Mice immunization with *Trypanosoma brucei gambiense* Translationally Controlled Tumour Protein modulates immunoglobulin and cytokine production, as well as parasitaemia and mice survival after challenge with the parasite

Running title : *Trypanosoma* TCTP immunization modulates parasitamia, mice survival and mice antibodies and cytokine production

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Abstract

Fighting trypanosomiasis with an anti-trypanosome vaccine is ineffective, the parasite being protected by a Variable Surface Glycoprotein (VSG) whose structure is modified at each peak of parasitaemia, which allows it to escape the host's immune defenses. However, the host immunization against an essential factor for the survival of the parasite or the expression of its pathogenicity could achieve the same objective. Here we present the results of mouse immunization against the Translationally Controlled Tumor Protein (TCTP), a protein present in the *Trypanosoma brucei gambiense* (*Tbg*) secretome, the parasite responsible for human trypanosomiasis. Mice immunization was followed by infection with *Tbg* parasites. The production of IgG, IgG1 and IgG2a begun after the second TCTP injection and was dose-dependant, the maximum level of anti-TCTP antibodies remained stable up to 4 days post-infection and then decreased. Regarding cytokines (IL-2, 4, 6, 10, INFγ, TNFα), the most striking result was their total suppression after immunization with the highest TCTP dose. Compared to the control group, the immunized mice displayed a reduced first peak of parasitaemia, a 100% increase in the time to onset of the second peak, and an increased time of mice survival. The effect of immunization was only transient but demonstrated the likely important role that TCTP plays in host-parasite interactions and that some key parasite proteins could reduce infection impact.

Keywords: *Trypanosoma brucei gambiense*, Translationally Controlled Tumour Protein (TCTP), host immunization, parasitaemia, cytokines, antibodies

1. Introduction

Human African trypanosomiasis (HAT), the well-known sleeping sickness, is caused by two protozoan species belonging to the genus *Trypanosoma*, respectively *Trypanosoma brucei gambiense* (*Tbg*) causing the chronic form of the disease in West and Central Africa, and *Trypanosoma brucei rhodesiense* (*Tbr*) causing the acute form in East Africa. These two trypanosome species are transmitted to humans by two *Glossina* (tsetse flies) species, *Glossina palpalis* and *Glossina morsitans*, respectively. Both forms of the disease have severe social and economic impacts on populations living within or near HAT foci.

Despite the disease has been described more than one hundred years ago, it has not been eradicated yet. Sleeping sickness is a vector-borne disease, thus control strategies may be focused either on the vector or on the patient. Regarding the latter, two complementary strategies are implemented, respectively, a curative one that needs the use of various drugs that often are not easily supported by the patients, or a preventive one which in general consists of the vaccination of populations that are at risk. Unfortunately this technique does not work in the case of sleeping sickness since the bloodstream form of the trypanosome is covered by a coat that consists of a glycoprotein called variant surface glycoprotein (VSG). Despite the trypanosome genome includes over 1,000 different VSG genes, due to a strict allelic exclusion, only one gene is expressed at any time so that the coat is composed of a single type of VSG. However, an antigenic variation, associated with successive waves of parasitaemia, occurs *in vivo* (in the infected human), which consists in the replacement of a given VSG type, for example VSG1 by a VSG2, resulting from the silencing of the VSG1 gene and the concomitant activation of the VSG2 gene (Dubois et al., 2005 ; Geiger et al., 2016). Since VSG2 is not recognized by the anti-VSG1 antibodies the patient may have produced, the trypanosome escapes the host defense and continues to multiply up to the next

wave of parasitaemia which induces the replacement of VSG2 by a VSG3. Thus another approach should be considered.

The idea is to immunize the patient not against the trypanosome itself, but against a major component of its pathogenicity. With this respect, a comparative inventory of the excreted / secreted proteins (ESPs) produced *in vitro* respectively by the procyclic form (hosted by the vector) (Atyame Nten et al., 2010) and the bloodstream form (pathogen for humans) (Geiger et al., 2010) of the parasite was carried out. More than 400 ESPs have been identified in the secretome of the bloodstream form of the trypanosome. Some of them have also been identified in secretion vesicles isolated from the serum of trypanosome-infected mice, demonstrating that these proteins are excreted not only *in vitro* but also *in vivo*. Furthermore, the total secretome has been shown to be involved in an immunosuppression mechanism impairing dendritic cells maturation, altering their function, leading to a dramatic reduction of the secretion of TNFα which plays a key role in regulating parasitemia along *Trypanosoma brucei* infection (Magez et al. 1999), interleukin 10 and 6 production as well as that of cytokines Th1 / Th2 (Garzón et al. 2013). It should be noted that *Trypanosoma* infection induces activation of polyclonal cells of B cells, clonal exhaustion of B cells, depletion of Marginal Zone B and Folicular B cells, and finally destruction of the memory compartment of B cells (Bockstal et al., 2011). Among the ESPs we identified number of proteases that may contribute to the parasite's virulence (Atyame Nten et al., 2010).

However our attention was drawn to the presence in the trypanosome secretome of a particular protein, the Translationally Controlled Tumor Protein (TCTP). TCTP is a highly conserved protein distributed ubiquitously in eukaryotes, whether plants or animals. It was reported to be involved in cellular processes such as stress responses (Brioudes et al., 2010), cell proliferation (Stierum et al., 2003; Arcuri et al., 2004), embryo development (Roque et

al., 2016), apoptosis (Susini et al., 2008) and physiological disorders (Kadioglu and Efferth, 2016; Seo and Efferth, 2016).

TCTP was not only identified in the secretome of the bloodstream form of the trypanosome, but also in the secretome of its procyclic form which is hosted by its *Glossina* vector (Atyame Nten et al., 2010). Moreover the corresponding gene (*Tbg*TCTP) was shown to be overexpressed in Tbg-infected *Glossina palpalis* flies as compared to non-infected flies (Hamidou Soumana et al., 2015) which could indicate TCTP to be involved in fly infection process, inasmuch the protein was also identified in total gut extracts from Tbg-infected flies (Geiger et al. 2015). Recently we have produced the recombinant r*Tbg*TCTP protein, which was shown to be able to modulate the growth of bacteria isolated from the gut of tsetse flies (Bossard et al., 2017).

Finally TCTP seems to be involved in various steps governing the interactions between the parasite and either its insect vector or its human host.

To attempt getting a better insight into the role of *Tbg*TCTP as a possible effector of the parasite pathogenic process, we have investigated the effect of the immunization of mice with the r*Tbg*TCTP on the modulation of immunoglobulins and cytokines/chemokines production in parallel with the evolution of parasitaemia and mice survival.

2. Material and methods

2.1. Recombinant rTbgTCTP protein production

The whole production process was described in Bossard et al. (2017). Briefly, the *Tbg tctp* gene was synthesized and cloned into the baculovirus vector pAcGHLT-A; the vector was then transfected into *Spodoptera frugicola* (strain 9) cells in which the gene was expressed. The recombinant r*Tbg*TCTP was recovered and purified using the AKTAprime plus system.

2.2. Mice immunization schedule

Mice were kept under strict ethical conditions according to the international guidelines for the care and use of laboratory animals. The experiments designed for this study were approved by the regional Ethic Committee for Animal Experimentation CEEA-LR 36 under project number 02187.01 and authorized by French Ministry for Higher Education and Research. The immunogenicity and antigenicity of r*Tbg*TCTP were evaluated on 6-weeks-old female mice (OF-1 mice) purchased from Charles River Laboratory, France. Three doses of r*Tbg*TCTP, 20µg, 40µg and 80µg, in 70µl of Complete or incomplete Freund's adjuvant were tested. For each dose, the immunization was performed with three successive sub-cutaneous injections separated by a 2-weeks interval. Controls were processed similarly with adjuvant alone or PBS.

The experimental design included 30 mice randomly distributed in 5 groups of 6 mice. The mice were then identified by numbering. The groups were the followings:

- PBS Control group (mice numbers 1 to 6): each mouse became three successive injections of 70µl PBS;

- Freund's control group (mice numbers 7 to 12): each mouse received a first injection of a mixture of 35µl of complete Freund's adjuvant and 35µl of PBS. Second and third injections, were of 70µl incomplete Freund's adjuvant;

- in the three groups called "20TCTP" (mice numbers 13 to 18), "40TCTP" (mice numbers 19 to 24) and "80TCTP" (mice numbers 25 to 30), the mice received three successive injections of 20, 40 or 80µg r*Tbg*TCTP protein, respectively, emulsified in 70µl of either complete Freund's adjuvant (first injection) or incomplete Freund's adjuvant (second and third injections).

2.3. Mice infectious challenge with trypanosomes

Eighteen days after the last injection, each mouse was infected intraperitoneally with 3000 trypanosomes belonging to the *T. b. gambiense* species (ABBA strain) in a total volume of 100µl blood from one previously infected mouse. The ABBA strain has been isolated in Côte d'Ivoire, in 1983, from a patient suffering HAT. After infection, each mouse was monitored every day in order to evaluate parasitaemia (microscopic observation) and survival. Once a week, hematocrit was measured (200µl) and the serum was collected and stored at -80°C until cytokine and antibody analysis.

2.4. Cytokine quantification

A commercial BD CBA mouse Th1/Th2 cytokines kit (Becton Dickinson, USA) was used to quantify IL-10, IL-6, IL-4, IL-2, TNF-α and IFN-γ in the sera using the bead array technology as described by the manufacturer. Briefly, a mixture of seven capture beads (with distinct APC fluorescent intensities) coated with capture antibodies specific for each cytokine was used. Fifty µl of the bead mixture to which 50 µl of an unknown serum sample (or of a recombinant standard), and with 50 µl of phycoerythrin (PE) detection reagent were incubated to form sandwich complexes. The concentration of each particular cytokine was measured by

the intensity of PE fluorescence of each sandwich complex. After washing, samples were processed on the BD Canto II dual laser (488 and 633 nm) flow cytometer and data were analyzed by FCAP Array software. Serial diluted standards were first analyzed to generate calibration curves and the sample cytokine concentrations were calculated with reference to these curves.

2.5. ELISA: immunoglobulin detection

Each well of 96 Maxisorp plates (Thermofisher) was coated overnight at 4°C with 0.1µg r*Tbg*TCTP diluted in 100µl. The uncoated antigen was then removed as follows: the plate was saturated with 150µl of PBS containing 5% nonfat milk and 0.1% Tween 20 (blocking buffer solution), and incubated during 45 min at 37°C. Blocking buffer was removed and replaced by mouse serum sample each diluted at 1:100 in blocking buffer solution (100µl per well), and incubated 45 min at 37°C. Plates were then washed 4 times with PBS containing 0.1% Tween 20 (washing buffer solution). Horseradish peroxidase-conjugated sheep anti-mouse IgG, IgG1, IgG2a, IgG2b secondary antibody (Jackson) was added to each well (100µl) at 1:2000 dilution in blocking buffer solution, and incubated 45 min at 37°C. Each immunoglobulin was then tested independently. The conjugate was discarded, and the plates were washed 4 times with the wash buffer solution. Kblue substrate (Neogen Corp.) was then added (100µl per well) and plates were incubated 30 min in the dark at room temperature. Absorbance at 620nm was measured using a spectrophotometer (Enspire, Perkin Elmer Inc.).

