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SPECIAL ARTICLE

Vaccine model of antiphospholipid syndrome induced by tetanus vaccine

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Successful induction of antiphospholipid syndrome (APS) in two different non-autoimmune prone mouse strains, BALB/c and C57BL/6, was achieved by tetanus toxoid (TTd) hyperimmunization using different adjuvants (glycerol or aluminium hydroxide), and different adjuvant pretreatments (glycerol or Complete Freund's Adjuvant (CFA)). APS had different manifestations of reproductive pathology in BALB/c and C57BL/6 mice: fetal resorption (as a consequence of extreme T-cell activation obtained in the course of pretreatment), and lowering of fecundity (as a consequence of polyclonal B-cell stimulation), respectively. In BALB/c mice fetal resorption coincided with glycerol and CFA pretreatments, while in C57BL/6 mice lowering of fecundity was most obvious in CFA-pretreated mice immunized with TTd in aluminium hydroxide. Both molecular mimicry and polyclonal B-cell activation occur in APS induction, with molecular mimicry effects being dominant in BALB/c mice, and polyclonal cell activation being dominant in C57BL/6 mice. Confirmation of molecular mimicry effects, which in the condition of T-cell stimulation generated fetal resorptions in the BALB/c strain, was achieved by passive infusion of monoclonal antibody (MoAb) T-26 specific for TTd and anti- β_2 -glycoprotein I obtained after TTd hyperimmunization. High polyclonal B-cell activation in C57BL/6 mice prevented fetal resorption but induced fecundity lowering, as was the case in passive administration of MoAb T-26 in this mouse strain. Passive infusion of anti-idiotypic MoAb Y7 into C57BL/6 mice induced fetal resorptions and confirmed the above suggestion on the protective role of polyclonal B-cell stimulation in fetal resorptions. *Lupus* (2012) 21, 195–202.

Key words: anticardiolipin antibodies; antiphospholipid syndrome; pregnancy

Introduction

Antiphospholipid syndrome (APS) is a systemic autoimmune disease that can have various clinical manifestations (thrombocytopenia, thrombosis, reproductive pathology). It is considered that this disease appears due to an increase in anti-phospholipid (aPL), anti- β_2 -glycoprotein I (β_2 GPI) antibodies (Abs) and/or β_2 GPI-dependent aPL Abs. Manifestations of APS¹ in the mouse model include a decrease in fecundity and/or fertility. Various data that connect exposure to microbial antigens, either during infections^{2–5} or vaccinations,^{6–10} to autoantibody production have been

published in recent years. Experimental evidence for infectious origins of APS came from mice, immunized with a panel of microbial preparations, which developed anti- β_2 GPI Abs.¹¹ Pathogenic anti- β_2 GPI Abs directed against the hexapeptide TLRVYK epitope are produced in mice upon immunization with common bacteria (*Haemophilus influenzae* or *Neisseria gonorrhoeae*), viruses, yeast and tetanus toxin.¹²

Our APS model differs from those previously described as it is generated through tetanus toxoid (TTd) hyperimmunization. Pathogenic anti- β_2 GPI Abs in our model are thought not to be specific for the linear TLRVYK sequence present in TTd, but instead recognize TTd conformation, which is similar to that of β_2 GPI. Anti- β_2 GPI Abs per se are not necessarily pathological, but we were able to show their pathological effects on reproduction. The multifactorial aetiopathogenesis¹³ of APS is

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stressed in our model, as it is shown to involve responses of both adaptive and innate immunity, and is supported by a genetic background and triggered by environmental factors.

Environmental factors

Experimental design

To induce APS, mice were pretreated with Complete Freund's Adjuvant (CFA) or glycerol (Glyc) before TTd application (Table 1). One week after pretreatment, the TTd hyperdose (200 µg per dose) mixed with aluminium hydroxide (Al) or Glyc was injected into the mice.

