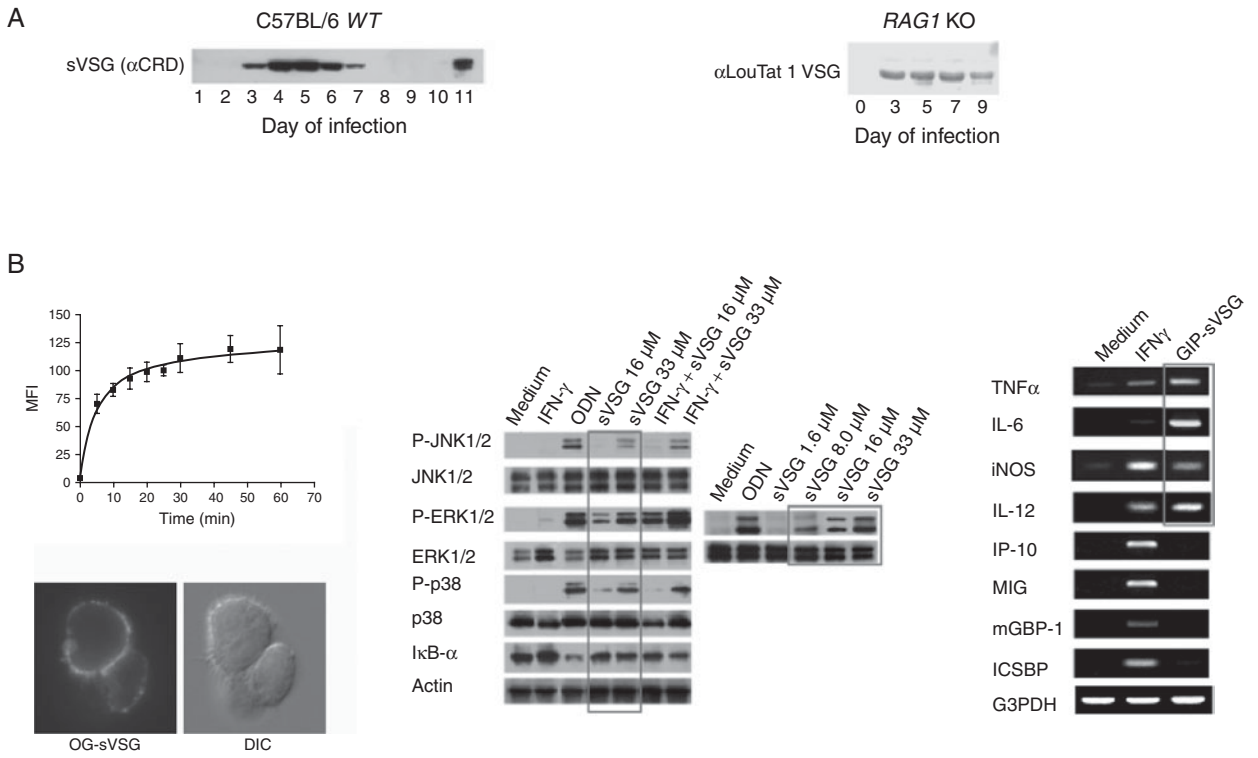

Fig. 2. (A) Detection of GIP-sVSG in the sera of *wt* and immunodeficient mice infected with *Trypanosoma brucei rhodesiense* LouTat 1. Western Blots with Ab specific for the cross-reacting determinant (CRD) of the GIP anchor residue in *wt* mice or against VSG in RAG1 KO mice (Paulnock and Coller, 2001; Coller *et al*. 2003). (B) Binding of fluorochrome labeled GIP-sVSG to MP membranes with subsequent downstream signaling events and activation of pro-inflammatory genes (adapted from Leppert *et al*. (2007) and Lopez *et al*. (2008)). Activation of MPs and DCs is dependent upon SR-A mediated, clathrin-dependent internalization of GIP-sVSG and delivery to late endolysosomal compartments. Aadapted from Leppert *et al*. (2007) and Mansfield and Paulnock (2010).

subsequently triggers internalization and targeting of the receptor-GIP-sVSG complex to an endolysosomal compartment. This event initiates a cascade of subcellular signaling leading to activation of NFkB and MAPK pathways and expression of a subset of pro-inflammatory genes (Fig. 2B) (Coller and Paulnock, 2001; Paulnock and Coller, 2001; Coller *et al*. 2003; Leppert *et al*. 2007; Lopez *et al*. 2008). These signaling events are modulated by additional factors, including (1) Traf6-, MyD88- and TLR-dependent down-regulation of GIP-sVSG-induced NFkB responses (Leppert *et al*. 2007); (2) augmentation of the response in the presence of IFN-γ (Coller *et al*. 2003; Leppert *et al*. 2007; Lopez *et al*. 2008); and (3) modulation by CpG DNA released from senescent or damaged trypanosomes (Harris *et al*. 2006, 2007).