2.6. Statistical analysis

Statistical analyses were carried out using R studio software. Data from parasitaemia and mouse serum (ELISA and BD^{TM} CBA mouse Th1/Th2 Cytokine Kit) were compared between groups using non-parametric tests (Kruskall-Wallis and Wilcoxon test). The R package

Survival was used to analysis survival with the Kaplan-Meier estimate (Harrington and Fleming, 1982).

3. Results

3.1. Production, purification of r*Tbg*TCTP

r*Tbg*TCTP protein purification was processed as reported in Bossard et al. (2017), and allowed to recover approximately 174 mg of recombinant protein (95% purity rate) per liter of insect cells culture medium. Based on the r*Tbg*TCTP specific sequence, the B linear epitopes prediction software [Bepipred 1.0 Server (Larsen et al., 2006)] predicted epitopic structure covering 46 % of the total protein sequence, thus showing theoretically a large antigenic potential.

3.2. Kinetics of T. b. gambiense infection

We have investigated the possibility that mice immunization with r*Tbg*TCTP could have an impact on the parasite multiplication rate (parasitaemia evolution) and, consequently on mice survival.

3.2.1. Parasitaemia in mice

The level of parasitaemia is classically used as a diagnostic parameter to evaluate the evolution of the disease itself in humans (and in animals) (Stanghellini and Roux, 1984). Figure 1 shows the mean fluctuation of the parasitaemia in mice of the six different groups after having been challenged with trypanosomes at day(0). The evolution of the control groups (PBS and Freund) was characteristic for *Tbg* infections (Giroud et al., 2009; Parra-

Gimenez and Reyna-Bello, 2019). Parasitaemia increased from day(5) to reach a first peak at day(7) to day (8). Thereafter it decreased to a base-line and increased again to reach a second parasitaemia peak between day(14) to day(15). A third peak occurred roughly at day(+19) / day(21). Regarding the groups of immunized mice, differences were noticed with reference to the control groups. While the first parasitaemia peak for the group 20TCTP (5.10^8 parasite/ml) was similar to that of the controls (4 to 5.10^8 parasite/ml), those of groups 40TCTP ($2{,}5.10^8$ parasite/ml) and 80TCTP (2.10^8 parasite/ml) were 1.5 to 2 times lower. Nevertheless the main significant difference consisted in the absence of the second parasitaemia peak in the mice of the three immunized groups as compared to the control groups. However, from day(17/18) post infection, parasitaemia increased in mice from all the 5 groups whether control or TCTP immunized. In addition, for the TCTP20 and TCTP80 groups, the 2^{nd} parasitaemia peak appeared and increased at the time the 3^{rd} peak of the control mice was increasing.

3.2.2. Mice survival

Figure 2 shows mice survival in the different groups. During the first 6 days following mice infection with *T. b. gambiense*, 100% of the individuals survived. At day 7, we observed a loss of 50% of mice from the PBS-control group, while 80% survived in the four other groups whether Freund-control or the three groups of TCTP-immunized mice. From day(15) survival rate decreased progressively and regularly except in the 80TCTP group where a 80% mice survival lasted up to day(20), then decreased similarly to the other groups.

3.2.3. Hematocrit and weight of the mice

Mean hematocrit value evolution was similar in all the 5 groups (data not shown). It was stable at 55 % along the 48 days of immunization. Then, it decreased to 50% during the 4 first days post infection and finally to 40 and to 30%, depending on the considered group, between day(4) and day(8) and remained at these low levels until day(18) post infection.

No particular effect was noticed regarding the weight of mice, which did not vary along the experiment, regardless of the group (data not shown).

3.2.4. Mice antibody and interleukine responses to immunization with r*Tbg*TCTP

Note regarding the Figures: the day(0) corresponds to the day of mice inoculation with the trypanosomes. The negative and positive values indicate the number of days preceding (period of mice immunization) and following the day(0) of the infection, respectively. Each mouse was identified thus allowing the individual data to be presented in the different Figures (3 to 12) together with the statistics representation.

3.2.4.1. Antibody response

IgG, IgG1, IgG2a and IgG2b antibodies production was measured in order to evaluate the r*Tbg*TCTP antigenic potential. The assays were performed on sera taken from the mice at day(-48), just before the first injection of the PBS, Freund's adjuvant or of the three doses of r*Tbg*TCTP; the next assays were performed at days(-33) and (-18), then at day(0), just before performing parasite inoculation, and at days(+4), (+8) and (+12) post infection. Results, including individual data, are shown in Figures 3-6.

Regarding IgG (Figure 3) the seroconversion against r*Tbg*TCTP did not occur before day(+4) in each control group (PBS and Freund) whereas it started before infection, at day(-33) in the 3 TCTP groups, although the results were only statistically slightly significant ($p = 0.02$), at this day, in 80TCTP. In the PBS, 20TCTP and 40TCTP groups, seroconversion occurred significantly (respectively, $p = 0.0001$; $p = 0.002$ and $p = 0.02$) at day(+4). Finally, IgG production increased from day(0) to day(+8) in all the 5 groups with exception for the 80TCTP group where it was highest from day(0) to day(+8) and then decreased. It is important to note the dispersal of individual data and individual reactions of some mice compared to other mice from the same group. This is, for example, the case of mouse n° 22 from the 40TCTP group; this same mouse was also singled out, although less clearly, for the

production of IgG1, IgG2a and IgG2b. Finally, the only statistically significant difference was recorded when comparing the "80TCTP" group with control group: IgG production began earlier and the levels were much higher.

Regarding the IgG1 subclass (Figure 4), we could not detect any production in both control groups (PBS and Freund) throughout the experiment. In contrast seroconversion occurred either at day(-18) (40TCTP / $p = 0.0001$; and 80TCTP / $p = 0.0008$) or at day(0) (20TCTP), this latter being however not significant ($p > 0.05$) because of the high data dispersal. Maximum amount of IgG1 was recorded at day(0) (80TCTP) or at day(+4) (40TCTP) and then decreased until day(+12).

IgG2a (Figure 5), only 40TCTP ($p = 0.02$) and 80TCTP ($p = 0.03$) produced this immunoglobulin at day(-18), i. e. before infectious challenge with the parasite. From this time up to day(+8), IgG2a production increased slowly and finally decreased drastically at day(+12).

Finally, regarding IgG2b (Figure 6), its level was low throughout the experiment, whatever the group. The weak variations observed in 80TCTP were not statistically significant. One may notice that group 20TCTP included a mouse (n° 13) whose behavior seemed distinctly different from the 5 other members of the group which was not reliable with any clinical signs

3.2.5. Cytokines responses

IL-2, IL-4, IL-6, IL-10, TNFα and IFNγ levels were measured in sera recovered from mice respectively at day(-48), just before immunization, at day(0), just before mice challenge with trypanosomes, and at day(+4), day(+8) and day(+12) post infection. Results are shown in Figures 7 to 12.

IL-2, IL-4, IL-6 and IL-10 data were most often highly dispersed. Amounts of IL-2 (Figure 8) and IL-4 (Figure 9), respectively, were similar over time and experimental design. Whatever

the groups, IL-6 (Figure 10) and IL-10 (Figure 7) levels significantly decreased after infection. IFNγ (Figure 11) and TNFα (Figure 12) production, their levels increased at day (0) compared to day(-48). Finally, the rates of IFNγ (Figure 11) and TNFα (Figure 12) decreased significantly after infection. It is interesting to note that for the 80 TCTP group, all the cytokine productions were very low or totally absent all over the whole time of the experiment.

4. Discussion

The most widely used method, as it is usually the most effective for preventing an infectious disease, is vaccination, the extension of which to the largest number of individuals in a population protects the entire population itself. This approach unfortunately has exceptions, for example, when epitopes of the pathogen are only weakly immunogenic, or when frequent mutations occur that modify the structure of epitopes or when, through an appropriate mechanism, they are regularly renewed. This latter case occurs in the infectious form of trypanosome; that is why it escapes the immune defenses of the host, human or animal. This is also why the principle of vaccination is currently inapplicable because it is not known which of the hundreds of possible variants of VSG will cover the parasite at the time of infection, nor in which sequence (preprogrammed or random) the various genes encoding the variants will be successively activated. The vaccination approach has however not definitively to be discarded, it may be focused on invariant proteins that are essential either for the survival of the parasite or for the expression of its pathogenicity. In this context, the choice of TCTP was based both on data from the literature and on those resulting from our previous researches.

From a general point of view results recorded under some experimental conditions were too dispersed to be statistically validated, mice immunization with r*Tbg*TCTP was working, although limited in intensity and time. In fact, immunization resulted, in a dose-dependent manner, in the production of specific immunoglobulins, in the stimulation of the production and/or in the inhibition of certain cytokines, and finally in modulating parasitaemia kinetics and mice survival.

Regarding this latter aspects, four criteria were measured, the weight of the mice, their hematocrit, their survival throughout the experiment, and the evolution of their parasitaemia after having been trypanosome-infected. The criteria included hematocrit, since anemia was shown to be associated with *T. congolense* and *T. vivax* infections of cattle (Mbewe et al., 2015).

In this study, neither the evolution of the weight in the immunized / infected mice nor that of the hematocrit did differ significantly from that of the control mice. In contrast, a dose-dependant effect of immunization clearly appeared on mouse mortality since immunization with the highest dose of r*Tbg*TCTP (80 µg per mouse) allowed the survival of 80% of individuals at day(+20) post-inoculation, while less than 40% of individuals survived in the other groups of mice at the same time. However, the effect of r*Tbg*TCTP only delayed mice death by 7 days.

The evolution of mice survival can be explained by that of their parasitaemia depending on the different groups. In the two control groups, parasitaemia evolved along the classical pattern of *Tbg*-infected mice: a first peak 7 to 8 days after infection, a second around day(+14) to day(+15) and finally a third one at day(+22) after which all the surviving mice died. In the immunized mice, the first peak occurred also 7 to 8 days post-infection, but it was approximately 50% lower in the mice that were immunized with either 40 or 80 µg of r*Tbg*TCTP, than in the control groups. However, the most remarkable was the increased time

that separated the second peak from the first: 15 days (group 80TCTP) versus 7-8 days in the control groups. This result clearly demonstrated the mice immunization with r*Tbg*TCTP to provide an inhibitory, although transient, effect against *in vivo Tbg* blood stream forms multiplication.

