Induction and regulation of *Trypanosoma brucei* VSG-specific antibody responses

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SUMMARY

The review addresses how infection with *Trypanosoma brucei* affects the development, survival and functions of B lymphocytes in mice. It discusses (1) the contributions of antibodies to trypanosome clearance from the bloodstream, (2) how B lymphocytes, the precursors of antibody producing plasma cells, interact with membrane form variable surface glycoprotein (VSG), i.e. with monovalent antigen that is free to diffuse within the lipid bilayer of the trypanosome plasma membrane and consequently can cross-link B cell antigen specific receptors by indirect processes only and (3) the extent and underlying causes of dysregulation of humoral immune responses in infected mice, focusing on the impact of wild type and GPI-PLC$^{-/-}$ trypanosomes on bone marrow and extramedullary B lymphopoiesis, B cell maturation and survival.

Key words: *T. brucei*, antibody clearance, B cell activation, B cell deletion.

VSG COAT

Trypanosoma brucei inhabits the blood plasma, lymph and interstitial fluids, fully exposed to humoral immune attack. Its primary defence is a cloak of membrane-bound glycoprotein that does not elicit production of opsonins or assembly of membrane attack complexes in the absence of antibody, and which is subject to antigenic variation. On each *T. brucei*, the surface coat is made up of about 10 million identical copies of variable surface glycoprotein (VSG), encoded by a single gene and organized as a more-or-less contiguous array of 5 million non-covalent VSG homodimers (Mehlert *et al.* 2002). The expressed VSG gene is selected from a large archive of silent VSG genes and pseudogenes arranged as sub-telomeric arrays (Marcello and Barry, 2007), and is periodically varied, during which process the trypanosome transitorily has a mosaic coat comprised of 2 distinct VSG homodimers. VSG genes are expressed in a somewhat orderly manner resulting in recurring waves of parasitaemia as new variant antigenic types evade clearance by on-going protective antibody responses. Irrespective of the VSG gene that is expressed, each VSG is held in the outer leaflet of the plasma membrane by a dimyristoyl glycosyl phosphatidylinositol (GPI) anchor and VSG homodimers are each free to diffuse in the plane of the plasma membrane, which has important implications for antibody and trypanosome interactions and for trypanosome and B cell interactions as discussed below. In addition, the VSG coat can, under hypo-osmotic and other stress conditions that breach the plasma membrane (Cardoso de Almeida *et al.* 1984; Rolin *et al.* 1998), be cleaved from the parasite surface by a GPI-phospholipase C (GPI-PLC). The GPI-PLC is a virulence factor (Webb *et al.* 1997) that enhances inflammation, possibly through the action of soluble VSG (sVSG) and remnant GPI (Magez *et al.* 1998) and, as discussed below, is associated with profound dysregulation of B cell lymphopoiesis, maturation and activation to antibody production.

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TRYPANOSOMA BRUCEI AND ANTIBODY INTERACTIONS

Clearance of African trypanosomes from the bloodstream is mediated by VSG-specific, antibody-dependent phagocytosis in the liver and other tissues (Macaskill *et al.* 1980; Dempsey and Mansfield 1983; Pan *et al.* 2006). This occurs only at a permissive concentration of plasma antibodies, which in the case of *T. brucei* is typically achieved after most blood stream form organisms have switched to non-dividing stumpy forms (Newson *et al.* 1990; McLintock *et al.* 1993). Various immunoglobulin classes have been implicated in phagocytic removal and killing of the parasites. 'Immunoglobulin M (IgM) antibodies to VSG, which are the first and predominant class of trypanocidal antibodies in the infected host', cause