An additional key component of MP and DC activation during infection is interaction of IFN-γ with the IFN-R. This receptor-ligand association generates the formation and phosphorylation of STAT1 homodimers and activation of downstream pro-inflammatory genes (Paulnock, 1992, 1994, 2000; Paulnock *et al*. 2000). Studies focused on understanding the nature of the interaction between the host and parasite factors in terms of innate cell activation have shown that the relative timing of MP and DC exposure to GPI substituents and to IFN-γ is critical for the ability of cells to become activated for APC functions and, for MPs, to kill the parasites. If there is coincident exposure to significant levels of both IFN-γ and GPI, augmentation of cellular activation and transcription of pro-inflammatory genes occurs (Magez *et al*. 1998; Coller *et al*. 2003; Lopez *et al*. 2008; Freeman, Mansfield and Paulnock, unpublished observations); this event may predominate in early infection with IFN-γ derived from Th1 cells and, potentially, NK cells (Demick, Suresh and Mansfield, unpublished observations) and may help to control the early explosive growth of trypanosomes within host tissues so the host survives the first wave of infection. However, if cells are exposed to GPI substituents prior to, or in the absence of, sufficient levels of IFN-γ, STAT1 is not phosphorylated in response to IFN-γ and the production of trypanocidal factors as well as APC functions are inhibited (Coller *et al*. 2003; Dagenais *et al*. 2009*b*; Freeman, Mansfield and Paulnock, unpublished observations). These events likely occur as the infection progresses, when increasing numbers of parasites release GIP-sVSG prior to sufficient activation of new T cell responses for production of IFN-γ. Finally, there is nascent evidence that not only does activation of the innate immune system trigger a potent

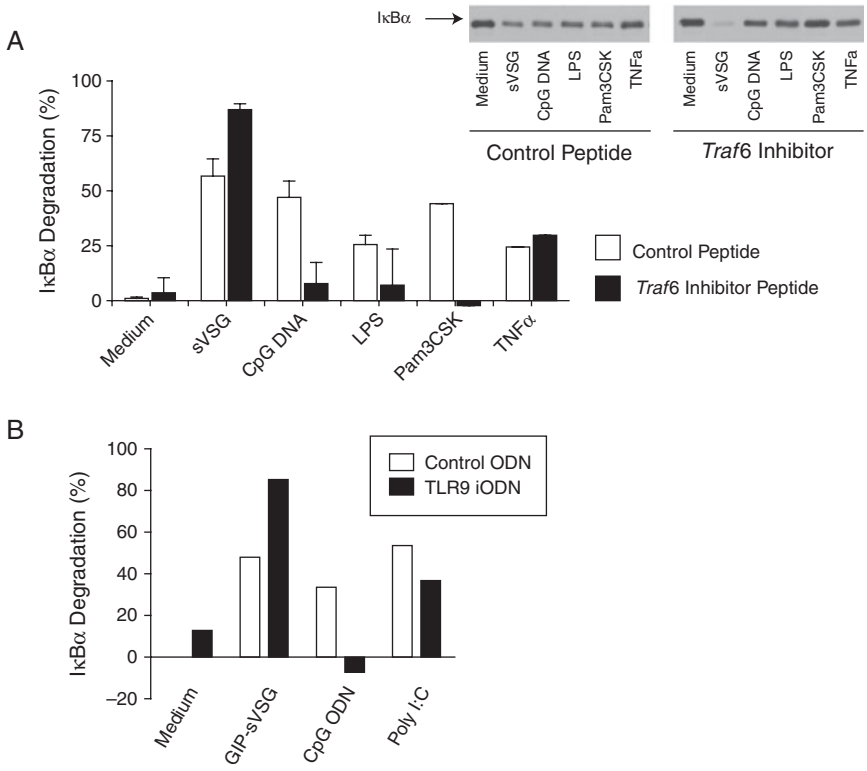

Fig. 3. GIP-sVSG-induced NFkB signaling is enhanced in the absence of TLR signaling (A): irreversible *Traf*6 inhibitor; (Leppert *et al*. 2007) and TLR9 inhibitory ODN (B): TLR9 iODN 2088; (Mansfield and Paulnock, 2010).

IFN-α response but that this response ultimately negatively impacts on the host ability to produce IFN-γ during infection (Lopez *et al*. 2008). The sections below explore some of the primary data and their extrapolations that relate to the information presented above.