Regarding immunoglobulin productions (IgG, IgG1, IgG2a and IgG2b), a first comment concerns the reality of immunization with r*Tbg*TCTP. Whereas in the control groups the level of immunoglobulins was almost failing, their production was stimulated between days(-33) and (-18) (i. e. after the 2nd injection of r*Tbg*TCTP), and their production increased very significantly between the 3rd injection and the day(0) which corresponded to the mice inoculation with trypanosomes. The dose-dependent effect also appeared very clearly. The ELISA tests being very specific, the IgG tested were anti-r*Tbg*TCTP immunoglobulins, demonstrating: a) that TCTP is really immunogenic, and b) that the immunization procedure was working.

Post-infection stimulation of immunoglobulin production in the mice control group logically meant that the trypanosomes secreted TCTP *in vivo*, in the infected mouse, in amounts sufficient to stimulate the mice immune response. Theoretically, this interpretation was in agreement with previous results that have shown, among others: a) that TCTP is part of the trypanosome secretome proteins (procyclic and bloodstream forms), b) that it is excreted *in vivo* and released into the bloodstream of the mouse, c) that the corresponding TCTP gene is also overexpressed in flies infected by the parasite. This effect may also be significant in the 20 and 40TCTP mice immunized with the lowest doses; it was lesser significant in the 80TCTP mice immunized with the highest dose.

The question was why does the level of free IgG decrease from 4 or 8 days post-infection in immunized mice? Immunization had only a partial effect on the evolution of parasitaemia; it lowered the importance of the first peak but did not suppress it; it also significantly decreased

the concentration / density of surviving trypanosomes during the period following the parasitaemia peak, but did not completely eliminate them. It may be thought that the first peak of parasitaemia weakens the mouse reaction capacities, reducing, maybe suppressing, the production of total IgG, thus decreasing the free IgGs in the blood, while the molecules still present were mobilized via agglutination with the TCTPs that continued to be produced and excreted by the parasite. Knowing that B lymphocytes play an important role in parasite clearance (Magez et al., 2008), the lack of free IgG availability could probably be due to clonal depletion of B cells or even destruction of the memory compartment of B cells as described (Bockstal et al., 2011) and thus allowed a new and fatal increase in the trypanosome population, even with the highest dose of TCTPs.

Besides global IgG analyses, we tested also the subclasses IgG1, IgG2a and IgG2b. In the control groups and compared to the immunized groups, IgG1 were not produced at all even after mice infection; this could mean that the *in vivo* TCTP secretion by the infecting trypanosomes was too low to induce an IgG1 response from the mice. In mice that had been immunized, a dose-dependent effect was shown regarding IgG1 production.

In mice, there are two IgG2 sub-classes, IgG2a and IgG2b. IgG2b were very weakly produced, while IgG2a production evolved quite similarly to IgG1, except that a weak production can be observed in control mice after they have been infected by trypanosomes.

To define the orientation of the immune response using the IgG1/IgG2 ratio, we used only the IgG2a sub-class response, as the production of IgG2b was extremely weak. Before infection, the orientation was towards Th2 type through the production of IgG1. After infection, orientation of the immune response switched to a Th1 type response, but the cytokine productions in our study did not seemed to correlate.

Indeed, concerning the Th1, Th2 and inflammatory cytokines tested (INFγ, TNFα, IL-2, IL-4, IL-6 and IL-10), and except for the 80 TCTP group which is discussed further, pre-

immunization with TCTP did not induce any specific modulations of cytokine levels when comparing with Freund control group, whereas infection induced a modulation. Once more, the modulation is approximately the same between groups. All groups showed similar variations in the levels of the cytokines before infection with the parasite, whether they received the molecule or not. The decrease of levels of IL-6, IL-10, TNFα and IFNγ after infection was observed in all groups, and we assumed that was due by the presence of the viable parasite in animals. Some previous study showed that, in *T. b. rhodesiense*-infected mice, IFNγ production correlates with a decrease in parasite load and is implicated in resistance to infection (Hertz et al., 1998). It has also been shown that, during *T. congolense* infection, massive production of IFNγ appeared to have a deleterious effect on the liver (Shi et al., 2003). In our experiment, the rapid decrease in IFNγ production after infection was observed for all groups, without any significant difference in pre-immunization with TCTP or not, and therefore cannot explain the relative lengthening of the survival for the TCTP groups.

The TH1/TH2 immune response and its balance may differ depending on the considered parasite. In helminths, for example, the TH2 response is favored (Cortés et al., 2017). In contrast, in Leishmaniae, the Th1 response will be preponderant; nevertheless it has also been shown that in *Leishmania major* the Th1 response can switch to a Th2 response (Louis et al., 1998). The immune response against parasite is a TH2 response implies IgG1 production together with IL-4 and/or IL-10. In our study, IgG and IgG1 production were only observed in animals which received r*Tbg*TCTP protein with a maximum at day(0) and day(+4) although we didn't find significant levels in IL-4 and IL-10 productions in these animals.

Levels of IL-6 and IL-10 were very low (similar than those observed in PBS groups), and no significant modulation of these cytokines was observed, whatever the groups. The IL-6 cytokine was described to have a role in protection against neuropathogenesis of the parasite (Lejon et al., 2002). In addition, it is known that IL-10 plays a role in the immunological

balance between protection and pathology of immune response in Human African Trypanosomiasis (Shi et al., 2003, Namangala et al., 2001; Bosschaerts et al., 2010; Bosschaerts et al., 2011; Guilliams et al., 2009). The presence or absence of these cytokines in our study may not have an impact in delayed mortality in the TCTP groups.

Compared to the other groups, an extinction of cytokine production is measured in the group injected with 80 µG TCTP. Knowing the strong immunomodulatory properties of this molecule (Grébaut et al., 2009; Bommer and Thiele, 2004), we hypothesized that the dose injected is very or even too high and that the cells of the immune system are overwhelmed or anergic, and cytokine production was abolished.

Regarding this latter aspects, four criteria were measured, the weight of the mice, their hematocrit, their survival throughout the experiment, and the evolution of their parasitaemia after having been trypanosome-infected. The criteria included hematocrit, since anemia was shown to be associated with *T. congolense* and *T. vivax* infections of cattle (Mbewe et al., 2015).

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The evolution of mice survival can be explained by that of their parasitaemia depending on the different groups. In the two control groups, parasitaemia evolved along the classical pattern of *Tbg*-infected mice: a first peak 7 to 8 days after infection, a second around day(+14) to day(+15) and finally a third one at day(+22) after which all the surviving mice

died. In the immunized mice, the first peak occurred also 7 to 8 days post-infection, but it was approximately 50% lower in the mice that were immunized with either 40 or 80 µg of r*Tbg*TCTP, than in the control groups. However, the most remarkable was the increased time that separated the second peak from the first: 15 days (group 80TCTP) versus 7-8 days in the control groups. This result clearly demonstrated the mice immunization with r*Tbg*TCTP to provide an inhibitory, although transient, effect against *in vivo Tbg* blood stream forms multiplication.

To conclude, the results presented here assess the antigenic properties of the r*Tbg*TCTP protein. Injected in mice before infection with trypanosomes, it modulates immunoglobulin production, associated with biological effects such as limitation of parasitaemia level and mice survival. Although the role of TCTP in the parasite's interactions with its host during disease development is still not known, its blocking by immunization-induced immunoglobulins seems to impact directly parasitaemia i.e. the multiplication of the parasite within its host. TCTP may display a higher involvement than calreticulin [another trypanosome secreted protein (tested on *T. congolense* infected mice) (Bossard et al., 2016), owing that mouse immunization against TCTP, seems to have higher protective effects, despite only transient, than calreticulin immunization. Therefore research on TCTP deserves pursuing in combination with other trypanosome excreted/secreted proteins in order to improve host protection against parasite infection.

Declarations

Ethics approval and consent to participate

The experiments designed for this study were approved by the regional Ethic Committee for Animal Experimentation CEEA-LR 36 under project number 02187.01 and authorized by French Ministry for Higher Education and Research.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

GB and AG conceived and designed the experiments. GB, VR and ET conducted the experiments. GB and VR analyzed the data. GB and AG contributed reagents, materials and analytical tools. GB, VR, ET and AG wrote the manuscript. All authors read and approved the final manuscript.

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Figure Legend

Figure 1. Kinetics of *Trypanosoma brucei gambiense* parasitaemia. Three groups of OF-1 mice (n=6/group) were immunized three times (in 2-week intervals) by subcutaneous injection of r*Tbg*TCTP in Freund's adjuvant. Three amounts of protein were tested: 20 µg, 40 µg and 80 µg. Two control groups received either PBS or Freund's adjuvant in three successive injections. After immunization, each mouse was infected with 3,000 *T. b. gambiense* parasites (ABBA strain). Control groups were processed similarly. Parasitaemia was inspected daily by counting trypanosomes with the aid of a microscope. This figure presents the average of the data collected daily for each of the five groups.

Figure 2. Survival curves of mice infected with *Trypanosoma brucei gambiense* (ABBA strain). Mice had either been immunized with r*Tbg*TCTP protein (20TCTP, 40TCTP, or 80TCTP) or were non-immunized (PBS or Freund's controls). Survival was monitored daily.

Figure 3. IgG immunoglobulin production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Indirect ELISA targeting of r*Tbg*TCTP was performed. Total IgG was quantified in mice sera recovered at days (-48), (-33) and (-18), corresponding to the period of immunization; at day (0), corresponding to mouse challenging with *Trypanosoma brucei gambiense*; and at days (+2), (+4), (+8) and (+12) post-infection, which was obtained during immunization followed by infection with *T. b. gambiense.* Mean optical density (620 nm) with standard deviation for 3 to 6 mice per condition is shown; data were analyzed using non-parametric tests (Kruskall-Wallis and Wilcoxon tests; $P<0.05$).

Figure 4. IgG1 immunoglobulin production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Indirect ELISA targeting of r*Tbg*TCTP was performed. Experimental design was as in Figure 3.

Figure 5. IgG2a immunoglobulin production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Experimental design was as in Figure 3.

Figure 6. IgG2b immunoglobulin production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Indirect ELISA targeting of r*Tbg*TCTP was performed. Experimental design was as in Figure 3.

Figure 7. Cytokine production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Cytokine production of IL-10 was measured using the CBA assay procedure on mice sera recovered during immunization on day (-48), on day (0) of infection with *Trypanosoma brucei gambiense*, and days (+4), (+8) and (+12) post-infection. The data represent mean ± SEM for 3 to 6 mice per condition and were analyzed using non-parametric tests (Kruskall-Wallis and Wilcoxon tests; $P<0.05$).