Irrespective of which adjuvant pretreatment was used, the immune system was prepared to respond in the scope of dangerous self-signal threat. As for the question of adjuvant use, our results show that TTd-specific and autoreactive immune responses (which developed in tandem) are differently regulated: the immunogen-specific immune response was primarily shaped by the adjuvant used (whether Al or Glyc), while the dynamics and the intensities of self-directed immune responses were linked to the administered pretreatments.

The effect of adjuvants

As stated above, adjuvants used for pretreatments were CFA and Glyc. The innate immune system stimulatory potential of CFA comes from its numerous Toll-like receptor (TLR) agonists originating from *Mycobacterium tuberculosis*.^{14,15} By driving specific Th1 (activation of TLR4, TLR9, TLR3, TLR7)^{16,17} or Th2¹⁸ (activation of TLR2) differentiation, TLRs act as the crucial link between innate and acquired immunity.

It has already been reported that the *M. tuberculosis*-derived TLR agonist mixture, which constitutes CFA, could also stimulate innate receptors of B-1 cells and marginal zone (MZ) B cells, and thus generate a natural Ab response, as well as influence the generation of specific Abs. Different distribution of TLRs on dendritic cells as well as T and B cells in C57BL/6 and BALB/c mice greatly influences different manifestations of APS in these

mice strains. Since B-1 and MZ B cells represent the main source of natural Abs (NAb),¹⁹ CFA constituents act by triggering NAb response, and in addition, when TTd in Al or Glyc adjuvant is injected, prepare the milieu for generation of specific Ab response.

In the case of Glyc pretreatment and its use as an adjuvant, it has been shown that it positively affects the antigen-presenting potential of antigen-presenting cells (APCs), and that, as a T-cell stimulator, favours the Th1 type of immune response.²⁰

The most pronounced changes related to fecundity disturbance induced in C57BL/6 mice were shown to be dependant on the Al adjuvant, irrespective of pretreatment applied. Although both pretreatments, as explained above, generate a predominantly Th1 response, which is a natural milieu for this mouse strain, TTd injected in Al predominantly favoured the Th2^{21,22} response. In the BALB/c mouse strain, registered changes related to fertility were shown to be dependent on Glyc pretreatment and Glyc adjuvant. In the case of TTd application in Glyc, and especially when combined with Glyc adjuvant pretreatments, an adaptive immune response dominated by T cells was evidenced.

The effect of tetanus toxoid antigen

Innate immune memory to tetanus toxin could not be obtained owing to its high toxicity (1.4 ng/kg induces death in mice). This is the reason that its toxoid derivate, obtained through formaldehyde detoxification of the tetanus toxin, is used in vaccine production. The effect of TTd on APS induction could be observed through the effects of the administered dose and the characteristics of the antigen.

When soluble antigens are studied, the decision of whether a Th1 or Th2 response will be generated is dependent on the antigenic epitopes made available to the T-cell receptor (TCR) during priming. Tetanus toxin is amenable to otherwise limited proteolysis,²³ and in the form of TTd, a highly denatured toxin, its enzymatic degradation in macrophages and dendritic cells is even more restricted. Thus, a high density of identical peptide epitopes is presented and TCR ligation is low, which results in a strong Th2 response.

Table 1 Immunization schedule and experimental groups

Pretreatment (single dose: 1 st week)	Without pretreatment		Complete Freund's Adjuvant		Glycerol	
Treatment (3 doses: 2 nd , 4 th and 6 th week)	Aluminium	Glycerol	Aluminium	Glycerol	Aluminium	Glycerol

In addition, at the level of short peptide sequences, tetanus toxin shows similarity with proteins of higher organisms such as β_2 GPI,¹¹ laminin²⁴ and IL-1,²⁵ all of which implies that it could represent a potential inducer of autoimmune disease by a mechanism of molecular mimicry.