Negative regulation of GPI-mediated signaling

As noted above, GIP-sVSG is delivered to an intracellular endolysosomal compartment of MPs and DCs by SR-A-mediated uptake. SR-As are expressed primarily but not exclusively on cells of the monocyte/MP lineage, some DCs, and on other cells; they are trimeric integral membrane glycoproteins that demonstrate broad ligand-binding properties including altered self proteins and many different types of microbial PAMPs. While scavenger receptors were once thought to serve simply as non-opsonic phagocytic receptors for many ligands, it is now clear that there are direct signaling events generated by ligand engagement of these receptors, including SR-A [as shown in our earlier parasite-unrelated studies; (Coller and Paulnock, 2001) and by others (Todt *et al*. 2008; DeWitte-Orr *et al*. 2010)]; tyrosine phosphorylation and activation of MAPK and NFkB pathways appear to be hallmarks of SR-A-mediated signaling. Further, SR-A and other scavenger receptors have now been shown to interact functionally and synergistically with other pattern recognition receptors (PRRs) of the innate immune system, including TLRs, to generate fully functional innate immune responses to different microbial pathogens (Bowdish and Gordon, *et al*. 2009; Bowdish *et al*. 2009; DeWitte-Orr *et al*. 2010; Stewart *et al*. 2010; Yew *et al*. 2010).

PAMP recognition by combinatorial activation of multiple PRRs appears to be an evolutionarily conserved but poorly understood and complex phenomenon (Amit *et al*. 2009). To date it is clear that TLR-independent signaling is triggered by GIP-sVSG binding to SR-A, and that NFkB and MAPK signaling pathways are activated after internalization (Leppert *et al*. 2007; Freeman, Mansfield and Paulnock, unpublished observations; Mansfield and Paulnock, 2010). This GIP-sVSG–SR-A ligand/receptor interaction activates subsequent TLR-dependent signaling events that then down-regulate the initial response (Leppert *et al*. 2007; Mansfield and Paulnock, unpublished observations). While scavenger receptor interactions in other studies have been shown to promote positive TLR-mediated signaling effects by catalyzing activation, dimerization or multimerization of TLRs, our work is the first to show that SR-A-mediated signaling triggers a negative regulatory TLR signaling cascade that modifies the initial SR-A signaling event (Leppert *et al*. 2007). We know that TLRs are not required for SR-A induced signaling because the absence, inhibition or ablation of TLR signaling results in enhanced NFkB/MAPK signaling by GIP-sVSG [see examples in Fig. 3; (Leppert *et al*. 2007)].

However, the biological relevance of the SR-A-mediated activation response *alone* is not entirely clear and must be elucidated; currently we presume it to be a positive and protective effect early in infection but a counter-protective effect as infection progresses (Coller *et al*. 2003; Mansfield and Paulnock, 2005).

Our recent preliminary studies show clearly that TLR9 is the intracellular receptor to which GIP-sVSG is delivered and that directs the negative regulatory effect on GIP-sVSG induced signaling. Using TLR-deficient cells and known TLR inhibitors, we have ruled out the engagement of TLR2, TLR4 and other factors in this negative regulation, and have shown that TLR9 is the relevant receptor engaged by GIP (see Fig. 3B) (Mansfield *et al*. unpublished observations). It is not known which substituent of GIP serves as the TLR9 ligand. An early concern was that contamination of sVSG preparations with trypanosome CpG DNA was the active factor since CpG DNA released during infection contributes to MP activation (Harris *et al*. 2007), but HPLC purification of GIP-sVSG, the use of DNase treatments, and the knowledge that trypanosome CpG DNA is stimulatory only in significantly high concentrations (Harris *et al*. 2006; Leppert *et al*. 2007), revealed this was not the case. It is our current hypothesis that the phosphate residue of GIP, exposed as part of the GPI anchor residuum after GPI-PLC cleavage of GPI-mfVSG, is the most likely candidate for TLR9 interaction, based on recent studies elaborating such interactions in other systems (Gangloff and Gay 2008).

