CHALLENGED IMMUNE SYSTEM IMPROVES COGNITIVE-BEHAVIORAL RESPONSES IN HOMEOSTASIS AND RECOVERS MALARIA-INDUCED COGNITIVE IMPAIRMENT IN MICE

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ABSTRACT

The immune and nervous systems can be categorized as cognitive plastic systems for their ability to know and recognize “real world objects”, including microbes, and because of their prerogative of constant self-reorganization as they live learning experiences. After each antigenic or sensory occurrence, vertebrate organisms experience changes in the cellular connections of their immune and nervous systems altering their abilities and structures. Elements of the immune machinery are necessary for neurocognitive function, and the pattern of the immune response triggered by different stimuli may induce regulator or deregulator signals for the nervous functions. Here we show, for the first time, that the immune modulation with anti-inflammatory stimuli can positively regulate the behavior of healthy mice and mitigate the cognitive-behavioral deficits induced by a mild infection of C57BL/6 animals with *Plasmodium berghei* ANKA.

INTRODUCTION

There is considerable evidence for the existence of strong interactions between the immune and nervous cognitive systems[1-7]. Immunomodulation of the nervous system does exist and can either be physiological or pathological. The maturation and homeostasis of the nervous cognitive abilities require the participation of elements of the immune system[6-7]. Exogenous immune stimuli may also have positive or negative effect on the nervous system, depending on the nature and intensity of the immune response elicited[1-4,6]. Different studies, aiming to assess the effect of immune stimuli on brain function, reported i) the influence of maternal immune stimulation impairing the neurocognitive

performance of offspring[8-9], ii) both beneficial and harmful effects of neonate vaccination on neuronal plasticity and cognitive function in adulthood[10], and iii) the damaging impact of inflammatory stimuli on the cognitive function of adult mice[11-12]. Some infections may also cause neurocognitive dysfunction in human and experimental models[13-27].

Cerebral malaria (CM), the most severe complication of falciparum malaria, can be accompanied by neurocognitive sequelae, including severe motor deficit, behavioral alterations and severe learning difficulties[15]. Long-term sequelae, responsible for child ineptitude, are more common in Africa, where the prevalence of *falciparum* malaria and of CM is higher[28]. Some of these sequelae are also observed in *Plasmodium berghei* ANKA (*Pb*A) infected C57BL/6 mice, the most classical model of experimental CM[27.] In recent years, cognitive impairment, mainly related to learning and memory, has also been reported in residents of endemic regions presenting non-cerebral malaria (non-CM) episodes, but has not been recorded in the common murine experimental models of non-CM[29-31].

In a previous work, we have unprecedentedly adapted the classical experimental model of CM (*Pb*A-infected C57BL/6 mice) to study neurocognitive alterations after a short-term episode of non-CM[32]. Using this model, we evaluated here the effect of immune stimuli on behavioral paradigms, such as memory and anxious phenotype, during homeostasis or after a mild infection in mice.

Considering the data accumulated so far showing the effect of immune system on the performance of neurocognitive functions, we hypothesize that some types of immune stimulation may improve cognitive performance. Our data suggest an inedited beneficial effect of immunization, with antigens that drive T2 immune responses, on healthy mice cognitive performance and the recover of the cognitive impairment associated to plasmodium infectious stress.

RESULTS

T2-immune stimuli improve long-term memory in healthy mice

The immune stimuli were categorized according to the profile of cytokine induced by the specific properties of the studied immunogens. Thus, pro-inflammatory immune stimuli trigger Th1 cell responses with predominance of IFNγ and TNF-α production whereas polarization to an anti-inflammatory immune response profile (Th2) is dependent on T-cell derived IL-4[33-47]. Also, levels of certain antibody isotypes may indicate targeting of the immune response profile, such as IgG1 and IgG2a production indicating Th2 and Th1 responses profiles, respectively[42]. Moreover, adjuvants added to immune stimuli also play an important role in directing the immune response[43]. To study the effect of immune stimuli on behavioral paradigms, we employed three immunization strategies according to their pro- and anti-inflammatory immune response profiles. T1 strategy was used to induce a pro-inflammatory immune response and T2 to elicit immune responses of type 2[33-47]. The Pool of antigens, for a combined T1 and T2 strategy, was also used.