Genetic background (BALB/c versus C57BL/6)

Th1/Th2 polarization

Multiple genetically predetermined factors that specify T-cell polarization can be classified as intrinsic and extrinsic. The former are defined through the *tpm1* locus on chromosome 11 (control of IL-12 and promotion of Th1 differentiation), and with a still unidentified locus on chromosome 16 (which controls the early shift of CD4⁺ T lymphocytes toward the Th2 subtype). The extrinsic factors encompass dendritic cells, costimulatory signals and cytokines (primarily IL-4 and IL-12, as well as IL-6 which stimulates Th2 and inhibits Th1 response).

Classical examples of mouse strains with Th1 and Th2 types of immune response, either in vivo or in vitro, are C57BL/6 and BALB/c, respectively. These two mouse strains were included in parallel, and were treated in the same manner as previously described. It is generally thought that differences in responses to pathogens or self-antigens by these two strains result from differences in Th cell polarization. Unlike C57BL/6 mice, the BALB/c strain requires IL-1 α and TNF as additional co-factors for optimal IL-12-driven Th1 development.²⁶ Genetic loci that regulate Th differentiation through extrinsic pathways (the predominant locus that is mapped in the middle of chromosome 18, but also on chromosomes 5, 12, and 14) may also contribute to differences in the immune responses to bacterial pathogens and/or self-antigens in C57BL/6 and BALB/c mouse strains.²⁷

Idiotypic/anti-idiotypic network

Due to its unusual complexity, the mouse immunoglobulin heavy chain (*Igh*) locus has yet to be fully characterized in terms of functional germ-line heavy chain V (Vh) genes. A lower representation of VhJ558 (VH1)²⁸ in BALB/c mice is observed in comparison with C57BL/6 mice.²⁹ As C57BL/6 mice most frequently use the VhJ558 (VH1) family (70–80%), whereas

BALB/c mice equally use the VhJ558 (VH1) and VhQ52 (VH2)²⁸ (20–30%),³⁰ this could explain the difference in usage of Ig genes of NAb as well.

In this way, natural anti-phosphatidylcholine (anti-PtC) Abs of C57BL/6 mice are coded by the VH1 (VhJ558 in earlier literature) gene family of the variable Ig region, while the VH2 (earlier VhQ52) gene family-coded natural anti-phosphatidylcholine (anti-PtC) Abs are present in BALB/c mice as well. Natural autoantibodies encoded by the significantly smaller VH2 family (VhQ52 gene family) display a greater diversity (N-region additions and variable length CDR3s), and thus may easily slip through the regulatory net of natural Id/anti-Id interactions.³¹

B cells

The distribution of B-cell subsets is also dependent on the mouse strain. There are no differences in the percentages of mature and T2 B-cells between these two strains in non-immune mice. However, T1 and MZ B-cell percentages were shown to be twofold higher in the C57BL/6 strain.^{32,33} In addition, this mouse strain is also characterized by the expression of TLR2 and TLR4 on these cells, which greatly contributes to registered differences in APS induction between C57BL/6 and BALB/c mouse strains (absence of fetal resorptions versus fetal resorptions, respectively). Furthermore, in vitro stimulation of B cells with TLR agonists induces the production of cytokines that are able to inhibit T-cell activation in the IL-10-dependent manner, whereas activation of dendritic cells in the same way produces a milieu that contains very little IL-10, and that is not able to control T-cell proliferation.³⁴ On the other hand, BALB/c characteristics of the immune B-cell population have a great impact on BALB/c-dependent reproductive pathology in our APS model. The percentage of germinal centre B cells in immune mice was shown to be significantly higher in BALB/c (due to increased survival rate) than in C57BL/6 mice, as a result of a higher activation state and greater production of IL-4. It was shown that IL-4 produced by BALB/c B cells plays a key role in survival in an autocrine manner, whereas the addition of this bioactive cytokine is necessary for rescuing C57BL/6 B lymphocytes from apoptosis.³⁵ In addition, spleen B lymphocytes from BALB/c mice show a significantly high ability to proliferate in vitro to specific and non-specific stimuli, and produce higher levels of IL-4 and IL-6 than those of C57BL/6 mice.¹¹