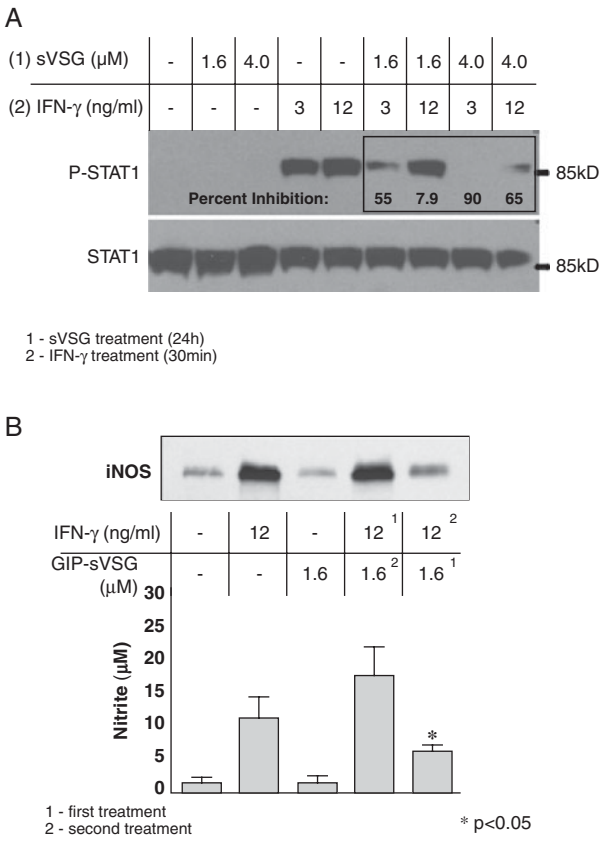

Fig. 4. (A) Inhibition of IFN-γ mediated STAT1 phosphorylation in MPs by prior exposure to GIP-sVSG. (B) Suppression of iNOS transcription and NO production in MPs exposed to GIP-sVSG prior to activating doses of IFN-γ. Adapted from Coller *et al*. (2003).

Down-modulation of IFN-γ-mediated MP activation

As mentioned above, the interplay and timing of parasite and host factors are critical for control of the parasite burden in host tissues and for regulation of host immune responses that provide such protection. Studies in our laboratory have shown that GIP-sVSG substituents are capable of blocking production of trypanocidal factors and parasite killing by interfering with IFN-γ-dependent activation of MPs and also by inhibiting APC function; these inhibitory events may be central to down-stream parasite evasion of host adaptive immunity.

Exposure of MPs to GIP-sVSG induces the rapid production of pro-inflammatory and protective factors including TNF-α, RNI and ROI and this response can be enhanced by initial or prior treatment of the cells with IFN-γ (Magez *et al*. 1998; Coller *et al*. 2001; Paulnock *et al*. 2001; Coller *et al*. 2003; Leppert *et al*. 2007; Lopez *et al*. 2008). However, the level and timing of exposure of MP to GIP-sVSG *vs* IFN-γ ultimately determines the MP response at the level of induced gene expression. Treatment of MPs with GIP-sVSG prior to IFN-γ exposure results in a marked reduction of IFN-induced responses, including transcription of inducible nitric oxide synthase and production of NO (Fig. 4) (Coller *et al*. 2003); this effect is mediated through a dose-related inhibition of STAT1 phosphorylation, which is required for signaling through the IFN-γ receptor (Fig. 4A). Thus, in this manner, regulation of the ability of MPs to respond to IFN-γ during infection is a central mechanism by which the parasites overcome host resistance.

These results suggest that cyclical exposure of innate immune cells to varying levels of GIP-sVSG as the infection progresses can result in changes in the MP response to IFN-γ during the course of infection. Given that infection with *Trypanosoma brucei* spp. can result in a chronic course of infection in relatively resistant animals and humans, this may be one mechanism not only for permitting parasites to escape from destruction in the tissues, but also for controlling host tissue pathology. However, a key question is when these changes in MP responses, and the accompanying changes in functional activity, occur and at what level they continue to fluctuate over the course of infection. Studies in a *Trypanosoma congolense* experimental model system as well as in human infections with *Trypanosoma brucei* spp. have

suggested that during the later stages of infection there is a permanent shift in the MP activation state from a 'classical' to an 'alternative' activation phenotype, with the latter state associated with production of anti-inflammatory cytokines including IL-10 (Uzonna *et al*. 1999; Kaushik *et al*. 2000; MacLean *et al*. 2001; Sternberg *et al*. 2005). A similar trend is observed in a *T. brucei brucei* model system, but only when GPI-PLC$^{-/-}$ parasites, which do not shed GIP-sVSG from their VSG coat, are used (Webb *et al*. 1997). Our work and the work of others (Stijlemans *et al*. 2007) suggests that it is unlikely that a full 'alternatively activated' MP response is induced in wild type *Trypanosoma brucei rhodesiense* infection, as a Th2 cell cytokine response, associated with alternatively activated MPs and capable of IL-4 production, has not been detected during chronic infection, even in IL-12 knockout mice that lack the ability to induce early Th1 polarization (Dagenais *et al*. 2009*b*). Thus, IL-10 may clearly have a non-Th2 origin in trypanosomiasis. However, MPs from chronically infected animals clearly are impaired in their ability to produce pro-inflammatory cytokines when they subsequently encounter stimulatory molecules such as trypanosome GIP-sVSG, CpG DNA or LPS, while at the same time IL-10 production is enhanced in both infected animals and humans (Uzonna *et al*. 1999; Kaushik *et al*. 2000; Namangala *et al*. 2000; MacLean *et al*. 2001; Sternberg *et al*. 2005; Harris *et al*. 2006; Barkhuizen *et al*. 2008; Lopez *et al*. 2008; Mansfield *et al*. unpublished observations); this impairment is seen in experimental mice by the time the second wave of parasitaemia appears, around two weeks post-infection.