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complement fragments to be deposited on the parasites (Pan *et al*. 2006) and the membrane attack complex that assembles proximal to antibody-VSG complexes can lyse the parasites *in vitro* (Guirnalda *et al*. 2007). In addition, the iC3b fragment of complement factor 3, which is a byproduct of antibody-induced complement activation as well as the alternatively activated complement cascade, expedites phagocytosis of IgM coated trypanosome *in vivo* through complement receptor CR3 (CD11b/CD18), in the process provoking tumor necrosis factor (TNF)-alpha production and possibly down-regulating NO synthesis (Pan *et al*. 2006). Immunoglobulin G (IgG) can also mediate phagocytosis of African trypanosomes (Takahashi *et al*. 1987; Kaushik *et al*. 1999) and there is clear evidence that IgG but not IgM antibodies clear *T. congolense* from the bloodstream of infected mice (Magez *et al*. 2006; Pan *et al*. 2006). Since trypanosome motility is likely to affect the efficacy of phagocytosis, damage of antibody-coated parasites by innate defence molecules released onto the parasites when in the embrace of the phagocyte (macrophages and neutrophils) may affect host control of parasitaemic waves. In this regard, some *T. brucei* are killed by TNFα (Magez *et al*. 2001) while others are not (Kitani *et al*. 2002) and NO has general trypanocidal activity (Duleu *et al*. 2004). Thus, under some circumstances IgM may make a substantial contribution to parasite clearance by enhancing TNFα production and expediting CR3-mediated phagocytosis. In other cases IgM may have lesser relevance and IgG (Fcγ)-mediated phagocytosis, expedited by the impact of diffusible oxygen radicals on the parasites, may hold the sway. Diffusible oxidative defenses include NO and H_2O_2 (Wang *et al*. 2002; Magez *et al*. 2006; Pan *et al*. 2006) the latter of which has been shown to deplete *T. brucei* ATP making the parasites moribund (Muranjan *et al*. 1997; Wang *et al*. 2002) putatively by driving glucose metabolism into a truncated (dead-end) pentose phosphate pathway (Cronin *et al*. 1989).

The accumulation of VSG-specific antibody on the trypanosome surface is slowed by endocytosis, which occurs via the trypanosome flagellar pocket. Endocytosis of VSG-bound antibodies is governed both by hydrodynamic sorting of the antibody complexes to the flagellar pocket under flow conditions created by the swimming organism, and by the rate of endocytosis (Engstler *et al*. 2007). Thus, VSG-bound antibody of the IgM class is cleared more rapidly than antibody of the IgG class reflecting their respective sizes and capacities to act as molecular sails, and bound antibody is cleared more rapidly from non-dividing stumpy form than dividing slender form *T. brucei* reflecting the respective endocytic activities of the parasites used in the studies (Engstler *et al*. 2007). In other cases, endocytic rates of slender and stumpy *T. brucei* have been reported to be similar and both several fold greater than those of procyclic (tsetse gut form) trypanosomes (Natesan *et al*. 2007).

Directional streaming of antibody-tagged VSGs on the trypanosome surface is inconsistent with extensive cross-linking of VSGs, which would rigidify the parasite surface. Rather, it is consistent with the binding of only one Fab (an antigen binding arm of an antibody) per trypanosome attached VSG homodimer, in agreement with constraints imposed by the molecular dimensions of the exposed face of VSG homodimers and the antigen binding footprint of antibody molecules. In this regard each *T. brucei* has 5 million VSG-GPI homodimers dispersed over a plasma membrane area of 165 μm^2 'in a (hypothetical) hexagonal grid (pattern) with spacing of 5·7 nm (corresponding to the exposed face of a VSG homodimer), a random displacement of 0·5 nm and a random rotation perpendicular to the membrane' (Mehlert *et al*. 2002). Because the antigen binding footprint of a single antigen combining site is in the order of 765 $Å^2$ to 1500 $Å^2$ (Karpusas *et al*. 2001; Lok *et al*. 2008), equivalent to squares with sides of 2·76 nm to 3·9 nm, and occupies about half of the antigen binding face of the Fab, only a single Fab of secreted or membrane form immunoglobulin (Ig) could dock on the exposed face of each VSG homodimer on a healthy *T. brucei*. Thus, antibodies as well as VSG-specific B cell receptors (BCR), which are bivalent membrane bound Igs, can react with this exposed VSG epitope and no other VSG epitopes so long as VSG is attached to the surface of healthy trypanosomes.