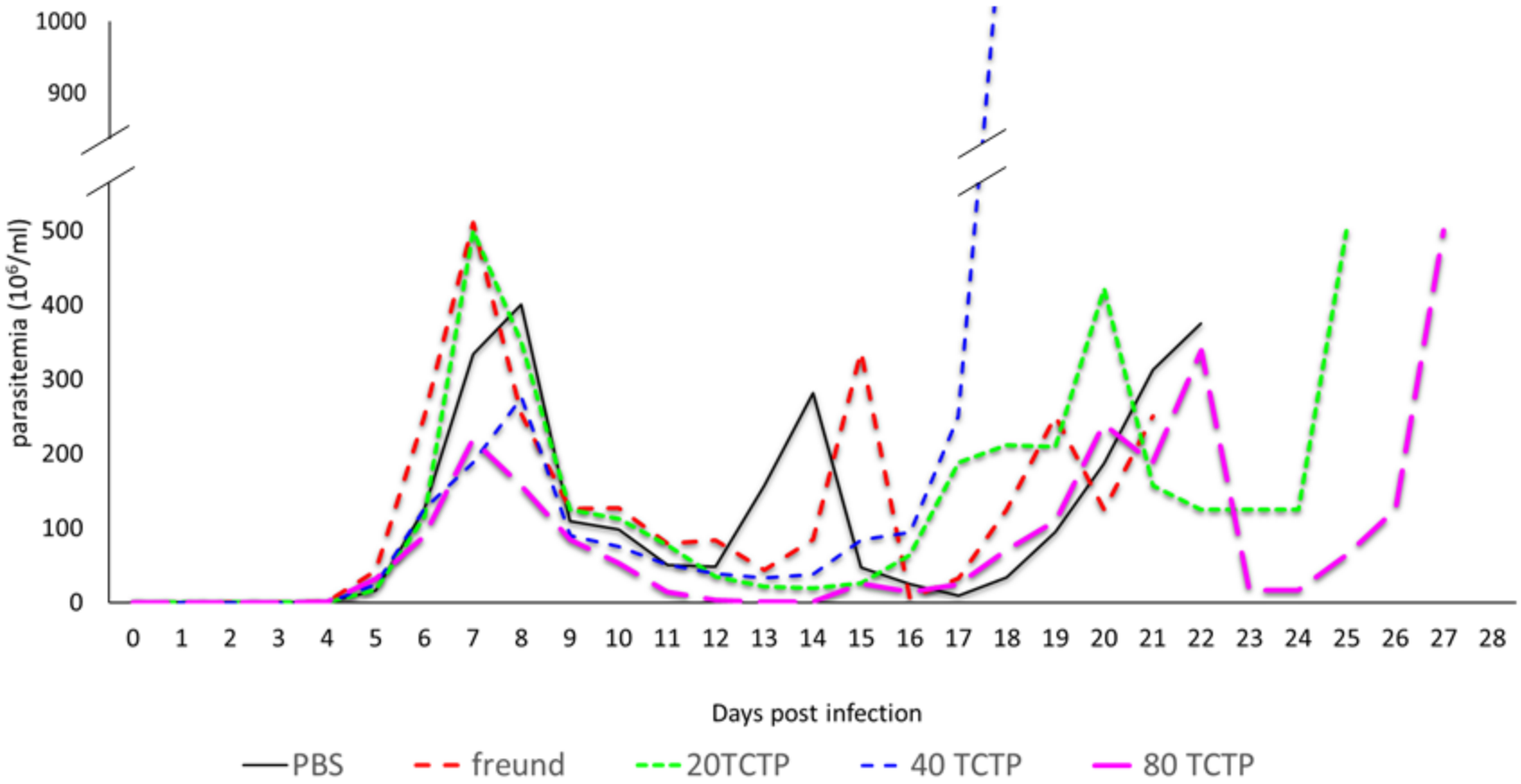
Figure 8. IL-2 cytokine production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Experimental design was as in Figure 7.

Figure 9. IL-4 cytokine production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Experimental design was as in Figure 7.

Figure 10. IL-6 cytokine production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Experimental design was as in Figure 7.

Figure 11. INF γ cytokine production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Experimental design was as in Figure 7.

Figure 12. TNFα cytokine production by r*Tbg*TCTP-immunized mice (20TCTP, 40TCTP and 80TCTP) and non-immunized controls (PBS or Freund's adjuvant). Experimental design was as in Figure 7.


1000
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300
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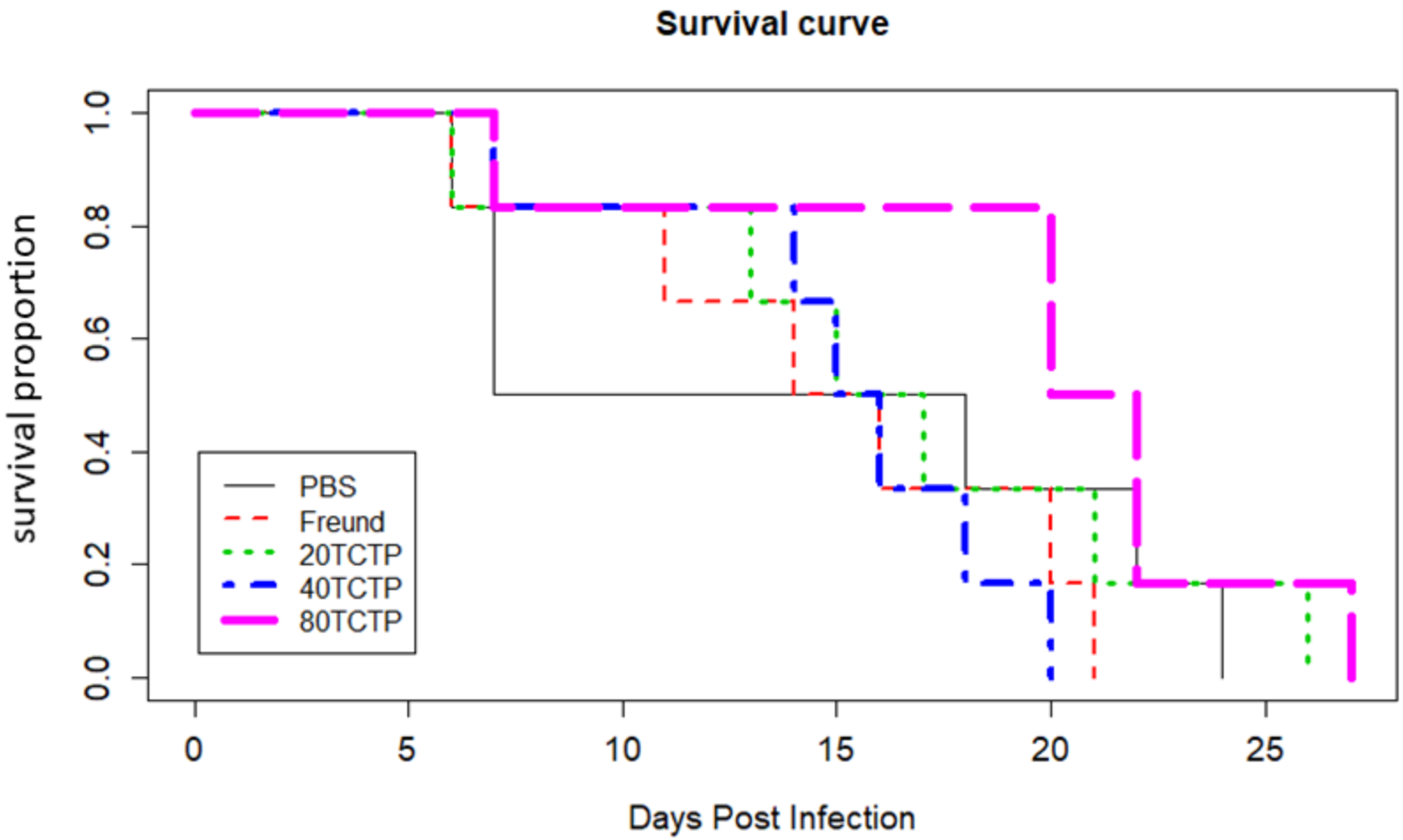
parasitemia (10⁶/ml)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Days post infection
PBS
freund
20TCTP
40 TCTP
80 TCTP


Survival curve
survival proportion
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0.6
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15
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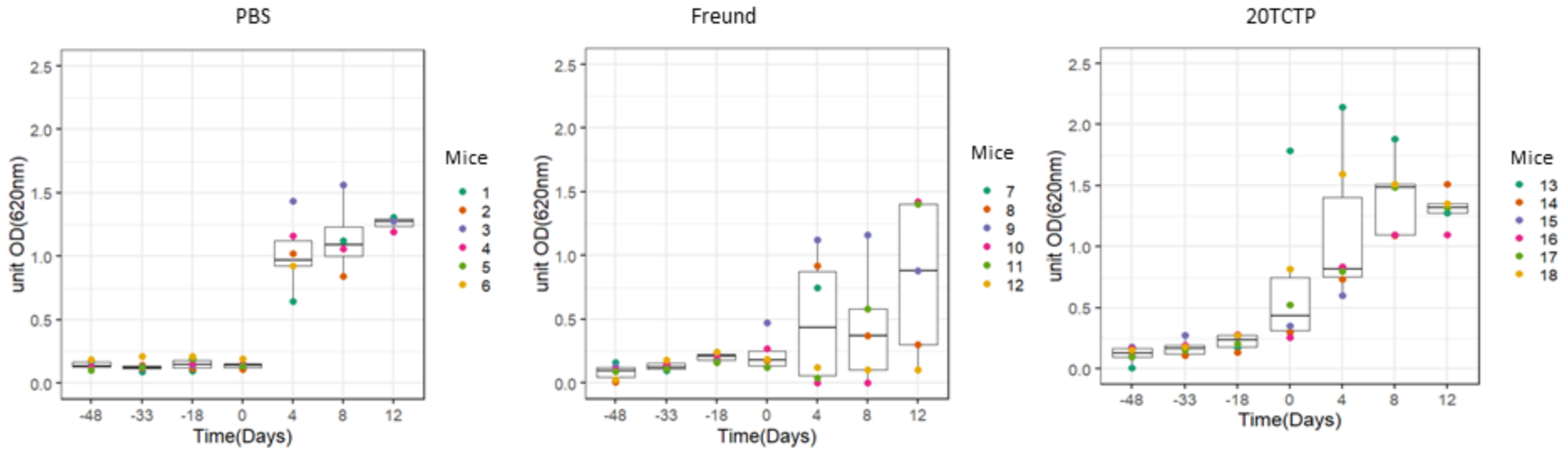
25
Days Post Infection
PBS
Freund
20TCTP
40TCTP
80TCTP

