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Processing and Presentation of Variant Surface Glycoprotein Molecules to T Cells in African Trypanosomiasis

Taylor R. Dagenais,* Bailey E. Freeman,† Karen P. Demick,*,† Donna M. Paulnock,† and John M. Mansfield‡*‡

Th1 cell responses to the variant surface glycoprotein (VSG) of African trypanosomes play a critical role in controlling infection through the production of IFN-γ, but the role of APCs in the induction and regulation of T cell-mediated protection is poorly understood. In this study, we have investigated the Ag presentation capabilities of dendritic cells (DCs) and macrophages during early trypanosome infection in relatively resistant responder and susceptible nonresponder mouse strains. Splenic DCs appeared to be the primary cell responsible for activating naive VSG-specific Th cell responses in resistant responder animals through the coordinated up-regulation of costimulatory molecules, secretion of IL-12, and presentation of VSG peptides to T cells in vivo. Splenic DC depletion and the down-regulation of costimulatory markers on splenic macrophages were observed in susceptible animals and may be associated with the inability of these animals to elicit a significant VSG-specific T cell response. In contrast to splenic APCs, peritoneal macrophages secreted NO, failed to activate naive Th cells in vitro, and presented relatively low levels of VSG peptides to T cells in vivo. Thus, VSG-specific Th1 cell responses may be determined by tissue- and cell-specific differences in Ag presentation. Additionally, all APCs from resistant and susceptible strains displayed a reduced ability to process and present newly encountered exogenous Ag, including new VSG molecules, during high parasitemia. Thus, initial uptake of VSG (or other trypanosome factors) may interfere with Ag presentation and have dramatic consequences for subsequent T cell responses to other proteins. The Journal of Immunology, 2009, 183: 3344–3355.

African trypanosomes express an immunogenic surface coat that is composed of variant surface glycoprotein (VSG) homodimers (1). VSG-specific B and T cell responses provide temporal variant-specific protection during infection by eliminating distinct variant antigenic types (VATs) present in the blood and extravascular tissues (2–5). However, trypanosomes may express up to 1000 different VSG genes and surface coats that ultimately enables the organisms to evade immune destruction (6, 7). The characterization of VSG-specific CD4+ Th cell responses, coupled with earlier functional and genetic studies on the failure of B cells to provide relative resistance, revealed the comparative importance of T cell responses in providing VAT-specific protection in trypanosomiasis (4, 5, 12–15). VSG-specific T cell responses of Trypanosoma brucei rhodesiense-infected animals were MHC class II (MHC II)-restricted and required processing of the VSG molecule by APCs to activate T cells (4); the production of IFN-γ by polarized Th1 cells was shown to contribute to early host resistance during infection (5, 16), likely through the activation of macrophages to produce trypanolytic factors such as ROI, RNI, and TNF-α (16–22). Recently we have demonstrated that VSG-specific Th1 cells generated during infection recognize peptides distributed throughout N-terminal domain sequences of the VSG molecule, but do not recognize peptide sequences from the conserved C-terminal domain (23).

Historically little is known about the role of APCs in the development of VSG-specific Th cell responses during trypanosome infection beyond MHC II restriction and processing-dependent T cell activation (4). However, the picture is much more complex due to evidence that cells of the innate immune system, including macrophages and DCs, may be dramatically altered in function following trypanosome infection. For example, macrophages are known for their ability to suppress, in a pan-specific manner, the adaptive T cell responses of infected animals and to release a number of immunomodulatory factors including NO, TNF-α, PGE2, and IL-10 (19, 24–38). Furthermore, macrophages and DCs are activated by GPI substituents present on the C-terminal domain of shed VSG molecules (39–42), and trypanosome CpG DNA released during infection also has immunomodulatory effects on cells of the innate immune system (43). Both parasite molecules also have been shown to down-regulate IFN-γ-induced activation of the innate immune system, however, dependent on timing of exposure to these trypanosome factors (15, 43–45), so it is difficult to envision how cells of the innate immune system process and present VSG peptides in a manner sufficient to activate host Th cells. Given that there are differences in VSG-specific Th1 cell activation in relatively resistant compared with susceptible mice, that there is tissue-specific regulation of Th cell responses during infection, that Th cell specificity is limited to variable region subsequences of the VSG molecule, and that there is gradual loss of T cell responsiveness during progressive infection, we hypothesize that APC functions are substantially altered during trypanosome infection.

Supportive evidence for this hypothesis includes an earlier study showing that macrophages from T. brucei brucei-infected mice

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3 Abbreviations used in this paper: VSG, variant surface glycoprotein; DC, dendritic cell; VAT, variant antigenic type; MHC II, MHC class II; HEL, hen egg lysozyme; MPI, mean fluorescence intensity.

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exhibited reduced presentation of exogenous (nonparasite) Ags to T cells; this was presumed to be due to a defect in the display of antigenic peptide/MHC II complexes (27). A similar reduction in Ag presentation for exogenous nonparasite Ag previously had been noted for T. brucei rhodesiense-infected mice (46). These types of studies point to infection-related changes in APC function, although it is still unclear how Ag presentation capabilities develop among different APC subsets during early infection and the extent to which they may affect VSG-specific T cell responses. Moreover, DCs and their functions have not been examined in the context of African trypanosome infection, despite extensive evidence that DCs are responsible for initiating naive T cell responses to many protein Ags.