APS pathology: the battle between natural and pathogenic autoantibodies

The role of molecular mimicry in the induction of APS pathology

The most cited mechanisms connecting microbial antigen stimulation and pathogenic aPL Abs secretion are molecular mimicry and polyclonal cell activation, although it is difficult to maintain a strict distinction between these two in the generation of pathogenic aPL Abs. Molecular mimicry, as the most likely mechanism, is characterized by cross-reactivity due to structural similarity between pathogen and host epitopes. However, in order for the autoimmune response to be successfully induced by the mechanism of molecular mimicry, host-like epitopes of pathogens must induce an adaptive immune response with T-cell involvement and consequent epitope spread in addition to the innate immunity activation. On the other hand, polyclonal cell activation, which is mostly dependent on innate immune receptor activation, can, if persistent, generate molecular mimicry-related consequences.³⁶

Two different mouse strains responded differently to the same TTd hyperimmunization approach, with both molecular mimicry and polyclonal cell activation being included (Table 2). BALB/c mice responded primarily by generating pathological anti- β_2 GPI Abs and fetal resorptions, favouring the influence of molecular mimicry.³⁷ In contrast, C57BL/6 mice responded primarily by generating a strong polyclonal Ab response and lowering of fecundity.^{38,39} In other words, the effect of molecular mimicry prevailed in BALB/c mice, while the effect of polyclonal cell stimulation prevailed in the

C57BL/6 strain. Both mouse strains developed anti- β_2 GPI Abs, thus making the role of molecular mimicry in governing the TTd-specific immune response more obvious.

Idiotypic network and natural antibodies

The results of our experiments have shown that TTd hyperimmunization in both mouse strains induced a significant rise in reactivity towards autoantigens, such as gangliosides, β_2 GPI and PLs.^{37,40} These autoantigens represent the common target of NAbs. The expression of natural idiotype (Id) on TTd-immunized mouse Abs during the course of immunization was tracked via the inhibition of interaction of a complementary Id pair: Id IgM DJ and its specific anti-Id murine monoclonal Ab (MoAb) Y7 (Figure 1). The Y7 Id was first described occurring on human IgM molecules (IgM DJ), and then classified as natural.^{41–45}

Whichever immunization protocol was performed, C57BL/6 sera collected during the post-immunization period were more potent inhibitors of MoAb Y7 F(ab)₂–IgM DJ interactions than were any of the normal sera, collected prior to intervention. Network perturbations in these mice were more intensive and occurred more rapidly amongst pretreated mice than amongst non-pretreated mice. In contrast, in BALB/c mice, TTd immunization induced a decrease in percentage of Y7⁺ NAbs, which was detected immediately after an increase in the concentration of IgG specific for mouse laminin, and coincided with an increase in anti- β_2 GPI IgG and fetal resorption in the CFA-pretreated Al-immunized group (Figure 1).

The titre of anti- β_2 GPI Abs in C57BL/6 mice was as high as 1:49,200 for the CFA-pretreated Al-immunized group, while the detected titre was 1:12,800 in the CFA-pretreated Glyc-immunized group.³⁹ The high titre of detected Abs was a result of the rise of low-affinity Abs, and extreme B-cell stimulation (probably B-1 and MZ B cells). In addition, the synchronous rises and fluctuations of the sera IgG specific for eight autoantigens (mouse laminin, PtC, phosphatidylserine (PtS), phosphatidylethanolamine (PtE), phosphatidylinositol (PtI), phosphatidylglycerol (PtG), cardiolipin (CL), disialoganglioside (G_{D1b}) and trisialoganglioside (G_{T1b})) in C57BL/6 mice⁴⁰ further indicates the polyclonal nature of produced IgG. These fluctuations were also most obvious in the CFA-pretreated Al-immunized group. On the other hand, the high levels of polyclonal Abs prevented the efferent pathological effect of anti- β_2 GPI on fetal resorption through network-dependent interactions,