This response phenotype is similar to that observed during induced tolerance to TLR ligands and certainly provides a significant advantage to the parasite in terms of survival (Medvedev *et al*. 2000). Overall, the response capability of innate immune cells clearly is diminished early in infection and may be the partial basis for subsequent alterations in APC function described below. Thus it is clear that MP plasticity during infection results in a wide range of activation responses at various times in trypanosomiasis. The ability of GIP-sVSG exposure to dramatically reduce the ability of MPs to respond to subsequent activation by IFN-γ may be the most important of these changes and has obvious implications for host resistance, since IFN-γ activation of MPs for the production of trypanocidal or trypanostatic factors in the extravascular environment is key for host protection.

Negative regulation of IFN-γ production

An additional outcome of GPI-sVSG induced cellular activation that impacts on IFN-γ-mediated host resistance is the induction of Type I interferon (IFN-$\alpha\beta$) production during infection. We have recently shown by microarray analysis of naïve MPs exposed to GIP-sVSG *in vitro* that a prominent component of the cytokine response induced by GIP-sVSG encompassed genes associated with the Type I interferon (IFN-$\alpha\beta$) response including IRF-7 and IFN-α (Lopez *et al*. 2008), suggesting that GIP-sVSG recognition by MPs results in modulation in the IRF family of transcription factors in a manner similar to the modulation of STAT1 signaling observed in earlier experiments. The subsequent production of IFN-$\alpha\beta$ (De Gee *et al*. 1985; Lopez *et al*. 2008) may set in motion the IRF-7- and IFN-dependent modulation of host resistance during late stage infection that inhibits IFN-γ production by Th1 cells (Lopez *et al*. 2008). The pattern observed in MPs treated with GIP-sVSG *in vitro* was similar to that which was observed in MPs taken directly from trypanosome infected mice within 72 h post-infection, and suggests a role for IFN-$\alpha\beta$ in GIP-sVSG-mediated MP activation events. Results of *in vivo* studies with trypanosome infection in IFNAR1$^{-/-}$ mice, which lack the receptor for Type I interferons, and UBP43$^{-/-}$ mice, which are hyper-responsive to Type I interferons, suggest that IFN-$\alpha\beta$ may play a pivotal role in host resistance. Results obtained using UBP43$^{-/-}$ mice are particularly informative, as these mice are more susceptible to African trypanosome infection than their wild type counterparts, initially displaying MP activation profiles that promote control of parasite burden during the early phases of infection but, as the disease progresses, promoted down-regulation of host IFN-γ production and host resistance (Lopez *et al*. 2008). These results correlate well with studies in a variety of other non-viral microbial systems that suggest a role for IFN-$\alpha\beta$ in modulating the course of infection, although the majority of the bacterial, fungal, and protozoan pathogen systems involve intracellular microbes.

Alterations in APC function during infection

For nearly a century the concept of trypanosome immunology encompassed a central paradigm in which Ab was the primary mechanism that protected the host against different parasite variant antigenic types (VATs) during infection. The old paradigm first began to unravel with publication of studies on resistant and susceptible bone marrow chimera mice in which Ab was not functionally linked to overall host resistance (De Gee and Mansfield, 1984). Subsequently classical genetic studies employing crosses between resistant and susceptible animals, as well as results from recombinant inbred strains, were used to demonstrate that Ab-mediated control of parasitaemia by itself was not genetically linked to the host resistance phenotype (De Gee *et al*. 1988). Thus,

although VSG-specific Abs provide an important mechanism for controlling trypanosome numbers in the blood (and perhaps also provide an unerringly specific means to select for new VATs in the process!), this event alone is insufficient to provide significant host resistance.

These seminal results laid the foundation for subsequent studies in which resistant and susceptible animals were examined for T cell-mediated immune responses to trypanosome antigens. Direct evidence for VSG-specific T cell responses came from our experimental studies with *Trypanosoma brucei rhodesiense* infections of mice (Schleifer *et al*. 1993*a*,*b*). VSG-activated T cells were shown to be $CD4^+$ lymphocytes that expressed the $CD3^+$ TCR membrane complex, were APC-dependent and MHC-II restricted (Schleifer *et al*. 1993*a*; Hertz *et al*. 1998; Schopf *et al*. 1998; Dagenais *et al*. 2009*a*). The cytokine profiles, both transcriptional and protein secretory, were those associated with highly polarized Th1 cell responses characterized by IL-2 and IFN-γ production without detectable IL-4 or IL-5 responses. Thus, VSG preferentially stimulates a polarized Th 1 cell cytokine response during trypanosome infection.