Thus, to better understand the role of macrophages and DCs in the development and specificity of VSG-specific Th1 cell responses, we have characterized phenotypic and functional parameters of Ag presentation during early T. brucei rhodesiense infection in both relatively resistant responder (B10.BR, B10) and susceptible nonresponder (C3HeB/FeJ, C3H) mice. These host strains have been previously characterized to show that B10 mice make VSG-specific Abs and develop VSG-specific Th1 cell responses, whereas C3H mice lack effective B cell or T cell responses to trypanosomes (3-5, 12). We examined multiple time points during early infection (before, during, and after the first wave of parasitemia) for APC function as well as the activation of VSG-specific Th1 cell responses within the lymphoid tissues. Overall, our results show that DCs play a pivotal role in orchestrating the early development of VSG-specific Th1 cell responses in resistant mice through up-regulation of surface costimulatory markers, secretion of IL-12, ability to process and present Ag for the activation of naive T cells, and presentation of VSG peptides during infection. However, altered functional capabilities were noted for infected mouse APCs in terms of processing and presenting exogenous nonparasite and parasite Ags to T cells, suggesting that exposure to trypanosomes in vivo may have dramatic consequences for Th cell reactivity to other Ags and, perhaps, to VSG determinants of new variants arising in infection.

Materials and Methods

Mice

H-2k compatible female B10.BR/SgSnJ (B10) and C3HeB/FeJ (C3H) mice were purchased from The Jackson Laboratory and used between 6 and 12 wk of age for studies of APC function. A colony of 3A9 TCR transgenic mice on the B10.BR background was established from a breeding pair provided by Dr. M. Sandor (University of Wisconsin, Madison, WI). Additionally, C57BL/6 mice (H-2b) that express a resistant VSG-responder phenotype when infected with trypanosomes of the T. brucei rhodesiense infection in both relatively resistant responder (B10.BR, B10) and susceptible nonresponder (C3HeB/FeJ, C3H) mice. These host strains have been previously characterized to show that B10 mice make VSG-specific Abs and develop VSG-specific Th1 cell responses, whereas C3H mice lack effective B cell or T cell responses to trypanosomes (3-5, 12). We examined multiple time points during early infection (before, during, and after the first wave of parasitemia) for APC function as well as the activation of VSG-specific Th1 cell responses within the lymphoid tissues. Overall, our results show that DCs play a pivotal role in orchestrating the early development of VSG-specific Th1 cell responses in resistant mice through up-regulation of surface costimulatory markers, secretion of IL-12, ability to process and present Ag for the activation of naive T cells, and presentation of VSG peptides during infection. However, altered functional capabilities were noted for infected mouse APCs in terms of processing and presenting exogenous nonparasite and parasite Ags to T cells, suggesting that exposure to trypanosomes in vivo may have dramatic consequences for Th cell reactivity to other Ags and, perhaps, to VSG determinants of new variants arising in infection.

Trypanosomes

T. brucei rhodesiense clones LouTat 1 and LouTat 1.5 were used for infections and were grown from frozen stables as previously described (47). Briefly, Swiss mice were immunosuppressed with cyclophosphamide treatment (300 mg/kg body weight; Sigma-Aldrich) (47) concurrent with i.p. injection of trypanosomes. After 5 days, blood was collected and diluted in PBS/1% glucose and experimental mouse groups were infected by i.p. injection of 10⁵ trypanosomes.

VSG purification

Trypanosomes harvested for VSG purification were passed over a DEAE cellulose column (DE52; Fisher Scientific) equilibrated with PBS/1% glucose to bind cellular components of host blood (48). Eluted trypanosomes were washed three times in PBS/1% glucose, and VSG purification was performed as previously described (4, 24, 45). Briefly, trypanosomes were resuspended in 0.3 mM zinc acetate containing 0.1 mM tosyl lysine chloromethyl ketone to 10⁶/ml and incubated for 15 min at 4°C before centrifugation at 3000 × g at 4°C for 10 min. Supernatant fluid was reserved and kept at 4°C. The pellet was resuspended in an equal volume of 10 mM phosphate buffer containing 0.1 mM tosyl lysine chloromethyl ketone incubated for 20 min at 37°C, then cooled to 4°C and spun at 10,000 × g for 15 min. Supernatant and previously reserved fluids were combined and spun at 300,000 × g for 1 h at 4°C. The resulting supernatant was concentrated by centrifugation through a Centriprep-30 column (Amicon) and passed over a DEAE-cellulose column equilibrated with 10 mM phosphate buffer (pH 8). VSG detected in the first peak eluted off the column was subjected to electrophoretic analysis and appeared as a single 62-kDa band on SDS-polyacrylamide gels under reducing conditions.

Isolation of macrophages and DCs

Adherent splenic and peritoneal macrophages were isolated from uninfected or infected mice at different times during infection. Splenectomized mice were disrupted in complete RPMI 1640 medium (RPMI 1640 Sigma-Aldrich) supplemented with 10% FBS; 2 mM l-glutamine, 16 mM HEPES, 50 μg/ml gentamicin) and passed through 40-μm nylon filters to obtain a single cell suspension. Peritoneal exudate cells collected in ice-cold PBS/ heparin (10 U/ml) and splenocytes were treated with ACK lysis buffer to remove erythrocytes. Cells were washed twice with complete RPMI 1640 medium, plated at 1 × 10⁶ peritoneal (or 2.5 × 10⁶ splenocyte) viable cells/ml, and incubated for 4 h at 37°C in 5% CO₂. Plates were washed thoroughly with PBS to remove nonadherent cells. Splenic macrophages were collected after incubation at 4°C for 15 min in ice-cold RPMI, followed by scraping with a rubber policeman. Peritoneal macrophages were lifted from plates using Accutase (Innovative Cell Technologies). Macrophages were washed and resuspended in complete DMEM (Sigma-Aldrich) supplemented with 10% FBS, 2 mM l-glutamine, 1% MEM nonessential amino acids, 16 mM HEPES, 50 μM 2-ME, and 50 μg/ml gentamicin before use.