IgG


PBS
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unit OD(620nm)
Time(Days)
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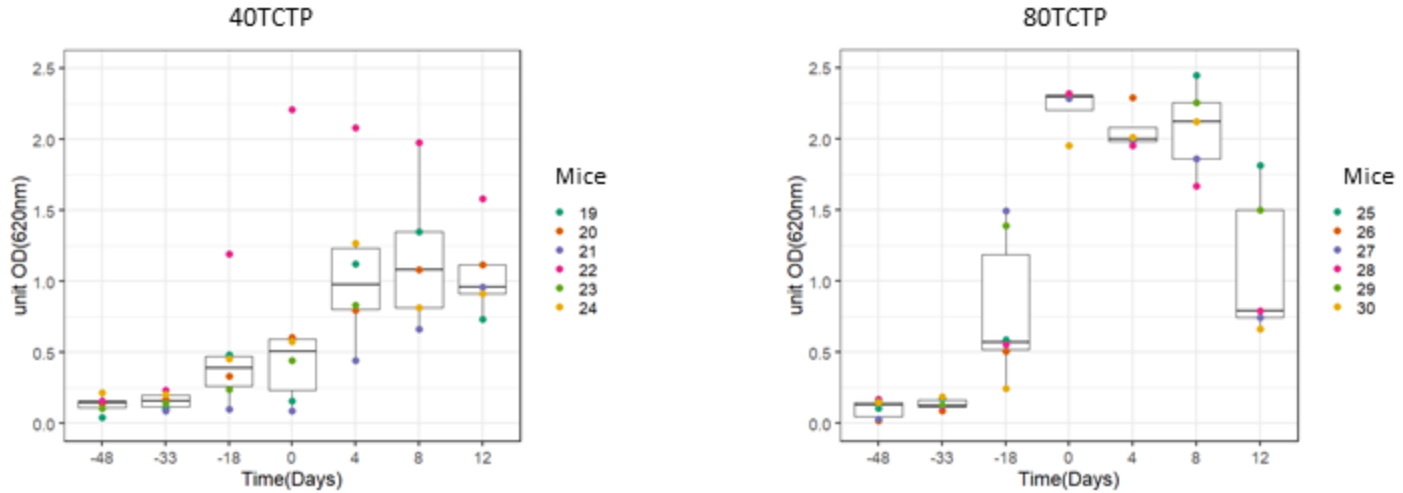
40TCTP
80TCTP
unit OD(620nm)
Time(Days)
Mice
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28
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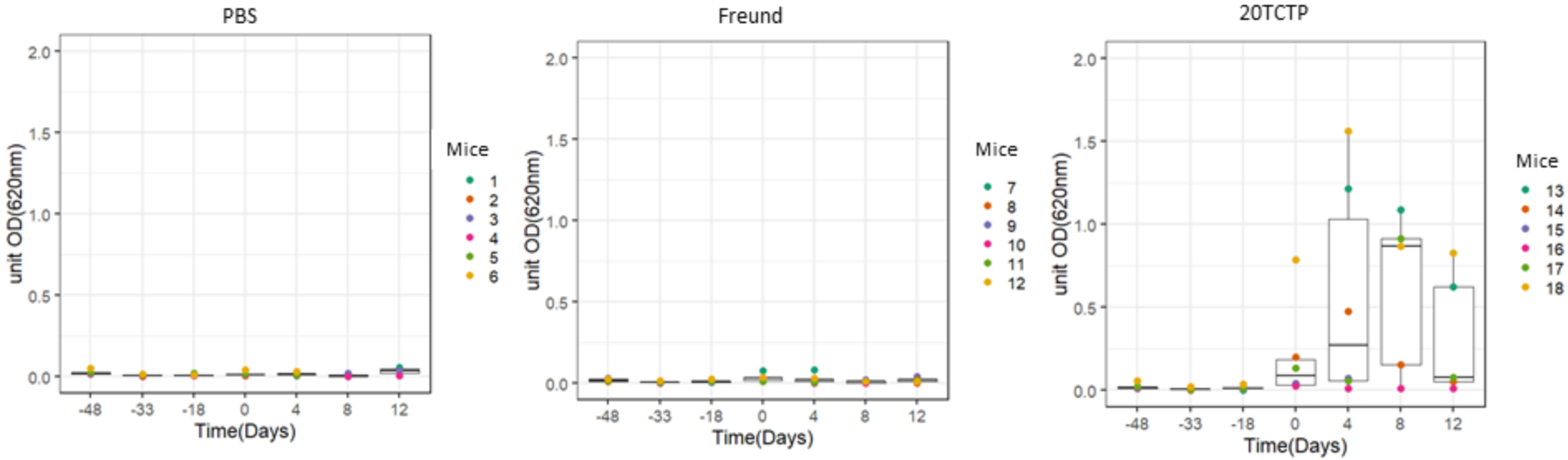
30

IgG1


PBS
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unit OD(620nm)
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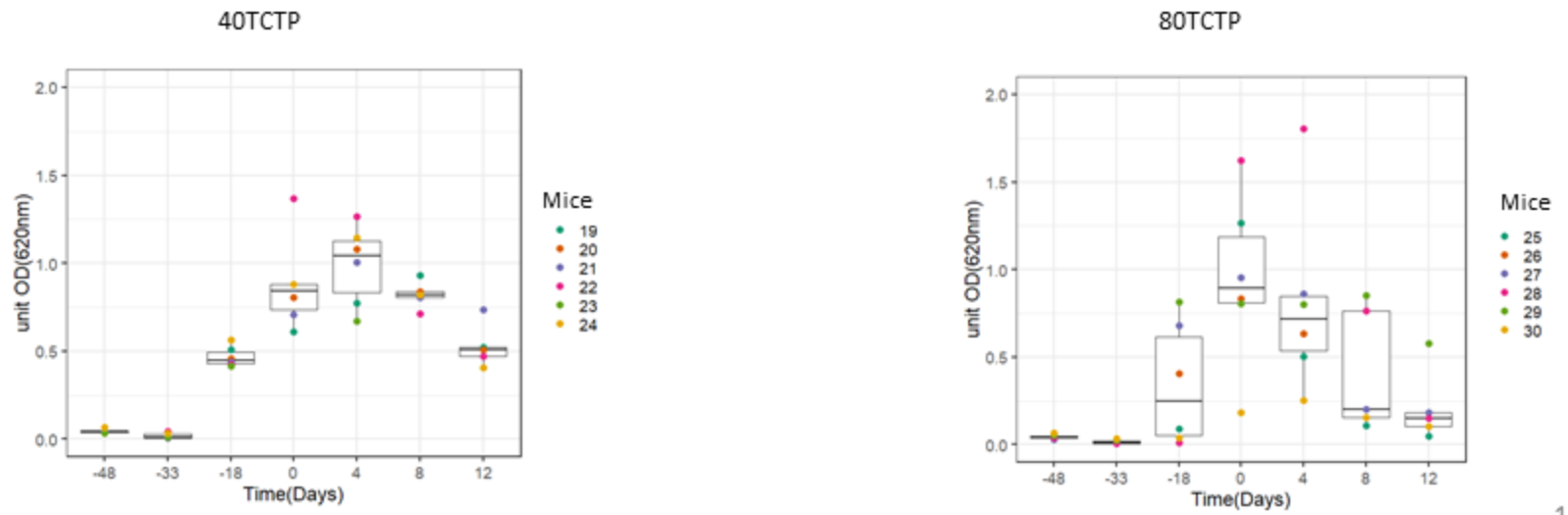
40TCTP
80TCTP
unit OD(620nm)
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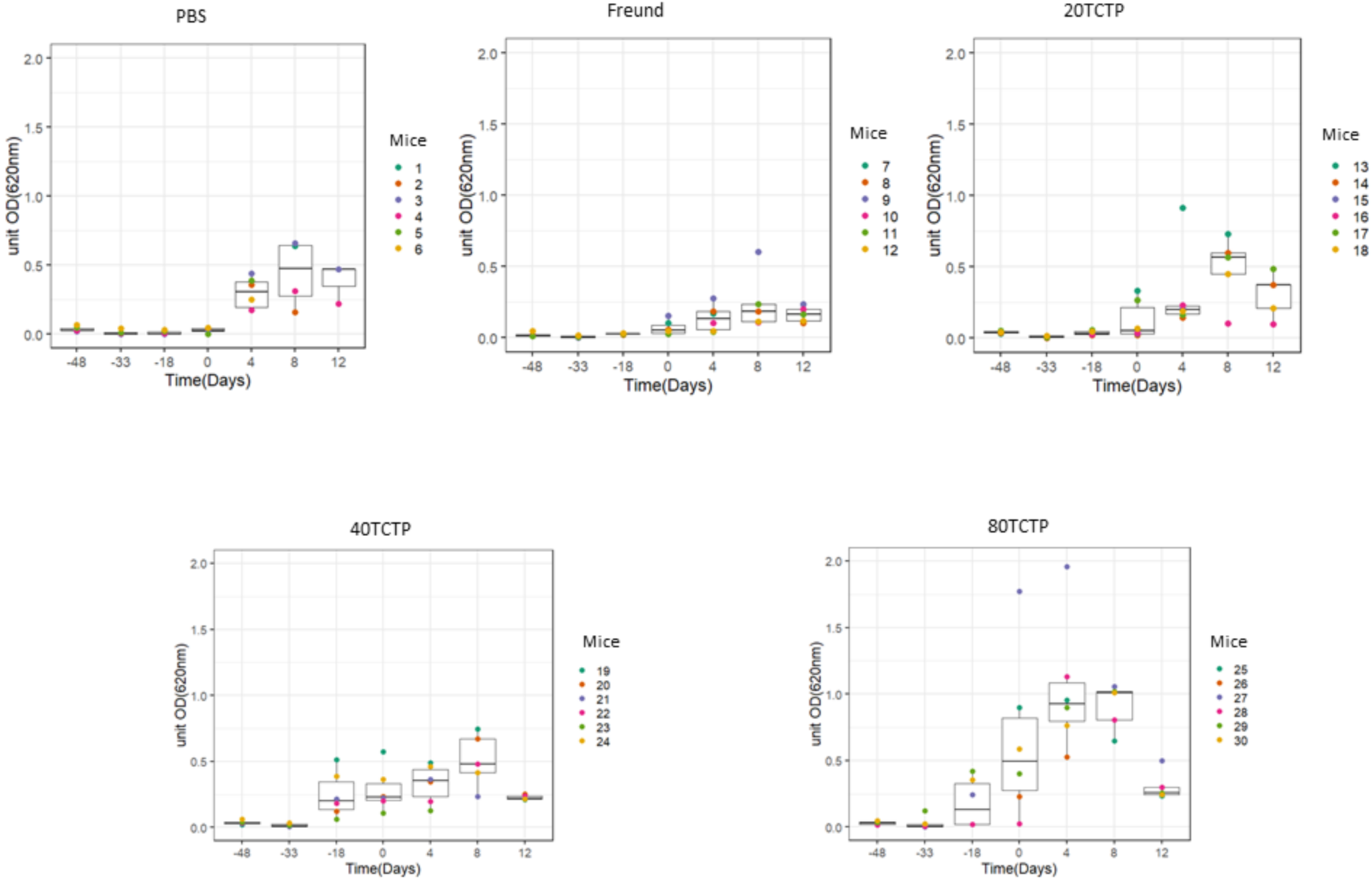
IgG2a