VSG-specific Th1 cells are the primary source of IFN-γ produced during infection. IFN-γ is linked to host resistance against *Brucei* group trypanosomes through macrophage activation events, as noted above, that lead to the release of factors such as TNF-α, ROI and RNI that are cytotoxic for trypanosomes (De Gee *et al*. 1985; Magez *et al*. 1993, 1997, 1999, 2006; Lucas *et al*. 1994; Hertz *et al*. 1998,1999; Drennan *et al*. 2005; Dagenais *et al*. 2009*a*). Infection of mice lacking the IFN-γ gene was shown to result in susceptibility similar to that of scid mice, which have no adaptive immune system, despite the fact that such IFN-γ KO animals made Ab responses that controlled trypanosomes in the bloodstream like *wt* mice (Hertz *et al*. 1998; Schopf *et al*. 1998). The current presumption is that IFN-γ activation of macrophages with concomitant production of microbicidal factors is responsible for control of the parasite burden within extravascular tissues where Ab is relatively ineffective in killing trypanosomes. Thus, the current paradigm is that VSG-specific Th1 cells and IFN-γ regulate the major component of host resistance to African trypanosomes.

Given that APC function is critical to the development, maintenance and memory responses of VSG-specific Th cells, and that GIP-sVSG has the capacity to alter both innate and adaptive immune responses, it is only natural that antigen-presenting cells have come under observation for functional abnormalities. To date, several studies have demonstrated that APC functions are significantly altered during experimental trypanosomiasis (Paulnock *et al*. 1989; Namangala *et al*. 2000; Dagenais *et al*. 2009*b*).

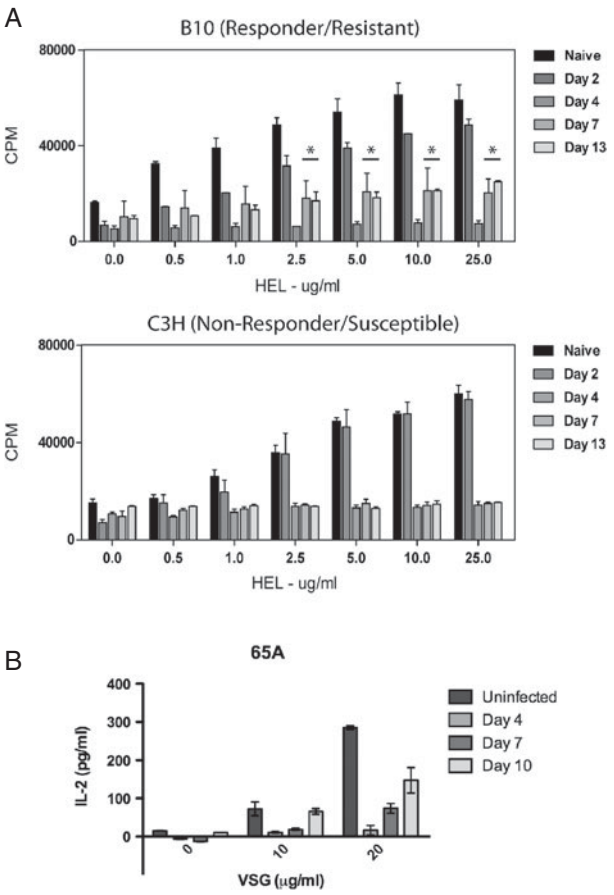

Fig. 5. (A) APCs from trypanosome infected mice were pulsed with HEL or HEL peptides and exposed to HEL-specific T cell hybridomas to assess processing and presentation of HEL antigen. (B) DCs from mice infected with one trypanosome variant, and which express the relevant MHC II-VSG peptides, are unable to process and present exogenous VSGs from other variants. Adapted from Dagenais *et al*. (2009*a*,*b*).

Recently a detailed analysis of APCs involved in the activation of naïve Th cells and induction of the polarized Th1 response in trypanosomiasis showed that DCs are the primary cell population capable of activating T cells to both parasite and non-parasite antigens (Dagenais *et al*. 2009b). However, a key finding was that, during the process of APC activation of VSG-specific T cells, APCs rapidly became unable to process or present additional exogenous antigens to T cells (see Fig. 5A) (Dagenais *et al*. 2009*b*). Subsequent analyses revealed that the uptake and internalization of exogenous antigens by APCs was not significantly altered; rather, it appeared that existing peptide (presumably VSG)-MHC II complexes were not being recycled from the membrane and that formation of new intracellular MHC II-peptide complexes was unstable (Freeman *et al*. 2010). Of central importance to the control of parasites was the inability of APCs to present antigenic peptides from newly encountered VSG molecules expressed by variants arising during

the infection (Dagenais *et al*. 2009*b*; Freeman, Mansfield and Paulnock, unpublished observations). In these studies it was of interest that all the relevant co-stimulatory molecules were highly up-regulated on the APCs, and that during the period of APC dysfunction these cells still expressed T cell activating MHC II-VSG peptide complexes in which the peptides were derived from the initial infecting variant type. While the mechanistic basis for this remains to be determined, the GPI substituents of VSG seem to be intimately involved in the process (Mansfield and Paulnock, 2005; Mansfield *et al*. unpublished observations). Thus, along with other host innate immune cell modulations induced by trypanosomes and their molecules, APCs are also affected by infection and contribute to the overall down-modulation of protective Th1 cell responses and IFN-γ-dependent activation of MPs that lead to trypanocidal activity.