Table 2 Different outcomes of TTd hyperimmunization documented in C57BL/6 and BALB/c mice

Type of treatment	C57BL/6		BALB/c	
	Pregnancies (%)	Numbers of live pups (a)	Pregnancies (%)	Numbers of live pups (a)
nc	83	6.85 ± 0.87	70	9.43 ± 0.61
ø//Al	56*	7.2 ± 0.90	61	7.80 ± 1.12
ø//Glyc	83	7 ± 0.03	67	6.20 ± 0.87
CFA//Al	50*	6 ± 1.23	61	5.55 ± 0.95*
CFA//Glyc	67*	6.25 ± 0.91	68	3.92 ± 0.50*
Glyc//Al	47*	6.12 ± 1.05	67	6.83 ± 1.05*
Glyc//Glyc	85	6.45 ± 0.93	70	5.14 ± 1.06*

*: significant results; nc: normal intact control; ø//: group without pretreatment; CFA//: group pretreated with Complete Freund's adjuvant; Glyc//: group pretreated with glycerol; Al: group immunized with TTd in aluminium adjuvant; Glyc: group immunized in TTd with glycerol adjuvant.

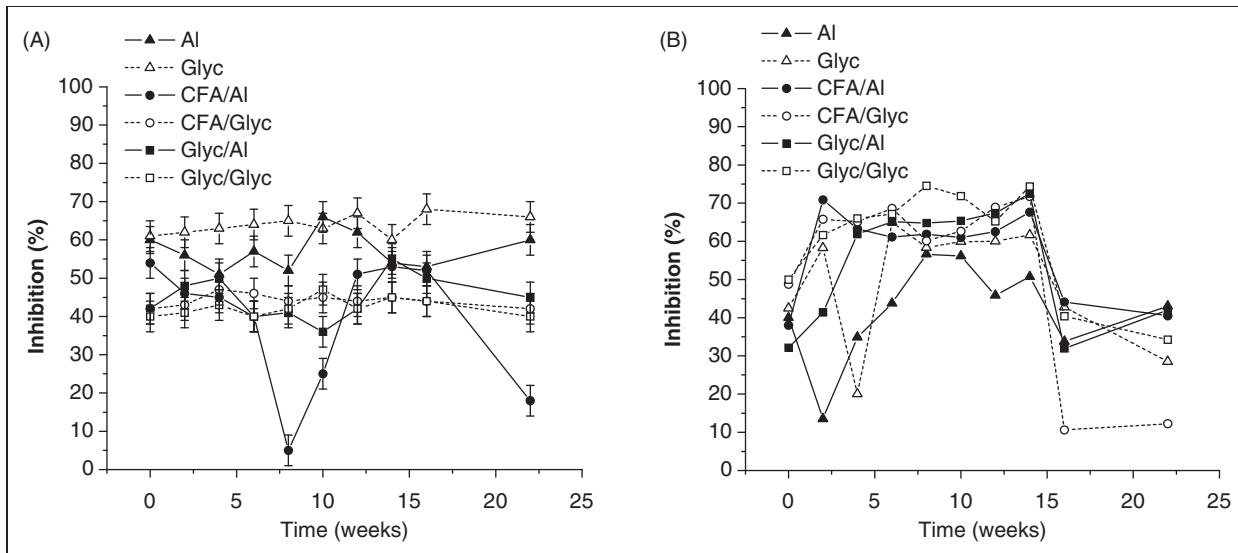


Figure 1 The expression of a natural idiotypic Y7 on sera Abs originating from TTd-hyperimmunized mice. The natural idiotypic expression was tracked by ELISA via the inhibition (presented as percentages) of interaction of IgM DJ and murine monoclonal Ab Y7.

which was, as stated above, registered as the rise in network connectivity.