Secreted IgG and IgM bind respectively to 2 and 10 VSG homodimers and, assuming a single bound Fab per VSG homodimer, will assort independently from each other and from other bound IgMs and IgGs. In the presence of high concentrations of antibody against their VSG coat, trypanosomes aggregate *in vitro*. However, the aggregates of trypanosomes disassemble over time by processes that do not include separation of immunoglobulin chains, or their proteolytic cleavage, or release of VSG from the trypanosome surface (O'Beirne *et al*. 1998) and most likely result from eventual sorting of bound antibody to single organisms and endocytosis. The lack of extracellular degradation of trypanosome-bound VSG-specific Ig suggests that interactions of trypanosomes with clonally expressed BCRs, would be similarly neutral with respect to impact on BCR chain integrity and gross structure.

TRYPANOSOMA BRUCEI AND B CELL ACTIVATION

Pleomorphic *T. brucei* readily induce antibody responses specific for exposed epitopes on trypanosome-attached VSG. Both T cell-independent and T cell-dependent responses are induced, with the former being largely responsible for clearance of

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parasitaemic waves (Mansfield and Paulnock, 2008). The T cell-independent antibody responses that arise during infection and cause remission of pleomorphic *T. brucei* parasitaemic waves are directed against exposed epitopes on parasite-attached VSG and do not react with sVSG (Sendashonga and Black, 1982). It is not known whether the antibodies are of too low an affinity to react with sVSG, or are limited by recognition of a conformational epitope that requires VSG-GPI membrane association, or are limited by both affinity and target epitope conformation. Given the inability of their antibody products to react with sVSG, it is likely that B cells responsible for producing these antibodies are also specific for epitopes present on the exposed face of trypanosome attached VSG only and thus that activation of the B cells requires their interaction with VSG presented on *T. brucei*.

T cell-independent B cell responses are readily activated by cross-linking of antigen-specific BCRs by multivalent ligands (Puffer *et al*. 2007). However, unlike multivalent ligands, VSG homodimers on trypanosomes cannot directly cross link BCRs because each VSG homodimer is free to diffuse in the outer leaflet of the trypanosome plasma membrane and, as discussed above, can accommodate binding of only one Fab. Recently it has been shown that antigen-lipid complexes bearing a monovalent B cell epitope and free to diffuse in a planar lipid membrane can activate B cells specific for that epitope (Tolar *et al*. 2009) suggesting that the same might be true of trypanosome-associated VSGs. Activation follows the development of BCR microclusters that form solely through intrinsic properties of the extracellular and transmembrane domain of the antigen-bound membrane Ig. It is hypothesized that following attachment of the IgM BCR to its specific ligand-lipid in the membrane, an interactive face is exposed on the Cμ4 domain and binds to the interactive face similarly exposed on a neighbouring antigen-bound BCR. With time, the zone of BCR interaction spreads resulting in BCR cluster and later synapse formation. While the inclusion of the adhesion molecule ICAM-1 in the planar membrane stablilizes BCR cluster formation and expedites synapse formation and B cell activation (Sohn *et al*. 2008), as does the inclusion of the B cell co-stimulatory molecule CD19 (Depoil *et al*. 2008), neither appear to be essential for synapse formation. Indeed, Tolar *et al*. (Tolar *et al*. 2009) showed that BCR interaction with a single species of monovalent ligand in an otherwise empty lipid bilayer leads to synapse formation, elicits a signaling cascade and activates the B cells. Thus, it would be expected that VSG on intact motile *T. brucei* would similarly activate B cells. However that is not the case.