Trypanosome virulence suppresses host resistance

The African trypanosomes undergo cellular differentiation and biological variation throughout their life cycle in which the organisms change surface coat glycoproteins, alter biochemical pathways, remodel cellular morphology and exhibit distinct host specificities. These changes are regulated by the expression of discrete genes and proteins (Vickerman and Luckins, 1969; Rifkin, 1978; Duszenko *et al*. 1985; Donelson, 1987; Hajduk *et al*. 1989, 1992, 1995; Cross, 1990; Van-der-Ploeg *et al*. 1992; Borst and Rudenko, 1994; De Greef *et al*. 1994; Steverding *et al*. 1994; Smith *et al*. 1995; Hager and Hajduk, 1997; Bitter *et al*. 1998; Borst *et al*. 1998; Xong *et al*. 1998; Milner and Hajduk,1999; Raper *et al*. 1999, 2001; Molina Portela *et al*. 2000; Navarro and Gull, 2001; Gerrits *et al*. 2002; Mussmann *et al*. 2003; Landeira *et al*. 2009; Mansfield *et al*. 2010). Many molecular elements of trypanosome antigenic variation, cellular differentiation, metabolism, morphology and host specificity may be defined as primary virulence factors for all African trypanosomes since parasites in which the relevant genes have been deleted, mutated or knocked down are unable to infect or survive in specific mammalian or insect hosts; for instance see Leal *et al*. (2001), Hoek *et al*. (2002) and Wang *et al*. (2003). A peripatetic focus of our laboratory, however, has been on *relative virulence* of trypanosomes for their mammalian hosts. It is well known that different isolates, species, subspecies and laboratory lines of trypanosomes exhibit remarkable variation in pathogenicity and virulence for genetically defined host species (McNeillage and Herbert, 1968; Mulligan, 1970; Clayton, 1978; Barry *et al*. 1979; Levine and Mansfield, 1981, 1984; Inverso and Mansfield, 1983). The underlying question has been whether such biological differences are *immutable* characteristics associated with genetically distinct populations of trypanosomes, or whether *intraclonal* biological variation occurs in trypanosomes that impacts on the course of infection within an individual host animal.

We addressed this question in earlier studies in which inbred mice individually were infected with a single *Trypanosoma brucei rhodesiense* LouTat 1 trypanosome (Inverso and Mansfield, 1983; Inverso *et al*. 1988; Mansfield and Paulnock, 2010).

Table 1. Microarray analyses of the immune transcriptome in mice infected with *Trypanosoma brucei rhodesiense* LouTat 1 (low virulence) vs LouTat 1A (high virulence). LouTat 1 induces the expression of all genes shown with the exception of CXCL19 (*), while LouTat 1A induces only a subset of the genes (underlined). Both organisms express the same VSG gene and surface coat, and exhibit identical GPI anchor residues (Inverso, De Gee *et al*. 1988; Inverso, Uphoff *et al*. 2010).

IFN-γ	CD5
IFN-β	IL-7R
TLR7	IL-12R
TLR13	ICAM-1
IRF1	Cathepsins C, D, S
IRF7	Beta-2 microglobulin
IFN-α inducible protein 1	IL1RA
IFN-α responsive protein	LPS BP
IFN-γ inducible protein 16	FcR IgG IIb
IFN-γ inducible protein 47	HSP1
IFN induced transmembrane protein 3	HSP60
ICSBP	CXCL1
ISG15	CXCL5
GBP2 (Mag-2)	CXCL6
IFN inducible protein 1	CXCL7
IFN inducible protein 35	CXCL10
IFN inducible protein MG11	CXCL13
IFN-γ induced GTPase	CXCL19*
IFN induced GTPase	RANTES
IFN related developmental regulator 1	MIP-1
IFN stimulated gene 12	MCP-3
IFN stimulated gene 203	Tachykinin 1
IFN stimulated gene 205	LPS inducible C-C chemokine
IFN dependent positive acting	LDLR related protein
IFN induced Mx2 mRNA	Mannose binding lectin
IFN inducible gene unknown	Protein H
STAT1	C1q
STAT2	C1r
STAT4	C1s
SOCS1	C4
Ubiquitin specific protease 12, 18	C2
PKR	C3
CTLA4	C8a
CD86	C8b
CD4	Properdin
IL-18BP	Complement factor I
MHCI	C-reactive protein
MHCII	