PBS
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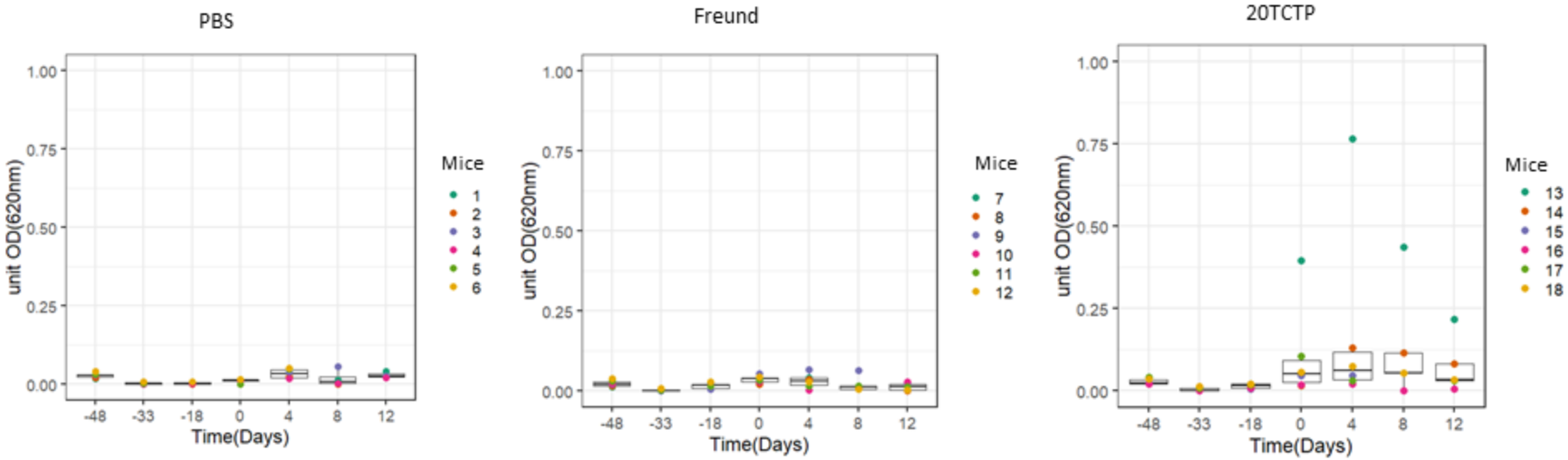
IgG2b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unit OD(620nm)
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Time(Days)
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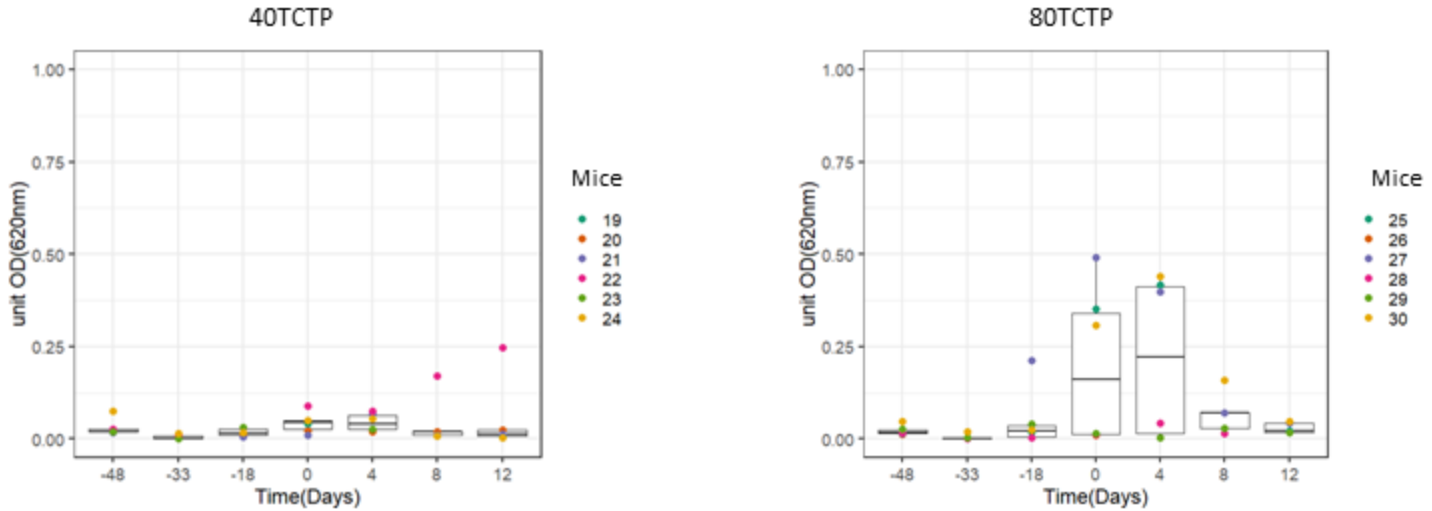
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Time(Days)
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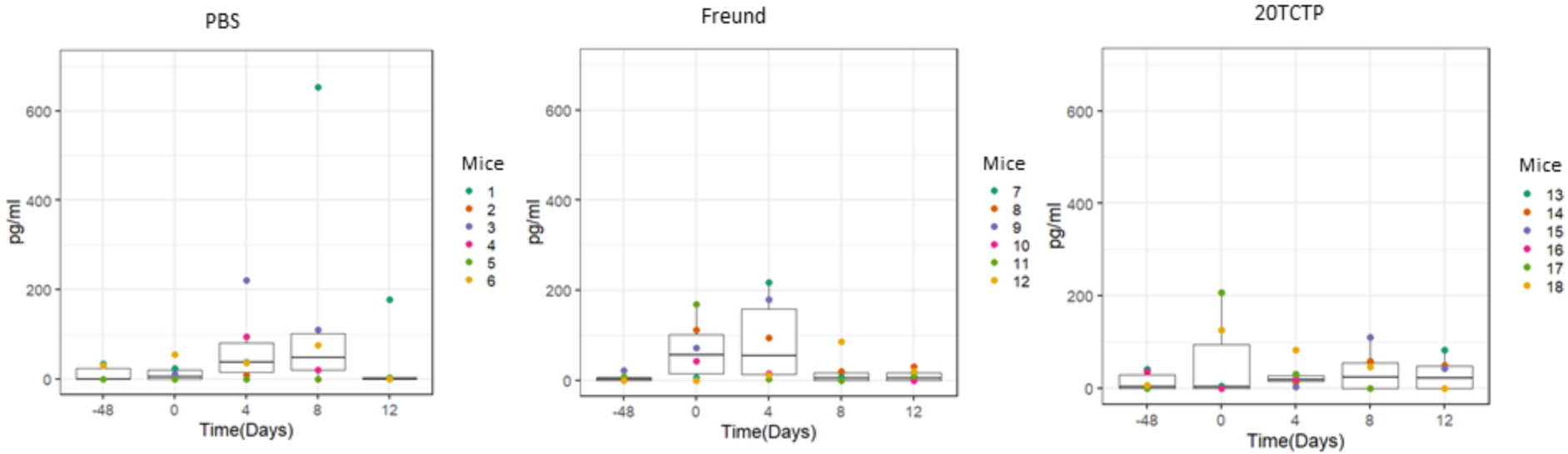
IL10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Mice
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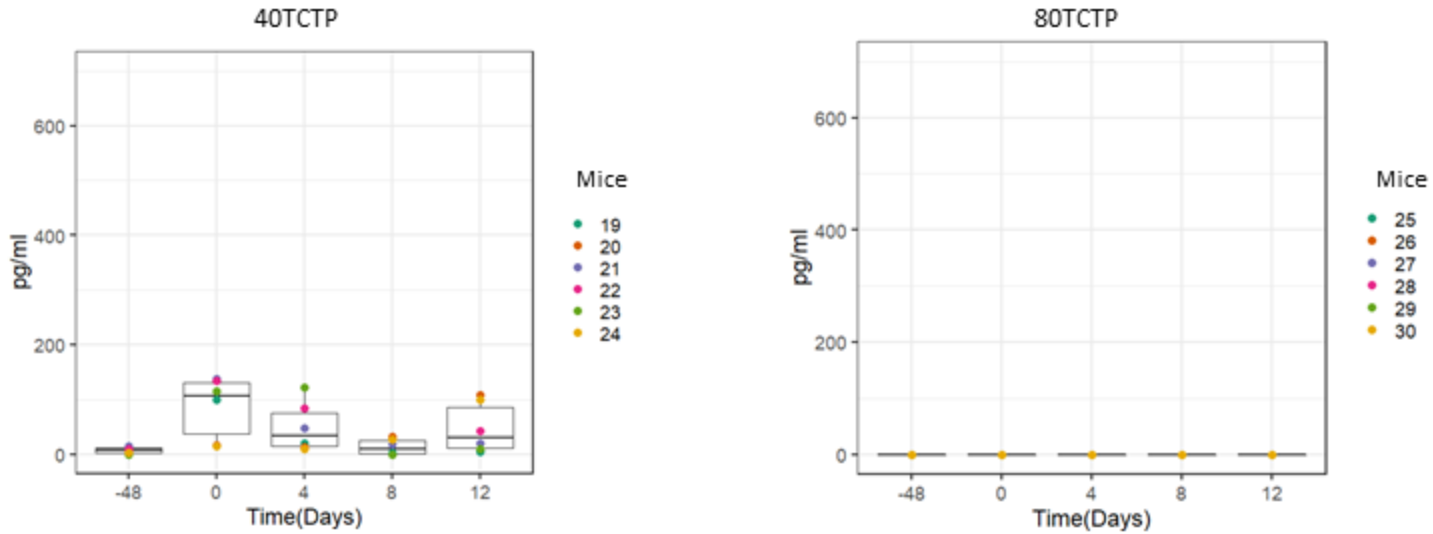