Anti-TTd Abs can also be observed from the angle of their participation in the idiotypic network. Namely, in each mouse group the increase of anti-TTd IgG sera levels was followed by an increase of anti-ganglioside IgG production.⁴⁰ G_{D1b} and G_{T1b} are receptors for the tetanus toxin, implying that the antigen-binding site of anti-ganglioside IgG must be an internal image of the anti-TTd Ab reactive to the ganglioside-binding site found on the tetanus antigen. In the context of idiotypic networks, anti-ganglioside Abs may be regarded as type Ab2. A comparison of time-dependent fluctuations in the levels of anti-TTd IgG and anti-ganglioside IgG confirmed that in all groups the appreciable rise in anti-TTd IgG production preceded the appreciable rise in anti-ganglioside IgG production. Analyses of specific IgG subclass contributions to the entirety of the anti-ganglioside IgG pools showed that, for all experimental groups, a significant moiety of these contributing Abs belonged to subclasses IgG1 and IgG2c.³⁸

Anti- β_2 GPI antibodies in C57BL/6 and BALB/c mice

Anti- β_2 GPI Abs can exert both beneficial (playing a homeostatic role) and deleterious effects, depending on their concentration, isotypic characteristics, affinity and fine epitope specificity.⁴⁶ We have already reported on the rise in the level of low-affinity

anti- β_2 GPI Abs, and the Th2 prevalence dependent on AI-induced mechanisms, as well as decrease in fecundity in TTd hyperimmunized C57BL/6 mice. Although CFA and Glyc used for pretreatment undoubtedly have some modulatory effects, AI exerted the dominant influence on the abovementioned properties. It is possible that AI can stimulate the production of low-affinity anti- β_2 GPI Abs independently on TLR activation of dendritic cells and T-cell stimulation. To answer which mechanisms are involved in these AI 'adjuvant-assisted' phenomena, more sophisticated investigations need to be conducted.

In BALB/c mice, the induced pathology was correlated by a rise in anti- β_2 GPI Ab affinities with a dominant effect of T-cell influence (highest affinity achieved in CFA pretreatments and Glyc adjuvant), as most of the pathological resorptions were observed in these groups. In addition, a rise in anti- β_2 GPI Ab affinity overlapped with the abundance of IgG that recognized β_2 GPI following interaction with PtS. The strong stimulation of T effector/memory cells is suggested to dominate in this response, with T regulatory cells being abrogated (IL-10 measured in spleen of immunized BALB/c mice, stimulated with β_2 GPI, was significantly lowered in groups with pretreatments; unpublished data). The effect of polyclonal stimulation cannot be completely excluded, as the highest titre of anti- β_2 GPI Abs detected in AI-immunized mice (1: 6400) is probably influenced partly by polyclonal stimulation. A strong T-cell stimulation

exerted on a small number of genes included in the PtC response, leading to a diminishing of network connectivity preceded by an enormous rise in anti-laminin Abs, which by definition have pathological attributes in mice,⁴⁷ was detected only in CFA-pretreated and AI-immunized groups. T regulatory cell abrogation and loss of network connectivity are the main characteristics of the BALB/c milieu, in which the pathological effects of anti- β_2 GPI Abs were observed. Although a clear correlation between the concentrations of complement-fixing Abs (IgG2a and IgM) and severity of pathology was not observed, the greatest abundance of IgG2a within the overall anti- β_2 GPI IgG in the CFA-pretreated Glyc-immunized mice implies their importance in genesis of pathology. In addition, given that IgG2a is a hallmark of the Th1 response,⁴⁸ it would seem that skewing of the immune response in a Th1 direction would be more deleterious.