Multiple VATs were isolated from the blood at intermediate and later times during the ensuing infection; these organisms were subcloned and characterized as to VSG phenotype and relative pathogenicity. Different VATs, which represented antigenically distinct daughter cell populations clonally derived from a *single* LouTat 1 'parental' cell in a single animal, were then used to infect resistant mouse strains. The surprising result was that each daughter cell population exhibited a different virulence phenotype compared to the parental clone. For example, infection with the parental LouTat 1 caused death in approximately 62 days post-infection, while infection with LouTat 1.3, LouTat 1.4 and LouTat 1.5 caused death in approximately 44, 30, and 28 days, respectively; trypanosomes taken from terminal peaks of parasitaemia were highly virulent, causing death in less than 10 days (Inverso and Mansfield, 1983; Inverso *et al*. 1988; Johnson *et al*. 2010).

These results clearly demonstrated that VATs arising during trypanosome infection expressed virulence phenotypes dramatically different from the infecting VAT. In essence, daughter cells arising within a trypanosome population expressed the capacity to transcend host genetic resistance characteristics and render a relatively resistant animal into a more susceptible one. Such virulence expression appears to be a progressive phenotype in chronic infections since low virulence trypanosomes always give rise to more virulent trypanosomes. Thus, trypanosomes appear to have evolved a molecular 'Virulence Rheostat' that is inherent to every trypanosome cell and that, at least in experimental model systems, ultimately kills the infected host (Inverso and Mansfield, 1983; Inverso *et al*. 1988, 2010; Mansfield 2006; Johnson *et al*. 2010). This mechanism may have evolved in order to provide trypanosomes the ability to ultimately escape destruction regardless of the disparate genetic resistance traits displayed by animal or human hosts upon which infected tsetse flies feed (Mansfield, 2006; Arnold *et al*. 2007).

We have developed a tractable model system for the molecular exploration of trypanosome virulence in which we compared low virulence parental cells to intermediate and high virulence daughter cells within the *T. b. rhodesiense* LouTat 1 serodeme. Several important features have emerged: First, virulence is not linked to the VSG coat, the mechanism of VSG gene expression or the specific chromosome telomeric VSG gene expression site (Inverso *et al*. 1988; Inverso *et al*. 2010). Second, high virulence expression results in the abrogation of measureable host innate and adaptive resistance (Mansfield and Paulnock, 2005; Mansfield, 2006; Johnson *et al*. 2010; Mansfield *et al*. 2010); for example, while a spectrum of relative resistance is observed when genetically distinct mouse strains are infected with low virulence organisms, such as LouTat 1 (Levine and Mansfield, 1981, 1984; De Gee *et al*. 1988), infection of these animals with high virulence trypanosomes derived from the low virulence parental cells such as LouTat 1A (Inverso and Mansfield, 1983; Inverso *et al*. 1988, 2010; Johnson *et al*. 2010) eliminates this spectrum and results in rapid death. Similarly, infection of immunodeficient scid mice with low virulence trypanosomes reveals a basal level of innate resistance, but this resistance is also ablated when such animals are infected with more virulent trypanosomes. Third, measureable and repeatable differences in the pattern of intraclonal parasite gene and protein expression occur as trypanosomes become more virulent with time of infection (Inverso *et al*. 2010; Johnson *et al*. 2010), and some genes and proteins are predicted to impact on host immunity. This pattern is, however, not reversible in infection of the mammalian host but must, somehow, be reset to a low level of virulence in nature, perhaps in the intermediate host and vector, the tsetse fly. Fourth, and relevant to the present discussion regarding the innate immune system, trypanosomes displaying an elevated virulence phenotype do not activate the same pattern of genes in the infected host as do low virulence organisms (see Table 1). Microarray analyses of immune system genes activated early in infection reveal that while virulent organisms activate some of the same genes as less virulent organisms (Lopez *et al*. 2008), it is a small subset of the total array of genes normally activated [Table 1; (Lopez *et al*. 2008; Johnson *et al*. 2010; Mansfield and Paulnock, 2010)]. Thus, virulent trypanosomes do not trigger the same danger signals and activation events as those triggered by less virulent organisms; this is not related to GPI residues expressed by high *vs* low virulence trypanosomes, as these appear to be identical in composition and biological activity *in vitro* (Mansfield and Paulnock, 2010). In summary, in addition to the disparate and multiple mechanisms that trypanosomes have evolved to alter host immunity, the trypanosome has another trick up its sleeve with the intraclonal expression of virulence which renders all animals highly susceptible to infection.

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