Splenic DCs were isolated by enzymatic digestion of tissues with Dnase 1 (Invitrogen/Liberase (Roche Applied Science) in complete RPMI 1640 medium for 30 min at 37°C, 5% CO₂. Dnase I and Liberase concentrations were empirically determined for each day of infection to account for changes in spleen size and composition. After 5 min incubation with EDTA (0.01 M final), spleen fragments were passed through 40-μm nylon filters and washed with PBS/1% MEM (Gibco)/EDTA); erythrocytes were lysed with ACK lysis buffer. The remaining cells were overlaid onto 14.1% Nycodenz (Sigma-Aldrich) and spun at 1900 rpm for 20 min at 4°C. For flow cytometry, low-density enriched cells were resuspended in PBS/0.5% BSA/2 mM EDTA/0.05% azide (flow buffer) and treated as described below. For DC isolation, low-density cells collected from the interface were washed twice with MACS buffer, labeled with anti-CD11c magnetic beads (Miltenyi Biotec), and passed over two consecutive MACS columns (Miltenyi Biotec) according to a standard protocol provided by the manufacturer. CD11c⁺ DCs were washed and re-suspended in complete DMEM.

Flow cytometry

FITC-conjugated anti-CD11c, anti-CD11b, anti-CD4, and anti-CD3; PE-conjugated anti-CD40, anti-CD54, anti-CD80, anti-CD86, and anti-1-Ak; and allophycocyanin-conjugated anti-CD8 were purchased from BD Biosciences. Alexa Fluor 647-conjugated F4/80 was purchased from Serotec. and allophycocyanin-conjugated anti-CD8 were purchased from BD Biosciences. Alexa Fluor 647-conjugated F4/80 was purchased from Serotec. Isotype-matched Abs with the appropriate fluorochromes were used as controls. Peritoneal cells, splenocyte suspensions, or low-density enriched cells were incubated with Fc Block (BD Biosciences) for 15 min on ice to block Fc-specific staining. Cells were then incubated with appropriate Ag surface Ags for 30 min, washed twice with flow buffer, and stained with a live/dead fixable stain (Invitrogen) according to manufacturer protocols. All cells were washed twice with flow buffer, fixed in 1% parformaldehyde (Polysciences), and analyzed immediately on an LSR II using BD FACSDiva (BD Biosciences) and FlowJo (Tree Star) software. Live macrophages and DCs were identified based on dead cell exclusion, SSC/PC profiles, and CD11c⁺/F4/80⁺/DC11c⁺ (splenic macrophages), or F4/80⁺/DC11b⁺ (peritoneal macrophages) expression. Rainbow fluorescent particles (Spherotech) were used to set flowchromes.
volumes so that quantitative analysis of MHC II and costimulatory molecule expression could be performed across multiple days of infection.

**TCR transgenic T cell isolation**

Naive T cells were enriched from the spleen and lymph nodes (inguinal, axillary, brachial) of H-2b-compatible 3A9 TCR transgenic mice by adherence to nylon wool as previously described. Cell depletion was performed on the T cell-enriched population using Low-Tox rabbit complement (Cedarlane Laboratories) and mAbs 16-1-2 (anti-H2k), 31M (anti-CD8α), and M1/70 (TIB128; American Type Culture Collection (ATCC)). Live cells were collected on a Percoll (Sigma-Aldrich) gradient and washed twice in complete DMEM before use.

**Cytokine assays**

Macrophages (2 × 10^5) or DCs (10^5) from B10 or C3H mice were incubated in 96-well plates in complete DMEM for 24 h at 37°C, 5% CO₂. Supernatants were stored at −20°C before analysis. For cytokine secretion and Ag presentation assays (see below), IL-2, IL-4, TNF-α, IFN-γ, IL-18, IL-12p40, and IL-10 were measured using OptiEIA ELISA kits (BD Biosciences) unless otherwise noted. NO was determined by adding an equal volume of supernatant to 1% sulfanilamide (Sigma-Aldrich) in 2.5% H₃PO₄ and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich) in 2.5% H₃PO₄ and measuring absorbance at 550 nm. A standard curve was prepared using sodium nitrite. The ability of infected wild-type and IL-12 knockout mice to generate VSG-specific Th1 responses was determined by preparing spleen cell suspensions and stimulating 10^5 viable cells with 50 μg of purified VSG for 48–72 h, as we have described previously (4, 38, 39); supernatant fluids were harvested, stored and tested as described for IL-12 levels.

**Ag presentation assays**

Irradiated splenocytes (10^5), macrophages (10^5), or DCs (5 × 10^4) from naive and infected B10 or C3H mice were incubated with complete nonhybrid cells, and proliferating cell wells were expanded. Hybridomas were selected for VSG reactivity and CD3/CD4 expression could be performed across multiple days of infection.

**Cytokine production**

Because local cytokine environment influences the activation and polarization of Th cells, we examined cytokine production by APCs from naive and infected mice cultured ex vivo. As seen in Fig. 3, all APCs produced IL-10 and IL-12p40 at varying levels throughout infection. The ratio of Th1-associated IL-12 to IL-10, a regulatory cytokine, demonstrates the significant phenotypic potential of DCs to elicit Th1 responses. The timing of IL-12 production by DCs also coincided with up-regulation of surface markers. Although the general pattern of cytokine production was similar between B10 and C3H animals, APCs from B10 animals generally secreted higher levels of IL-12 at earlier times of infection. Splenic macrophages produced less IL-12 but more IL-10 than DCs, and peritoneal macrophages produced little IL-12 or IL-10 but high levels of NO, which has been shown to suppress T cell proliferative responses to both trypanosome Ags and mitogens (26, 35, 38). TNF-α, IL-4, and IL-18 were not detected (data not shown). These quantitative and temporal differences in cytokine responses by APCs during infection may be a contributing factor to the relative ability of B10 animals to mount a polarized Th1 cell response. The role of IL-12 in contributing to the Th1 phenotype was clearly demonstrated using knockout mice on a resistant B10 and susceptible C3H background. As shown in Fig. 4, the early development and expression of VSG-specific Th1 cytokine responses (IFN-γ and IL-2) were absent in IL-12 knockout mice from day 4
through day 10 postinfection; there was no compensatory Th2 cell response in IL-12 knockout mice as measured by IL-4 secretion. Interestingly the VSG-specific Th1 cytokine phenotype emerged in IL-12 knockout mice by day 14 of infection, demonstrating that even in the absence of IL-12 there are significant alternative signals for mounting this protective response to infection.