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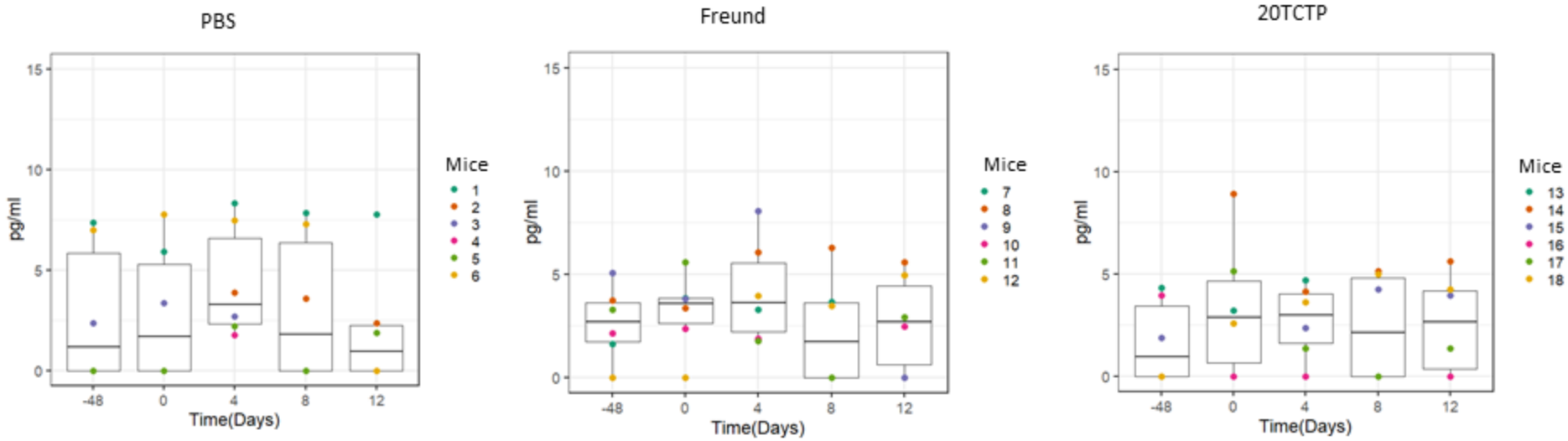
IL2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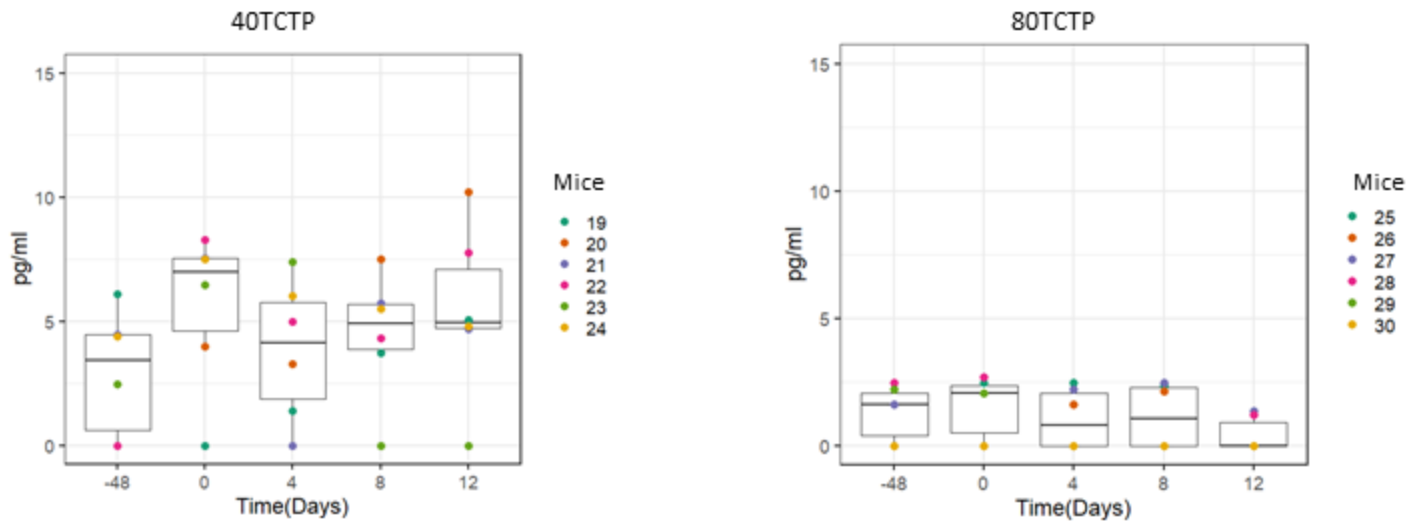
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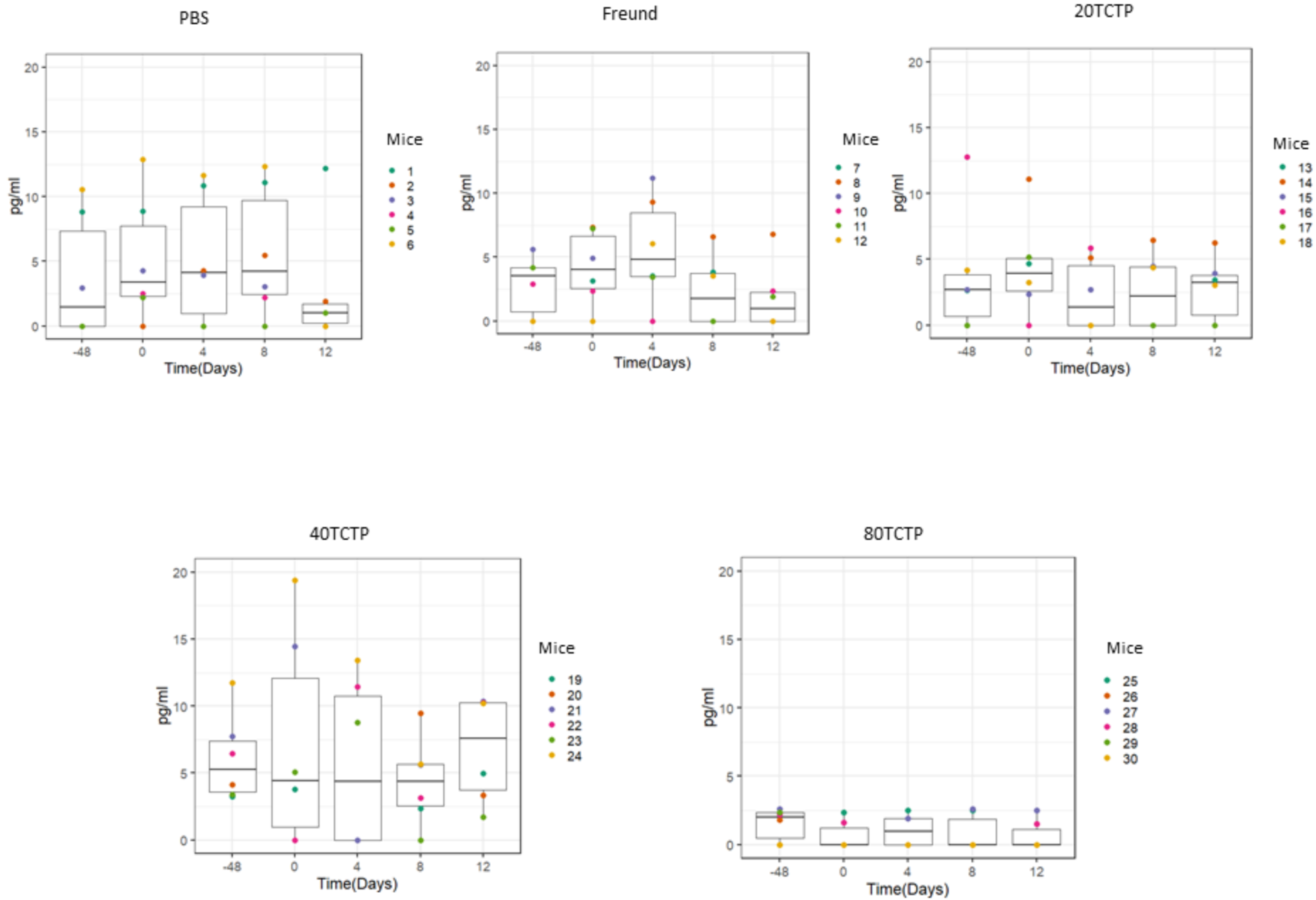
IL4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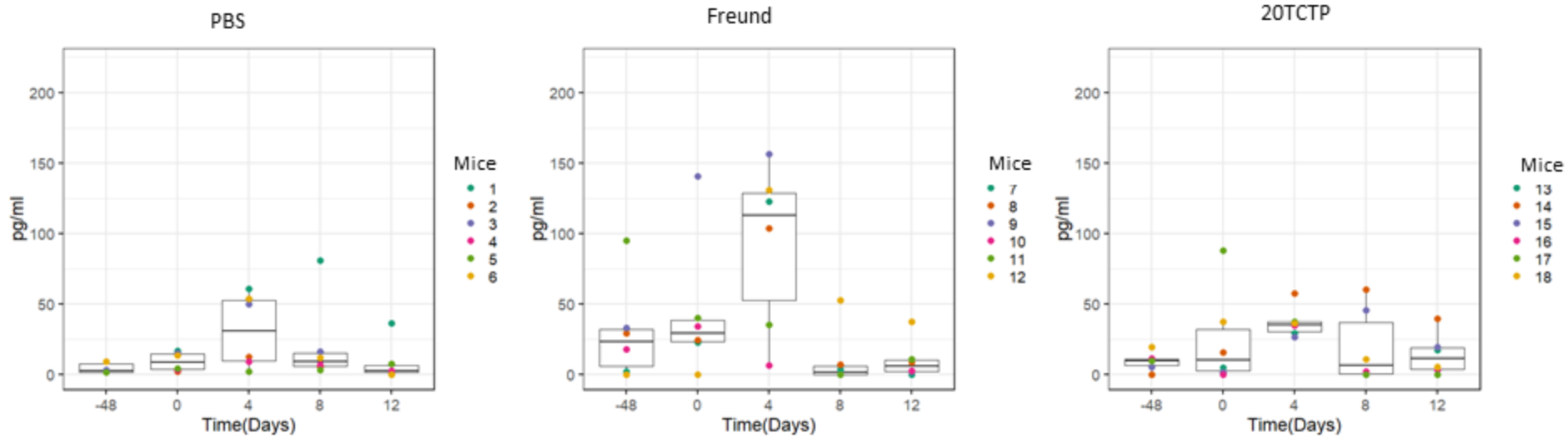
IL6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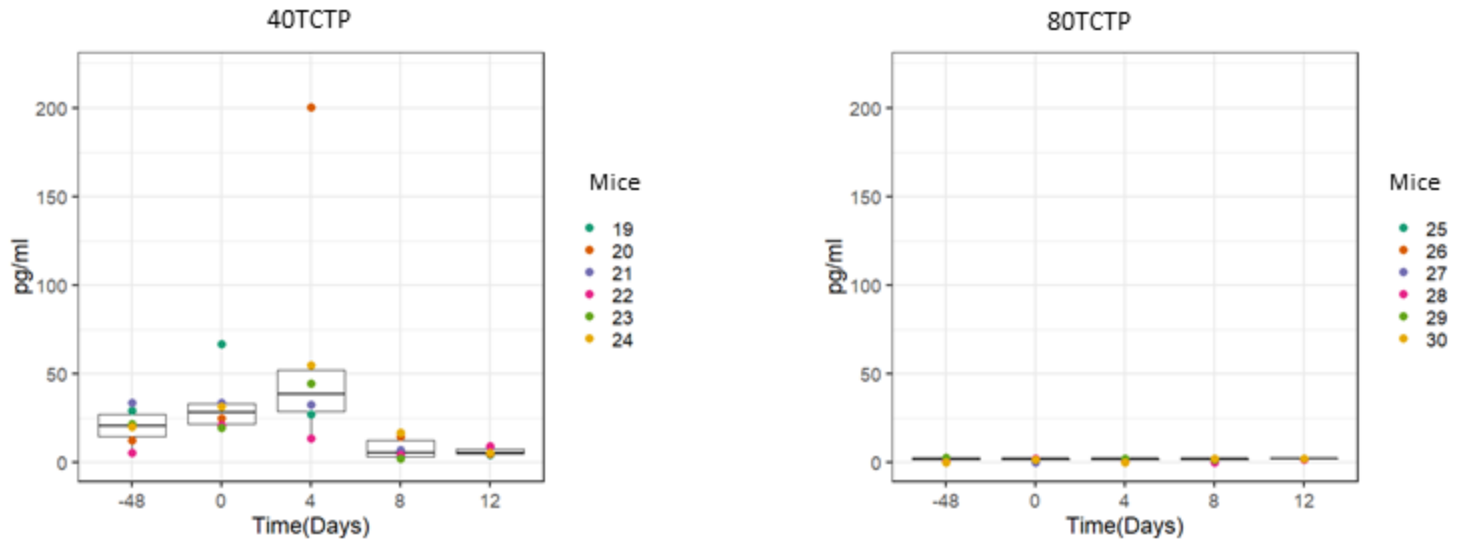
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80TCTP
pg/ml
Time(Days)
Mice
19
20
21
22
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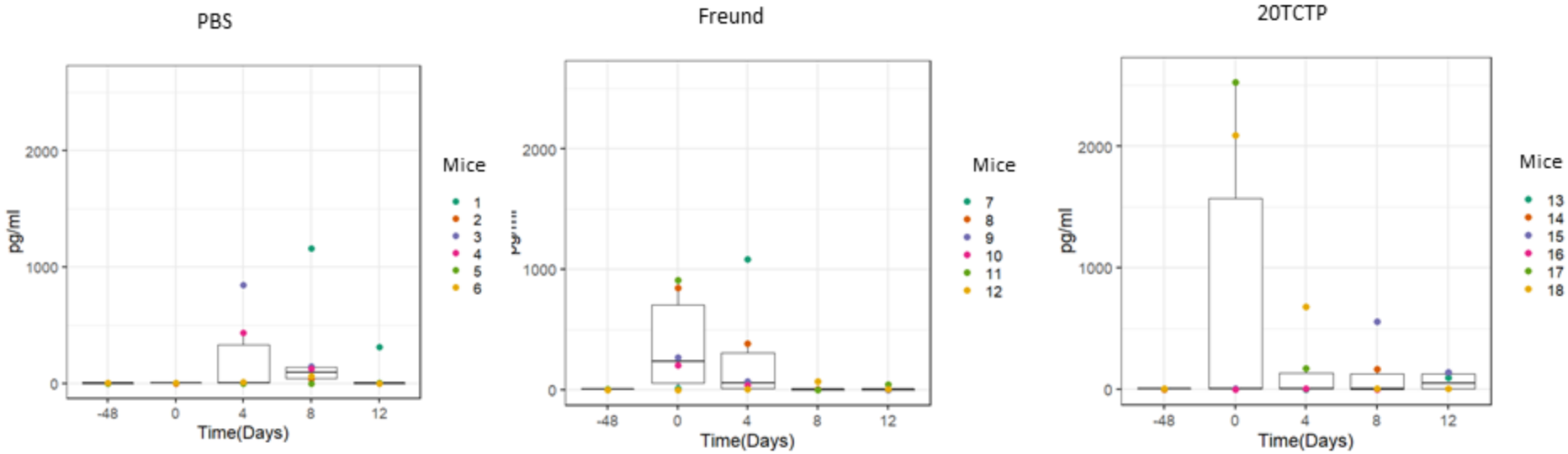
INFγ