Neither monomorphic *T. brucei* ILTat 1.4, which expresses a homogenous VSG, nor *T. brucei* LouTat 1, which is genetically engineered to express a mosaic of two distinct VSG homodimers on its surface, stimulates antibody responses against constituent VSG homodimers during infections in mice (Dubois *et al*. 2005; Sendashonga and Black, 1982). It is inconceivable that the failure of these trypanosomes to induce anti-VSG responses could result from a failure to bind BCRs. In this regard, Tolar *et al*. (2009) showed that 150 monovalent antigen-phospholipid molecules dispersed over an area of 1 μm^2 of planar lipid membrane were adequate to cause B cell activation, whereas the density of VSG homodimers on the surface of intact *T. brucei* is about 20 times higher than this. Furthermore, we calculate (legend Fig. 1) that the distance between antigen binding sites on a BCR is 6·3 nm to 15 nm and thus, when one antigen combining site on the bivalent BCR is bound to a VSG homodimer, the other could conceivably associate with any of 18 adjacent VSG homodimers (Fig. 1), or 9 of the surrounding VSG homodimers in organisms with equal amounts of 2 distinct VSG homodimers, given appropriate epitope orientation and the dimensions of the VSG homodimer hexagonal array proposed by Mehlert *et al*. (2002). We therefore conclude that cognate interaction between BCRs and exposed epitopes of VSG on intact viable trypanosomes is not enough to activate B cells to antibody production despite results obtained with model antigen conjugated to membrane phospholipid.

With respect to monomorphic *T. brucei* ILTat 1.4, which grows exponentially until death of its murine host, B cells of infected mice are neither stimulated to proliferate, nor to generate ILTat 1.4 VSG-specific antibodies (Sendashonga and Black, 1982, 1986) even in mice infected with a single organism and surviving for 9 days prior to death from overwhelming parasitaemia. Failure of the monomorphic ILTat 1.4 to activate specific B cells does not result from immunosuppression because the parasites do not interfere with the development of antibody responses against the VSG of a co-infecting pleomorphic *T. brucei*. Neither does the inability of monomorphic *T. brucei* ILTat 1.4 to stimulate an antibody response reflect an intrinsic lack of immunogenicity of exposed ILTat 1.4 VSG epitopes, because lethally irradiated *T. brucei* ILTat 1.4 effectively stimulates antibody responses specific for the ILTat 1.4 VSG on healthy organisms (Sendashonga and Black, 1982, 1986). It is possible that *T. brucei* ILTat 1.4 and mosaic trypanosomes discussed above fail to stimulate B cells simply because they break free of bound BCRs prior to BCR cluster and synapse formation, or that other, as yet unrecognized, properties of these cells prevent BCR microcluster formation, or in some other way prevent the activation of specific B cells.

Development of antibody responses to pleomorphic *T. brucei* coincides with the arisal of short-lived stumpy forms of the parasites, suggesting an association between trypanosome differentiation, or

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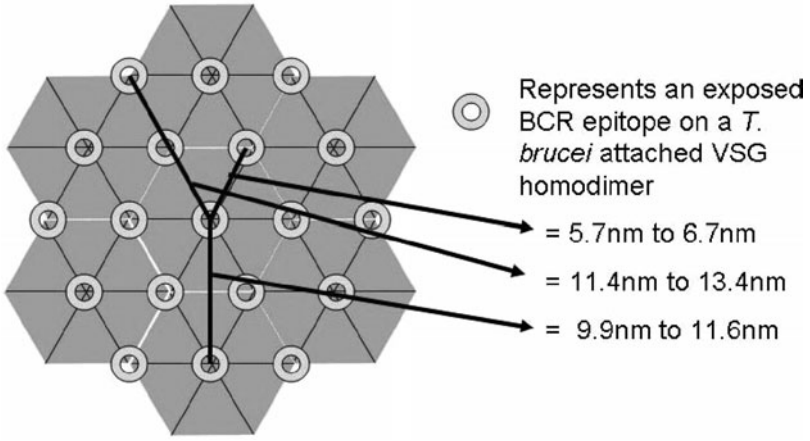