**APC function**

Cytokine secretion and MHC II/costimulatory molecule expression appeared to be temporally regulated in APCs during trypanosome infection. To relate APC phenotype to function, we first tested irradiated splenocytes for their ability to process and present a well-defined nonparasite Ag, HEL, to the HEL-specific 3A9 T cell hybridoma as a functional measure of Ag presentation capability. Splenic APCs isolated 2 days postinfection processed and presented HEL in a dose-dependent manner, similar to APCs from naive animals (Fig. 5). As infection progressed, splenic APCs elicited only weak T cell responses when combined with exogenous Ag, regardless of mouse strain tested. However, it may be worthwhile noting that APCs from resistant mice recovered a measurable ability to process and present exogenous Ag during the test period compared with APCs from susceptible mice.

To determine whether reduced Ag presentation extended to specific cell types or lymphoid compartments, DCs, splenic macrophages, and peritoneal macrophages were isolated from naive and infected animals and incubated with HEL and the 3A9 T cell hybridoma. As shown in Fig. 6, all APCs displayed dose-dependent HEL processing and presentation capability early in infection, whereas by day 4 or thereafter the ability to present Ag diminished. Ag presentation could not be rescued with high doses of Ag (up to 100 µg/ml concentrations) (Fig. 6). A reduction in DC processing and presentation of exogenous Ag during infection may be explained by their maturation state, as maturation halts endocytic activity. Our observations of increased IL-12 production and up-regulation of costimulatory molecules during the early period of infection support the contention that DCs undergo maturation during this time. Decreased endocytosis could not explain the decline in Ag-presenting function by macrophages, as FITC-dextran uptake was similar in both naive and infected animals (data not shown and B. E. Freeman, K. P. Demick, J. M. Mansfield, and D. M. Paulnock, manuscript in preparation).

Processing and presentation of exogenous Ag to T cell hybridomas reflects only the amount of HEL peptide:MHC II complexes expressed by APCs. To determine the additional contributions of costimulatory molecule expression or cytokine production, we tested the ability of APCs from control and infected animals to activate naive HEL-specific T cells from 3A9 transgenic mice. Similar to T cell hybridoma activation, naive TG HEL-specific T cells produced less IL-2 in response to APCs from infected animals (Fig. 7). DCs isolated at day 4 postinfection were, however, able to elicit high levels of IFN-γ secretion and T cell proliferation in the absence of IL-2. Splenic macrophages at day 4 elicited
IFN-γ production as well as IL-2 from T cells but were unable to induce proliferation. These data suggest that T cell proliferation observed in response to DCs may be IL-2-independent or, alternatively and probably, that rapidly replicating T cells in these 48-h cultures have taken up and used the IL-2 produced during activation. However, peritoneal macrophages were not capable of inducing T cell proliferation or cytokine secretion, and similar results were obtained with APCs from C3H mice (data not shown). APCs from infected animals preferentially polarized naive T cells toward a Th1 phenotype, as we did not detect IL-4 in the cell supernatant fluids of any cultures tested (data not shown); this is in complete agreement with earlier published data (4, 5, 49) and our findings with respect to polarization of the Th1 cell response in wild-type and IL-12 knockout mice. Overall, these results reveal that DCs

**FIGURE 2.** CD8α⁻ and CD8α⁺ DCs are selectively depleted in susceptible C3H but not resistant B10 mice infected with African trypanosomes. CD11chighCD8α⁻ or CD11chighCD8α⁺ DCs in live, low-density enriched splenocytes from B10 or C3H animals (n = 5 mice/group) on days 0–7 of infection are shown. The percentage of each subset is shown within the gated cell subpopulations. Results are of a representative experiment. The proportion for different time points of infection is as follows: B10.BR (Day 0) CD11chighCD8α⁻ (5%), CD11chighCD8α⁺ (2.8%), (Day 2) CD11chighCD8α⁻ (6.2%), CD11chighCD8α⁺ (2.6%), (Day 4) CD11chighCD8α⁻ (3.8%), CD11chighCD8α⁺ (1%), (Day 7) CD11chighCD8α⁻ (6.1%), CD11chighCD8α⁺ (1%); and C3H (Day 0) CD11chighCD8α⁻ (2.5%), CD11chighCD8α⁺ (0.5%), (Day 2) CD11chighCD8α⁻ (4.5%), CD11chighCD8α⁺ (1.1%), (Day 4) CD11chighCD8α⁻ (1.1%), CD11chighCD8α⁺ (0.4%), (Day 7) CD11chighCD8α⁻ (0.7%), CD11chighCD8α⁺ (0.03).