PBS
Freund
20TCTP
pg/ml
2000
1000
0
-48
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Time(Days)
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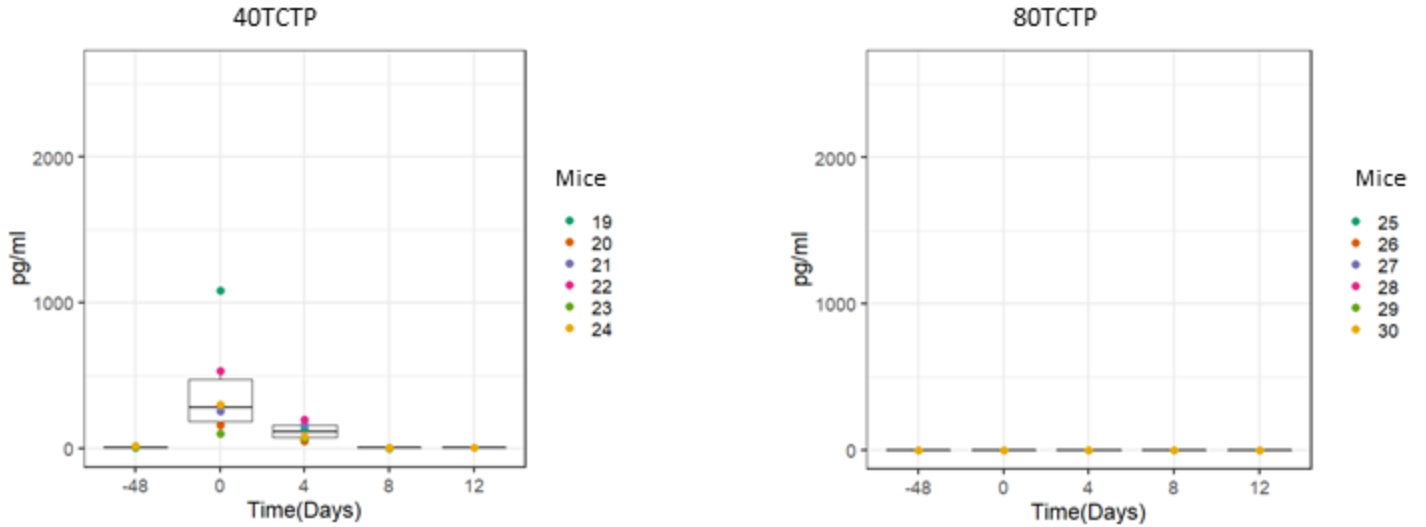
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Time(Days)
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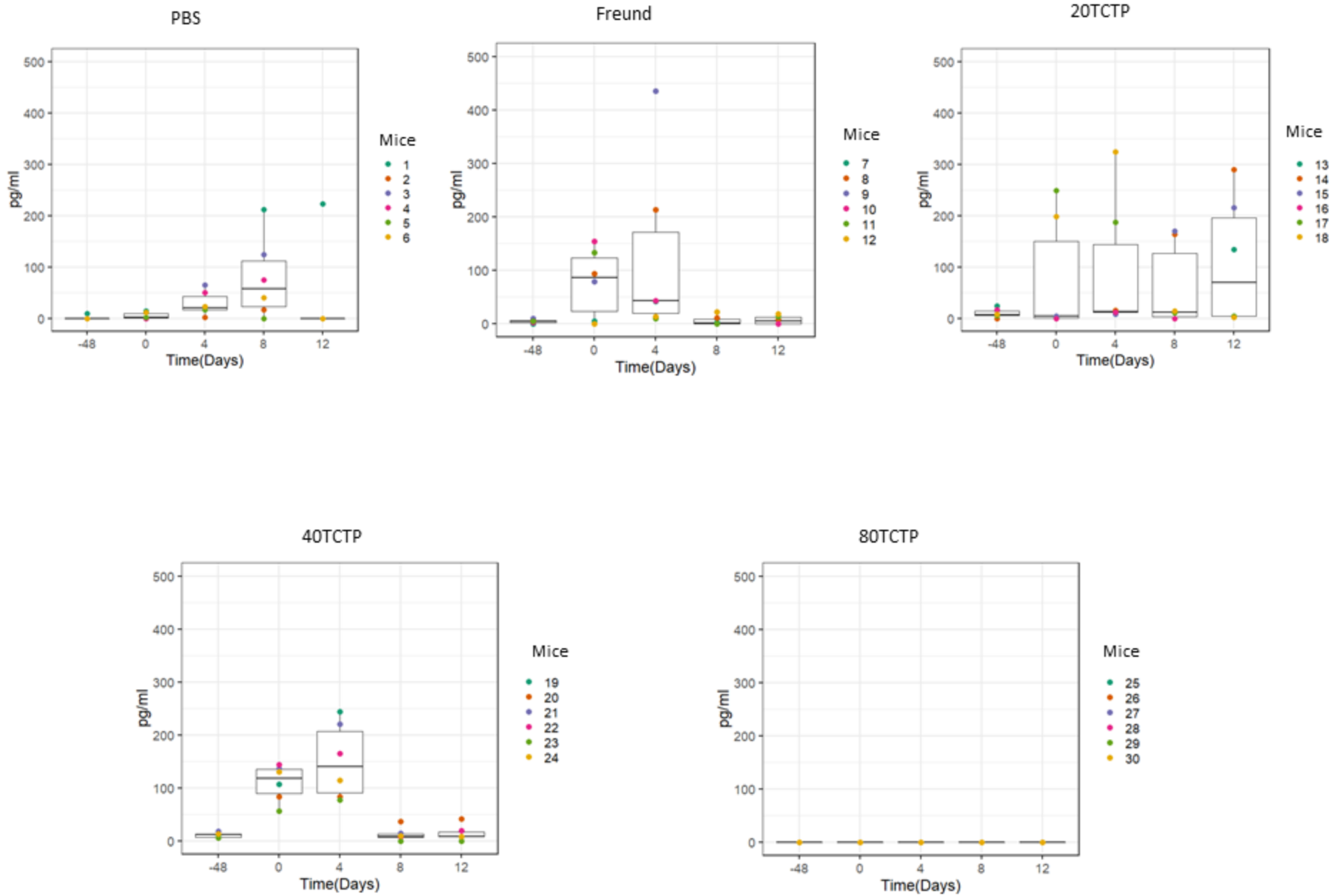
TNFα


PBS
Freund
20TCTP
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80TCTP
pg/ml
Time(Days)
Mice
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