Fig. 1. Hypothetical distribution of B cell antigen specific receptor (BCR) epitopes on VSG. BCR epitopes are shown on a small area of the VSG coat of an intact, healthy *T. brucei*. For the sake of simplicity each BCR epitope is positioned in the centre of the exposed face of a hypothetical VSG homodimer (not shown). The figure assumes that the VSGs are evenly dispersed and all have either 0 nm, or 0·5 nm, random displacement. Given a monomeric IgM BCR antigen binding site separation of 6 nm to 15 nm, BCRs on *T. brucei* VSG-binding B cells would be expected to form bivalent interactions with combinations of VSG homodimers that fall within that range. Thus, when a BCR Fab binds to the VSG homodimer in the centre of Fig. 1, the other Fab on the BCR could conceivably associate with any one of the 18 surrounding VSG homodimers, given adequate time, an adequate affinity of the bound Fab and an appropriate orientation of the target epitope. (The distance between antigen binding sites on a BCR is 6·3 nm to 15 nm calculated from a Fab to Fab angle of monomeric IgM of 105° +/− 56° (Roux *et al*. 1998), a Fab length of 7·6 nm and location of combining sites on the intersect between a straight line passing through the tips of the Fabs and connecting lines bisecting the Fabs.)

death, and induction of antibody responses. In this context, monomorphic *T. brucei* ILTat 1.4, which are non-immunogenic during exponential growth, induce VSG-specific and bystander antibody responses in mice when the parasites are lethally irradiated (Sendashonga and Black, 1982). These results are consistent with the possibility that BCR synapse formation leading to B cell activation results from the interaction of B cells with damaged trypanosomes possibly because they are not motile, or because they expose latent B cell activating components, or bind plasma components that contribute to B cell activation.

We have down-played the importance of conventional T cell dependent VSG-specific antibody responses in control of *T. brucei* parasitaemic waves. These follicular B cell responses require processing of VSG by antigen presenting cells and presentation of peptide–MHC class II complexes to specific T cells, are slow to develop in comparison to the T cell independent B cell responses and thus make a lesser contribution to rapid control of parasitaemia. In addition, the majority of antibodies made against soluble VSG are directed against epitopes that are not exposed on membrane-attached VSG and hence are not directly relevant to parasite elimination.

CELLULAR ORIGIN OF T CELL-INDEPENDENT VSG-SPECIFIC ANTIBODY RESPONSES

The rapid, T cell independent, VSG-specific antibody-mediated clearance of *T. brucei* from the bloodstream of infected mice is consistent with arisal from B1 B cells and, or, marginal zone B (MZB) cells. These cells have been shown in other systems to mount rapid, T cell-independent responses and to play critical roles in protection against blood-borne pathogens (Lopes-Carvalho and Kearney, 2004; Tung and Herzenberg, 2007; Alugupalli, 2008). B1 B cells arise early in ontogeny and self renew thereafter. They reside in the peritoneal cavity and can also migrate to the spleen. MZB cells are produced throughout life from stem cells in the bone marrow and after migration to the spleen reside in the marginal zone which separates the red and white pulp areas. The marginal zone houses marginal zone macrophages, which are ideally positioned to sample haemoparasites and introduce these to the MZB cells (Kraal and Mebius, 2006).

Mice of the C57 Bl/6 strain clear first wave parasitaemia by 6 to 7 days after infection with pleomorphic *T. brucei* AnTat 1.1E at which time 8% of total spleen cells are plasma cells. By 10 days after infection the number of plasma cells increases to close to 20% of total spleen cells, split evenly between IgM and IgG. Interestingly, a large number of these plasma cells produce antibodies that bind the hapten TNP but not the infecting trypanosomes, indicating a bystander, or polyclonal antibody response (Sendashonga and Black, 1982) and this response arises with the same kinetics as that against exposed epitopes on the VSG of infecting *T. brucei*. Our recent analyses of surface differentiation antigens on the parasite-induced plasma cells show that about

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50% of the plasma cells, defined by expression of CD138, express surface CD5, or CD11b, or both of these, indicative of the B1 B cell lineage. The remaining splenic plasma cells may derive from the B2 B cell lineage, or from B 1-derived plasma cells that lose their definitive markers. Plasma cells in the spleen remain elevated for about 25 days after infection but decline to almost background levels by the time of death of the infected mice, which occurs between 30 and 40 days post infection. The plasma cells do not accumulate in other organs hence their decline prior to death of the mice indicates depletion and lack of replacement.