IFN-γ production as well as IL-2 from T cells but were unable to induce proliferation. These data suggest that T cell proliferation observed in response to DCs may be IL-2-independent or, alternatively and probably, that rapidly replicating T cells in these 48-h cultures have taken up and used the IL-2 produced during activation. However, peritoneal macrophages were not capable of inducing T cell proliferation or cytokine secretion, and similar results were obtained with APCs from C3H mice (data not shown). APCs from infected animals preferentially polarized naive T cells toward a Th1 phenotype, as we did not detect IL-4 in the cell supernatant fluids of any cultures tested (data not shown); this is in complete agreement with earlier published data (4, 5, 49) and our findings with respect to polarization of the Th1 cell response in wild-type and IL-12 knockout mice. Overall, these results reveal that DCs

**FIGURE 3.** Splenic APCs produce IL-12 upon infection, whereas NO is produced by peritoneal macrophages. Cytokine production by DCs and peritoneal (PMP) and splenic (SMP) macrophages taken from naive and infected animals (n = 5 mice/group) and cultured ex vivo is shown. Supernatants were collected after 24 h, and IL-12p40 and IL-10 production were assessed by ELISA. NO was determined by Griess reaction. Results are mean ± SEM of triplicate wells in a representative experiment. *, p < 0.05 as compared with naive control; †, p < 0.05 as compared with same measurement in opposite mouse strain.
isolated at distinct times of infection from either resistant or susceptible mice are functionally capable of activating Th1 cells. Splenic macrophages may also contribute to the development of Th1 responses, but peritoneal macrophages from either B10 or C3H animals clearly lack the ability to activate Th cells. Thus APC function is selectively altered in specific tissues and at specific times of infection with African trypanosomes.

Processing and presentation of VSG to T cells ex vivo

Although DCs and macrophages displayed similar functional characteristics for nonparasite Ag regardless of host resistance phenotype, a protective VSG-specific Th1 cell response occurs only in resistant B10 and C57BL/6 mice. We might predict based on our findings, therefore, that only APCs from B10 mice process and present VSG during infection, or that APCs from resistant animals display a different VSG peptide repertoire than C3H mice. To survey the presentation of VSG-derived peptides displayed by APCs ex vivo from infected animals, we combined APCs with VSG-specific T cell hybridomas (originally derived from Th cells activated during infection) and monitored activation by IL-2 production; prior analyses had demonstrated that the Th cell hybridomas tested represent clones with unique TCRs specific for peptides distributed throughout the N-terminal domain of the clone LouTat 1 VSG molecule (23).

VSG-specific T hybridomas were combined with APCs from naive and infected animals to determine the extent and kinetics of VSG presentation during infection. Fig. 8 shows representative hybridoma responses as compared with wild-type control responses.

**FIGURE 4.** IL-12 induces the early generation and polarization of VSG-specific Th1 cells during trypanosome infection. Relatively resistant C57BL/6 mice with the IL-12p35 gene deleted were infected with *T. brucei rhodesiense* clone LouTat 1. SPC were harvested at the time points shown and tested for the secretion of IFN-γ, IL-2, and IL-4 following stimulation in vitro with purified LouTat 1 VSG. Identical results were obtained with IL-12p40 knockout (KO) mice and wild-type (WT) mice treated with neutralizing Ab to IL-12 (data not shown; manuscript in preparation). Results presented as ± SEM of three separate experiments. *p < 0.05 knockout mouse responses as compared with wild-type control responses.
10, the endpoint of the experiment. These patterns were observed in all of the VSG-specific T hybridoma responses to APCs (Fig. 8 and data not shown). Because parasite burden remains high and uncontrolled in C3H mice, whereas B10 mice exhibit Ab- and T cell-mediated clearance of VATs by day 5 or 6 of infection, we hypothesized that the failure to clear parasites, resulting in continuous exposure to VSG, led to an extended period of presentation due to greater levels of VSG peptide:MHC II complexes expressed. To test this hypothesis, APCs from B10 mice at day 10 of infection (following clearance of the first wave of parasitemia and at a time when these cells were expressing no or low VSG peptide: MHC II complexes) were combined with VSG-specific T hybridomas with or without administration of exogenous VSG. APCs from infected B10 animals were able to process and present additional exogenous VSG (Fig. 9), supporting the notion that VSG availability in vivo dictates a level of VSG peptide presentation. Additionally, the ability of APCs from infected animals to process and present exogenous VSG suggests a different mechanism of processing and presentation from that of HEL. DCs from B10 and C3H animals elicited IL-2 production from all but one VSG-specific T hybridoma (Table I), reflecting the variety of VSG epitopes that are presented for Th cell recognition. DCs also expressed comparatively higher levels of VSG peptide:MHC II molecules, as indicated by the number of hybridomas producing high amounts of IL-2 relative to responses from macrophages. Splenic macrophages from both B10 and C3H mice elicited IL-2 production from the same VSG-specific hybridomas highly activated by DCs, but IL-2 responses were moderate or low (Table I). Splenic macrophages failed to activate the remaining hybridomas and therefore by inference express fewer and less varied VSG peptide:MHC II molecules on their surface during infection compared with DCs. Regardless of host strain, DCs and splenic macrophages activated the same hybridomas to a similar extent during peak parasitemia (Table I and data not shown). Splenic APCs of both resistant and susceptible animals, therefore, appear equally capable of processing and presenting VSG during infection.

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** DCs and macrophages from infected animals display impaired processing and presentation of newly encountered Ag. DCs, splenic macrophages (SMP), or peritoneal macrophages (PMP) isolated from uninfected and infected mice (days 0–10 postinfection for $n = 5$ mice/group) were combined with the 3A9 T cell hybridoma and varying concentrations of HEL Ag. Supernatants were collected after 24 h and assayed for IL-2 production by ELISA. Results are mean ± SEM of triplicate wells in a representative experiment.
In contrast to splenic APCs, peritoneal macrophages activated comparatively few VSG-specific T hybridomas (Table I). The limited variety of peptides presented within the peritoneum may reflect the amounts or form of VSG encountered throughout infection. Indeed, peritoneal macrophages from infected animals supplied with exogenous VSG were capable of activating a wider array of VSG-specific T hybridomas than when tested ex vivo (Fig. 9 and data not shown). Additionally, some hybridomas were uniquely activated by peritoneal macrophages from either B10 or C3H mice, suggesting differences in peritoneal macrophage processing of VSG between strains (Fig. 8).