Supplementary results

Further confirmation of how molecular mimicry and polyclonal cell activation differently influence our model of APS induction by TTd hyperimmunization in two different mouse strains was obtained by the use of two MoAbs: T-26 and Y7. Passive infusion of MoAb T-26, specific for TTd and cross-reactive with β_2 GPI, obtained from BALB/c mice after TTd hyperimmunization, induces different reproduction failures in BALB/c and C57BL/6 mice. In addition to binding to β_2 GPI, MoAb T-26 was shown to bind to apoptotic cells in the presence of β_2 GPI, and was shown to induce fetal resorptions in BALB/c mice, due to its binding to the placenta's decidual tissue.⁴⁹ In C57BL/6 mice following i.v. application of MoAb T-26, the same pattern of reproductive pathology as for TTd hyperimmunized mice was observed. Namely, the reduction in fecundity in C57BL/6 mice was pronounced.³⁹

Confirmation of idotype network regulatory effects of possibly pathological anti- β_2 GPI Abs in C57BL/6 mice was ascertained after passive infusion of anti-Id MoAb Y7 into pregnant females, which resulted in reproductive failure and fetal resorptions.

Conclusion

In this paper we have explained our model of APS based on TTd hyperimmunization which supports

the concept of the mosaic of autoimmunity. In summary, C57BL/6 mice after CFA pretreatment and antigen applied in AI developed strong B-cell polyclonal activation, resulting in changes in fecundity (Figure 2). On the other hand, in BALB/c mice fetal resorptions were induced after CFA pretreatment and antigen applied in Glyc adjuvant, as a consequence of T-cell-boosted IgG production towards T-cell-dependent antigen (Figure 3). We also point out the importance of Abs in the manifestations of disease. Anti- β_2 GPI Abs (when possessing high affinities and specificities towards cryptic epitopes) are shown to be the main player in fetal resorptions. In addition, they are important in fetal resorption protection (when they are of a polyclonal nature), leading to fecundity lowering.

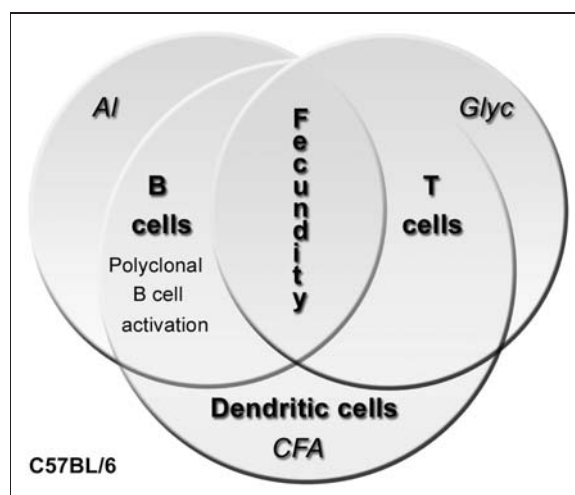


Figure 2 Antiphospholipid syndrome-related fecundity lowering recorded in C57BL/6 mice.

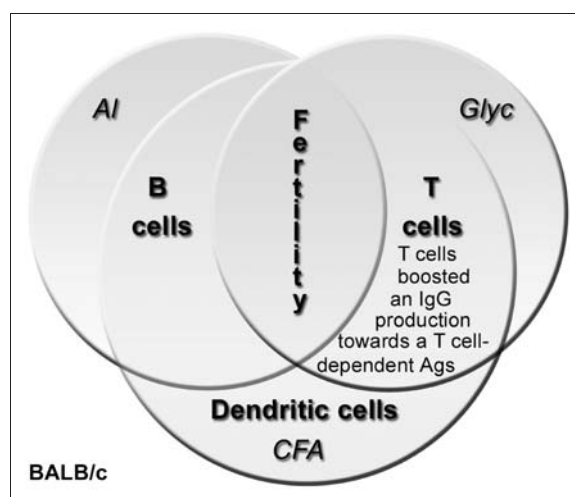


Figure 3 Antiphospholipid syndrome-related fertility disturbance recorded in BALB/c mice.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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