The massive, although relatively short-lived elevation in splenic plasma cells after infection of mice with pleomorphic *T. brucei*, is accompanied by loss of spleen structure. The marginal zone disappears (Radwanska *et al*. 2008) and thus so does the distinction between red and white pulp. Follicles and germinal centres, which are regions of the spleen that support T cell-dependent antibody responses of the follicular B (FoB) cells, also rapidly disappear and the spleen complement of FoB cells declines by 50% or more. Furthermore, loss of spleen structure is paralleled by loss of responsiveness to new antigens and of recall responses to previously encountered antigens, including trypanosome VSGs and vaccines (Radwanska *et al*. 2008). In addition, infections with African trypanosomes have been shown to prevent antigen-induced B cell proliferation in mice (Sacco *et al*. 1994), to partially prevent release of antibody from plasma cells (Black *et al*. 1986), to deplete marginal zone B (MZB) cells from the spleen (Radwanska *et al*. 2008) and to cause a general decrease in bone marrow cells (Clayton *et al*. 1980) consistent with a negative impact on lymphopoiesis and erythropoiesis.

INFECTION-ASSOCIATED DYSLYMPHOPOIESIS AND DEPLETION OF B2 B CELLS

Infections with wild type T. brucei

Our on-going studies (manuscript in preparation) show that B cell dysfunction is not restricted to the spleen of *T. brucei* infected mice. By 15 days post infection, development of immature B cells declines in the bone marrow (by >10 fold) as determined by multicolour flow cytometry using the combinations of cell surface differentiation antigens listed in Fig. 2. However, the decline in BM B lymphopoiesis is compensated by a dramatic increase in extramedullary B lymphopoiesis (Fig. 2). Thus, while few if any lymphoid-primed multi-potent progenitors (LMPP), common lymphoid progenitors (CLP) and early B cell developmental stages are detected in the normal spleen, these precursor cell types come to represent between 5% and 10% of cells in the spleens of infected mice. It is as yet unknown whether extramedullary haematopoiesis results from immigration of bone marrow stem cells to the spleen, or is mediated by a previously inactive spleen stem cell population, or both.

In spite of the massive increase in B cell developmental stages from CLP to Pre-B cells in the spleens of infected mice, there is a dramatic decline in splenic immature B cells and substantial depletion of transitional type 1 (T1) and Transitional Type 2 (T2) B cells (diagrammed in Fig. 3). Immature B cells differentiate to T1 B cells, which give rise to T2 B cells and subsequently either MZB or FoB cells dependent on environmental signaling through the BCR (Lopes-Carvalho and Kearney, 2004). Transitional type 3 B cells, which may be end stages because of BCR hyporesponsiveness, are also depleted. Truncation of B lymphopoiesis before the immature B cell stage, and depletion of leak-through transitional B cells results in an inability of infected animals to replenish MZB and FoB cells depleted during infection but does not affect development of B1 B cells.

Infections with PLC$^{-/-}$ T. brucei

Deletion of the gene encoding GPI-PLC from pleomorphic *T. brucei* AnTat 1.1E greatly reduces virulence (Webb *et al*. 1997). After an initial wave of parasitaemia that peaks at around 10^8 trypanosomes/ml blood, mice of the C57BL/6 strain suppress parasitaemia to levels of between 10^4 and 5×10^6 per ml blood for >95 days. Although C57BL/6 mice infected with wild type (wt) *T. brucei* AnTat 1.1 have a similar first wave parasitaemia to those infected with PLC$^{-/-}$ parasites, their capacity to control parasitaemia is lost by 30 to 35 days after infection and the mice die between 30 and 40 days after infection. Furthermore, mice infected with the pleomorphic wt *T. brucei* AnTat 1 develop much higher levels of serum interferon (Namangala *et al*. 2001), TNFα and tissue pathology (unpublished data) than mice infected with PLC$^{-/-}$ *T. brucei* AnTat 1.

Our on-going studies show that bone marrow B lymphopoiesis is retained in mice infected with the PLC$^{-/-}$ parasites, immature B cells and transitional B cells are not depleted and the marginal zone, follicles and germinal centres are retained in the spleen until at least 90 days after infection. In addition, loss of MZB cells from the spleen is only partial and short lived, and there is no depletion of FoB cells. Thus, unlike mice infected with the wt *T. brucei*, those infected with the PLC$^{-/-}$ organisms retain full potential to replenish the mature B2 B cell populations and to make immune responses against new trypanosome variant antigen types as well as other antigens.