**Processing and presentation of newly encountered VSG**

These results demonstrate that APCs from *T. brucei rhodesiense* clone LouTat 1-infected mice present LouTat 1 VSG peptides to T cells coincident with a failure to process and present additional exogenous nonparasite Ags such as HEL to T cells. These experiments also show that sufficient concentrations of VSG peptide: MHC II complexes, in which the peptides are derived from the surface coat of the infecting trypanosome strain, are presented only as long as the parasites remain in host tissues. What is not known is whether subsequent exposure to a newly encountered trypanosome VSG (e.g., that of a different VAT) results in processing and presentation, like the initial VSG, or whether such molecules are not processed or presented, similar to HEL. To test this question, B10 mice were first infected with LouTat 1.5, a distinct VAT of the LouTar serodeme that displays a unique VSG (51, 52); VSG-specific T cells from LouTat 1.5-infected mice do not cross-react with LouTat 1 VSG and LouTat 1 VSG-specific T cells from LouTat 1-infected mice do not cross-react with LouTat 1.5 VSG (4, 23 and data not shown). DCs were isolated from both naive and LouTat 1.5-infected animals and combined with LouTat 1 VSG-specific Th cell hybridomas in the presence or absence of exogenous LouTat 1 VSG. As shown in Fig. 10, DCs from LouTat 1.5-infected animals were unable to stimulate LouTat 1 VSG-specific T hybridoma cells (or HEL-specific T hybridomas (data not shown)) during the period of time when LouTat 1.5 parasite burden was high (days 4–7). These data show that active infection alters the ability of APCs to process or present exogenous Ags, including the VSG molecules of other trypanosome VATs. These data also reinforce previous evidence from this lab that VSG-specific T cells activated during infection do not cross-react with other VSGs and therefore do not contribute toward protective immunity against subsequent waves of infection, despite the presence of conserved sequences (4, 23, 49). The reason why conserved VSG sequences are not processed to peptides for presentation to T cells remains to be determined.

**Discussion**

Trypanosome VSG molecules are members of a protein superfamily that exhibits extensive variable sequences as well as defined conserved amino acid subsequences. Conserved invariant sequences permit VSGs to fold and orient in the same manner within the surface coat structure and also provide a scaffold for membrane anchoring via GPI residues; this preservation of structure is likely important to preserve a physical coat barrier that protects plasma...
membrane components from exposure to innate and adaptive immune attack during infection. However, most of the VSG molecule contains variable sequences that distinguish different VSGs and trypanosome VATs from one another. It has been suggested that B and T cell reactivity against VSGs resulted in selective pressure to alter amino acids within variable subregions of the molecule; thus antigenic variation of B and T cell epitopes within such regions provides a means to escape immune elimination. However, our recent studies of VSG-specific T cell responses in vivo demonstrated that infected responder mouse Th1 cells recognize multiple variant peptides distributed throughout the N-terminal domain of the VSG molecule (23), but there were no T cell responses detectable to invariant residues including those in the C-terminal domain.

**FIGURE 8.** APCs ex vivo from infected mice present VSG peptides to T cells. DCs or peritoneal (PMP) and splenic (SMP) macrophages isolated from uninfected and infected animals (days 0–10 postinfection for n = 5 mice/group) were combined with VSG-specific T hybridomas without the addition of exogenous VSG (50 μg/ml). IL-2 production was measured in 24 h supernatants by ELISA. Results are representative responses from four of 13 T hybridomas. The APC type and T hybridoma used are shown (Note: Unlike splenic DCs or macrophages, peritoneal macrophages from resistant and susceptible animals activated different subsets of T hybridomas). Results are mean ± SEM from triplicate wells in a representative experiment. *p < 0.05 as compared with naive control.

**FIGURE 9.** VSG availability contributes to the sustained presentation of VSG peptides during trypanosome infection. B10 APCs isolated at day 10 of infection (following clearance of LouTat 1 trypanosomes and at a time when such APCs ex vivo were no longer able to present peptides to the T hybridomas; see Fig. 8) were supplemented with additional (exogenous) VSG and cultured with VSG-specific T cell hybridomas. Two representative T hybridoma responses are shown. Peritoneal macrophages (PMP) were also able to activate these hybridomas when provided with exogenous VSG, though they were unable to do so at any time during infection without additional VSG added to cultures. IL-2 was measured by ELISA. Results are displayed as mean ± SEM of triplicate wells in a representative experiment.

**FIGURE 10.** DCs from mice infected with LouTat 1.5 display impaired processing and presentation of newly encountered variant LouTat 1 VSG. B10 animals were infected with LouTat 1.5, and DCs were collected on days 0, 4, 7, and 10 of infection. DCs were combined with different LouTat 1 VSG-specific T cell hybridomas (shown with each plot) and with or without the addition of LouTat 1 VSG. IL-2 was measured by ELISA. Results are displayed as mean ± SEM of triplicate wells in a representative experiment.

### Table I. VSG presentation by APCs during infection

<table>
<thead>
<tr>
<th>APCs</th>
<th>Max. Hybridoma Responsea</th>
<th>Hybridoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10/C3H DCs</td>
<td>+ + +</td>
<td>5.4, 38.6, 40.6, 28A, 130A, 65A, 14A</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.6, 17.5, 116A, 100A, Q7</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>12.2</td>
</tr>
<tr>
<td>B10/C3H SMPs</td>
<td>+ + +</td>
<td>38.6, 28A</td>
</tr>
<tr>
<td></td>
<td>+ +</td>
<td>40.6, 130A, 65A, 14A</td>
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<tr>
<td></td>
<td>+</td>
<td>5.4, 116A</td>
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<tr>
<td></td>
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<td>4.6, 17.5, 100A, Q7, 12.2</td>
</tr>
<tr>
<td>B10 PMPs</td>
<td>+ + +</td>
<td>n/a</td>
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<td></td>
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<td>28A</td>
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<td></td>
<td>+</td>
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<td>5.4, 40.6, 130A, 65A, 14A, 4.6, 17.5, 100A, Q7, 12.2</td>
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<tr>
<td>C3H PMPs</td>
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<tr>
<td></td>
<td>−</td>
<td>130A, 65A, 116A, 4.6, 17.5, 100A, Q7, 12.2</td>
</tr>
</tbody>
</table>

a The maximum hybridoma response elicited by DCs, peritoneal macrophages (PMPs), and splenic macrophages (SMPs) isolated from infected animals. + + + = >400 pg/ml IL-2; + + = 200–400 pg/ml IL-2; + = 40–200 pg/ml; − = <40 pg/ml. n/a, None activated.
suggesting that peptides may not be generated for MHC II binding and TCR presentation from these sites.

To examine the role of APCs in processing and presentation of VSG molecules during trypanosomiasis, and to address known issues regarding the activation and regulation of VSG-specific T cell responses during infection, we tested the hypothesis that APC functions are functionally altered by trypanosome infection. Thus we have broadly examined the phenotype and function of macrophages and DCs during experimental African trypanosomiasis in the context of activation of T cells for both VSG peptides and exogenous nonparasite peptides. Analysis of splenic APC phenotypes revealed some intriguing differences between responder B10 and nonresponder C3H animals that may contribute to the relative resistance and susceptibility of these strains. DCs and splenic macrophages from responder B10 animals up-regulated MHC II or costimulatory molecules and produced IL-12 that contributes to development of the Th1 cell phenotype during early infection. In contrast, splenic macrophages from nonresponder C3H animals down-regulated CD40, CD80, and CD86 by day 4, at which time their expression would be essential for providing proper costimulation to naive Th cells. Although some markers were up-regulated on DCs from C3H mice and cells produced IL-12 levels similar to responder B10 mice, both CD8α-DCs and CD8α+ DCs displayed decreased levels of CD80 expression, and CD8α-DCs continuously down-regulated MHC II during infection.

Perhaps most striking, we observed a reduction in the proportion of CD8α-DCs and complete depletion of CD8α-DCs by day 7 in C3H animals. Loss of CD8α- and CD8α+ DCs may be the result of changes in migration or apoptosis of DCs. Apoptosis-induced depletion of CD8α+ DCs by an unknown mechanism was recently observed in Plasmodium chabaudi-infected mice (53). A growing body of evidence suggests that mature DC are susceptible to MHC II-mediated apoptosis, resulting from interactions with other DCs, lymphocytes, or even microbial ligands (54, 55). MHC II-mediated apoptosis may therefore play a role in CD8α+ DC depletion, as CD8α+ DCs from C3H mice appear phenotypically mature before depletion. Parasite-mediated apoptosis may also drive DC death, a phenomenon observed in human DCs cultured in vitro with Brugia malayi microfilariae (56). Given the known activating and deactivating effects of GPI residues associated with the VSG molecule, it is possible that GPI residues preferentially promote DC maturation and augment apoptosis during infection of susceptible animals.

It is difficult to predict how the diminished proportion of CD8α+DCs or CD8α-DCs may affect the development of Th1 responses to trypanosomes, as we have not included functional assays for each distinct CD8 DC subset. However, the ability to process and present Ag ex vivo and present VSG peptides in vivo was similar between total CD11c+ DCs of B10 and C3H mice throughout infection. It is possible that CD8α-DCs remaining within C3H mice when CD8α+ have been depleted provide a similar functional contribution as the combination of subsets. Substantial evidence supports functional plasticity among DC subsets, such that environmental cues (e.g., microbial products such as GPI residues) can induce similar cytokine responses and Ag presentation capabilities from different subsets (57–59). Regardless of any subset-specific functions, a reduction in the number of DCs, combined with down-regulation of MHC II, will limit productive Th interactions and could impair the development of Th cell-mediated immunity in susceptible animals.

APC maturation and activation within the peritoneum seem to be regulated differently than in the spleen, perhaps because of differences in the local cytokine environment and the degree of interaction with parasites or parasite Ags. Unexpectedly, only susceptible peritoneal macrophages coordinately up-regulated both MHC II and costimulatory molecules at the time of splenic APC activation and peak parasitemia. However, we have observed that total peritoneal cells and the proportion of peritoneal macrophages in C3H mice decrease dramatically during early infection, whereas the number and proportion of peritoneal macrophages in B10 animals increases (our unpublished observations). A reduction in the number of peritoneal macrophages would thus significantly limit productive interactions with naive or effector Th cells. Aside from phenotypic differences observed, peritoneal macrophages produced little IL-12 and were unable to induce naive T cell proliferation or cytokine production when isolated from infected B10 or C3H mice. In fact, trypanosome infection may preferentially impair regulatory or suppressive capabilities on peritoneal macrophages. We observed high levels of NO production by peritoneal macrophages 1 wk after infection, at which time VSG-specific Th1 cytokine but not proliferative responses are consistently detected within the peritoneum; NO has been shown to contribute to the suppression of Th cell responses during early trypanosomiasis and may limit T cell clonal expansion (15, 24, 38).