PLC knock-in *T. brucei* AnTat 1 expressing reduced levels of PLC relative to the wt parasites are more virulent than PLC$^{-/-}$ but less virulent than wt *T. brucei* AnTat 1 (Webb *et al*. 1997). Because

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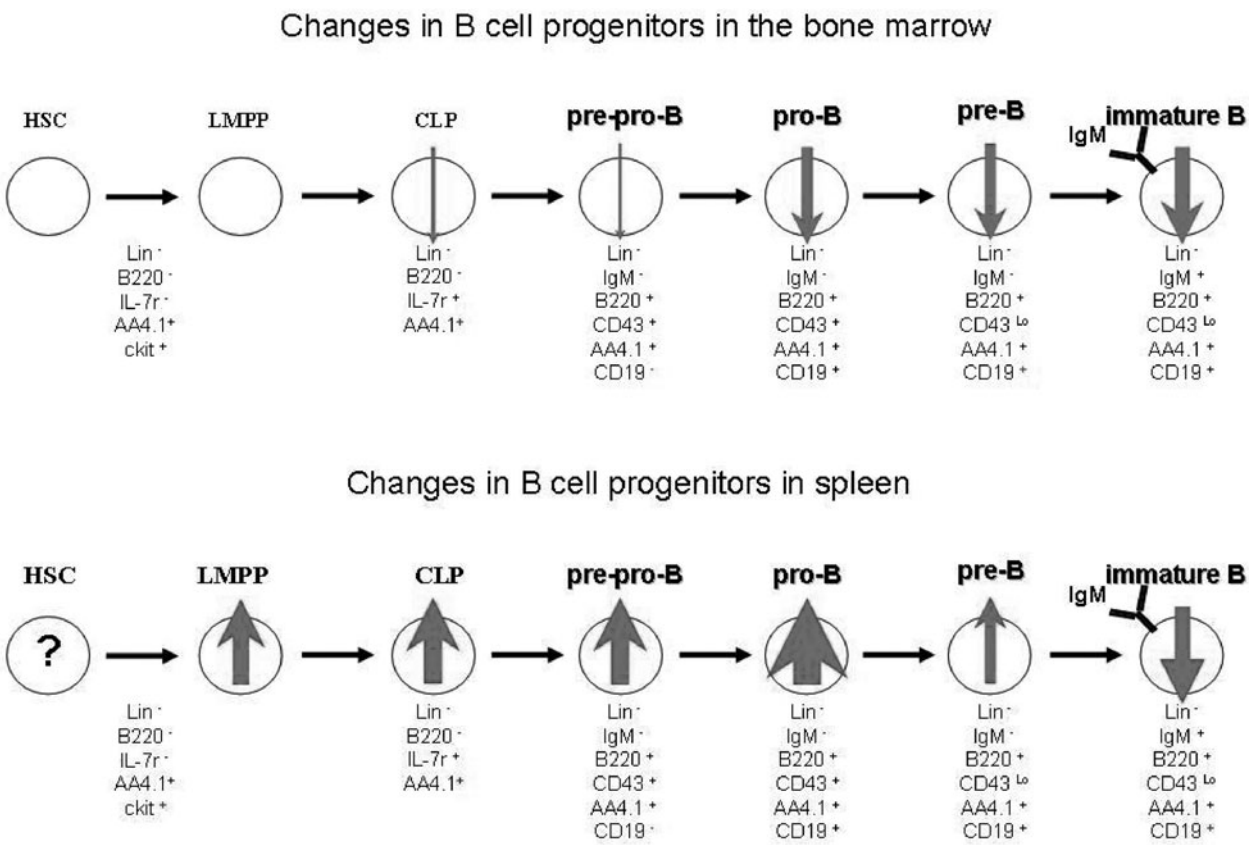