Further support for this theory on Th cell responses comes from our analysis of VSG presentation in vivo. Fewer (or different) VSG peptides are presented by peritoneal macrophages, in comparison to the broader variety and higher level of presentation of VSG peptides by splenic APCs, as noted by the selective activation of different VSG-specific T hybridomas. Restricted processing or presentation of VSG may thereby only elicit effector functions from a limited number of VSG-specific Th1 cells. We might speculate that a variety of VSG-specific Th1 cells initially becomes activated in the spleen or lymph nodes, but migration to the peritoneum results in suppression through soluble mediators (NO, PGE) that limit clonal expansion and reduce VSG peptide presentation in a site-specific context of reduced costimulation (resistant peritoneal macrophages) or fewer APC contacts (susceptible peritoneal macrophages). These compartment-specific differences in APC activation may underlie the limited Th cell responses that we previously have observed in resistant responder animals (4, 13, 38).

Regardless of cell type or mouse strain, APCs from infected animals displayed a reduced ability to process and present exogenous Ag to Ag-specific T cell hybridomas and naive T cells. This includes newly encountered exogenous VSGs, which may help explain why, despite the continuous generation of new VATs during infection and the potential for T cell priming by exposure to such minor VATs, the host adaptive Th cells “see” only the VSG expressed by the predominant trypanosome VAT. DCs may be taking up less Ag as a result of maturation, but altered endocytosis does not explain the reduction in Ag presentation by macrophages. Reduced presentation of exogenous nonparasite Ag has previously been observed in macrophages from T. brucei brucei-infected animals and was attributed to a potential defect in loading of exogenous antigenic peptides onto MHC II (27). It is possible that trypanosome Ags such as VSG with its GPI residues or trypanosome CpG DNA affect intracellular processing events. Preliminary experiments pretreating bone marrow-derived DCs with VSG or trypanosome DNA resulted in a reduced ability to present HEL to the 3A9 T cell hybridoma, though the mechanism of inhibition has yet to be defined (our unpublished observations). However, more recent results from our lab show that APCs taken from infected mice fail to generate new stable intracellular VSG peptide/MHC α- and β-chain complexes and that recycling of VSG peptide: MHC II complexes from the cell membrane is altered (B. E. Freeman, K. P. Demick, J. M. Mansfield, and D. M. Paulnock, Instability of peptide:MHC II complexes underlies modulation of APC function in African trypanosomiasis. Submitted for publication).
Differences in VSG processing and presentation among DCs and splenic and peritoneal macrophages are likely associated with the form or amount of VSG seen within different lymphoid compartments. We have established that splenic DCs and splenic macrophages from both B10 and C3H animals displayed a greater array of VSG-derived peptides than peritoneal macrophages. However, peritoneal macrophages from infected mice provided with additional exogenous VSG were able to activate T cell hybridomas that only splenic APCs could activate during infection, thus suggesting that VSG exposure or uptake differs among lymphoid compartments. One theory would be that splenic APCs encounter a greater proportion of VSG filtered from the blood than whole parasites, whereas peritoneal macrophages may phagocytize a greater proportion of parasites or take up less VSG. We also observed that peritoneal macrophages from B10 and C3H mice process VSG to present a different array of peptides. Because only peritoneal macrophages from B10 animals would encounter parasites opsonized with anti-VSG Abs, the route of Ag uptake may reflect differential processing. Extended encounter with the parasite or parasite Ags (or the GPI residues of VSG) in C3H mice may also alter proteolytic capabilities of peritoneal macrophages. Studies are currently underway to explore the trafficking of GPF-positive LouTat 1 trypanosomes in vivo to determine the extent of parasitemia within the tissues compared with the vasculature, so that we might better understand how parasites interface with cells of the innate immune system.

In conclusion, we have tested the hypothesis that trypanosome infection alters APC function using a variety of classical approaches. Our results reveal that APC phenotype and Ag processing/presentation capacity are altered by infection, but that the overall picture is quite complex and is dependent on multiple factors: APC type (DCs vs macrophages); tissue source (lymphoid organs vs peritoneum); maturation state relative to time of infection (surface expression of MHC II and costimulatory factors); availability of parasite Ag (levels of parasitemia in resistant vs susceptible mice at the time of sampling); and prior exposure to VSG GPI determinants or other parasite factors. In particular our results demonstrate that splenic DCs are the primary APC population responsible for eliciting Th1 cell responses during trypanosome infection in resistant responder B10 animals. DCs clearly undergo maturation within the first few days of infection, as evidenced by up-regulation of MHC II and costimulatory molecules, secretion of IL-12, and an enhanced ability to activate naive T cells to a Th1 phenotype. Coincident with their maturation, DCs present high levels of VSG peptides in vivo and are able to activate VSG-specific T hybridomas as well as infected mouse Th cells ex vivo. The timing of DC maturation and VSG presentation correlate with peak parasite tissue burden and the presence of VSG shed into plasma (44). DCs thus take up trypanosome Ag early in infection and present high and varied levels of VSG peptide:MHC II complexes, along with proper costimulatory and cytokine signals, for development of VSG-specific Th1 cells. Splenic macrophages may play only a supporting role in the development of T cell-mediated immunity by providing additional IL-12 and costimulatory signals for a select number or subset of VSG epitope-specific Th cells. Although VSG-specific Th cell responses have been detected within the peritoneum, peritoneal macrophages exhibit reduced Ag processing and presentation capabilities, secretion of cytokines and factors such as NO that inhibit T cell clonal expansion, and limited VSG presentation during infection that likely reflect regulatory or suppressive functions. The finding that, independent of the APC populations studied, cells expressing peak levels of VSG peptide: MHC II complexes are unable to process and present newly encountered exogenous Ag, including new VSG molecules is novel. This finding has far-reaching implications for control of a parasite population that constantly expresses new surface coats, and this observation may also impact on attempts to vaccinate trypanosome-infected hosts against any Ag.

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Disclosures

The authors have no financial conflict of interest.

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