Fig. 2. Impact of *T. brucei* on bone marrow and extramedullary B lymphogenesis. Changes in representation of B cell progenitors in the bone marrow and spleen of C57Bl/6 mice 10 days after infection with *T. brucei* AnTat 1.1. *HSC* = haematopoietic stem cell; LMPP = lymphoid primed multi-potent progenitor; CLP = common lymphoid progenitor; pre-pro-B cell to immature B cell = stages at with rearrangement of immunoglobulin gene segments generate first a functional immunoglobulin heavy gene, then a functional immunoglobulin light chain gene, resulting in the development of immature B cells which express monomeric membrane form IgM on their surface. ↓ = a decline in representation of the population ranging from 25% to 95% as indicated by the thickness of the arrow; ↑ = an increase in representation of the population ranging from 10 to 100 fold as indicated by the thickness of the arrow.

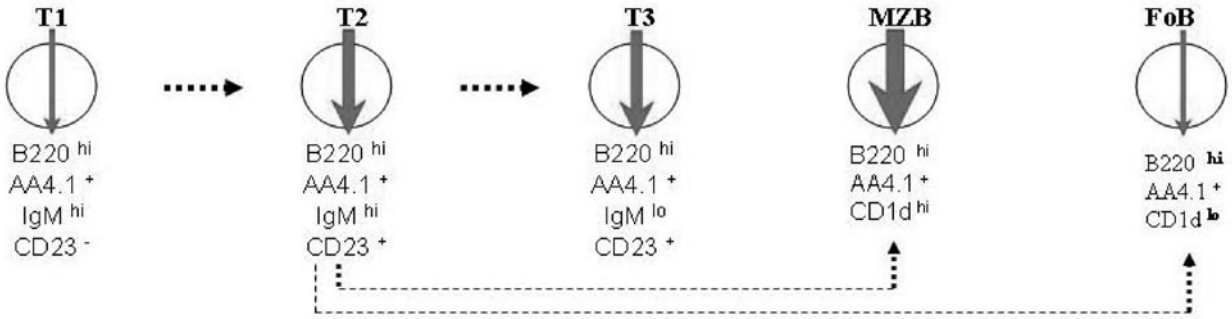

Fig. 3. Decline in splenic transitional and mature B cells. Relationships between and changes in, representation of transitional and mature B cells in the spleen of C57Bl/6 mice 10 days after infection with *T. brucei* AnTat 1.1. T1 = transitional stage 1 B cells; T2 = transitional stage 2 B cells, which are derived from T1 B cells; T3 = transitional stage 3 B cells; MZB = marginal zone B cells, which are derived from T2 B cells; FoB = follicular B cells, which are derived from T2 B cells; ↓ = a decline in representation of the population ranging from 25% to 92% as indicated by the thickness of the arrow.

PLC$^{-/-}$ *T. brucei* AnTat 1 has not been fully complemented by knock in of the *T. brucei* PLC gene it cannot be stated with complete confidence that the reduced virulence of this line is solely due to the absence of PLC. However, that is more likely than not. GPI-PLC has been localized to the cytoplasmic face of intracellular vesicles (Bolow *et al.* 1989), to glycosomes and the ER (Subramanya and Mensa-Wilmot, 2006) and most recently to a compartment lying between the flagellar and plasma membranes

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and parallel to the paraflagellar rod (Hanrahan *et al*. 2009). PLC gains access to membrane form VSG in bloodstream stage trypanosomes that are subjected to hypo-osmotic stress or other forms of membrane disruption (Cardoso de Almeida *et al*. 1984; Rolin *et al*. 1998) and cleaves this to yield soluble VSG (sVSG) and dimysteroylglycerol. Both agents activate macrophages expediting inflammatory responses (Tachado and Schofield, 1994; Magez *et al*. 1998; Mansfield and Paulnock, 2008). It is clearly of great importance to determine whether the reduced virulence of PLC$^{-/-}$ relative to wt *T. brucei* AnTat 1 results from reduced inflammation due to the absence of sVSG and dimyrstoylglycerol, or is due to a protective regulatory response elicited by intact VSG-GPI. That is a focus of the on-going research in our lab.

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