To address the effect of immune responses on locomotion and on long-term habituation, mice were submitted to the open field task (OFT), with a training and a test sessions, 24 hours apart, assessed in a well-established protocol[32]. Locomotion is evaluated when mice access the open field arena for the first time. At the training

session, a high rate of locomotor activity is commonly observed. Surprisingly, Pool and T1 immunized mice showed a reduced total OFT1 locomotion, when compared to non-immunized mice, and T2 immunized animals showed a clear trend towards a decreased locomotion (Extended data, Fig1a) Commonly, after the training session, the exploratory behavior decreases, as the stress related to the novelty disappears, and is significantly lower after 10 minutes of task performance[32,48-49]. Control and all immunized groups of mice presented a decreased locomotion in the test session (OFT2) as compared to the training session (Extended data, Fig1a). These results indicate that the immunization did not affect the long-term habituation memory accessed 24 hours after training.

Mice were then subjected to the novel object recognition test (NORT) in the same Open Field arena. On the training session, a similar exploratory activity of both familiar (FO1 or FO2) objects is expected and was observed in all groups (Control, Pool, T1 and T2) of mice (Fig.1a; Extended data, Fig.1c), exploring them for a mean of 25 seconds (data not shown). Remarkably, as we hypothesized, the groups of mice immunized with the Pool and T2 strategies presented significantly higher recognition memory performance in relation to the control group of non-immunized mice during the test session (Fig.1c; Extended data Fig.1e). These data indicate, for the first time, that an immune response classically categorized as anti-inflammatory (induced by T2 stimuli) may enhance long-term recognition memory in healthy mice.

Immunization of healthy mice did not influence their anxiety-like state

In addition to the exploratory activity, the OFT also allows the evaluation of phenotypes related to anxiety-like behavior through the dwell time or the locomotion rate in the center of the open field, during the first exposition to the apparatus. Immunized mice (Pool, T1 and T2) showed no difference in time (data not show) but presented a significantly reduced locomotion in the center of the open field in relation to non-immunized control group (Fig. 1e). However, these data may have been impacted by the total reduced mobility observed in these animals (Extended data, Fig. 1a), and no conclusion about anxious behavior can be scratched from these data. To address this issue, we used another approach-avoidance conflict test, the classical light-dark specific task, to evaluate the anxiety-like behavior. In this test, immunized mice (Pool, T1 and T2) groups clearly behaved similarly to mice of the control group, remaining during an equal time in the light zone (Fig.1g), implying that immunization did not change the anxiety phenotype.

T2-immune stimuli may recover the cognitive-behavioral damage caused by non-complicated *P. berghei* ANKA infection

About 92% of the world's malaria cases are due to *Plasmodium falciparum* infection, 1 to 2% of which progress to cerebral malaria that accounts for about 80% of deaths due to malaria in the world. Thus, about 90% of malaria cases globally are caused by a lethal species of *Plasmodium*, but occur without clinical complications[28] and may also present with cognitive impairment, indicating that non-complicated malaria can impact not only the children health but also their cognitive development[29-31].

The experimental model we have described uses *Pb*A-infected C57BL/6 treated at day 4 post-infection, before appearance of any clinical sign of CM. The main advantage of such a model is that it best mimics the human situation described above that

139 corresponds to the largest number of malaria cases in the world (uncomplicated
140 falciparum malaria treated timely)[28]. Using this model, we have been able to observe
141 a long-term cognitive-behavioral impairment related to memory and anxious
142 phenotype[32]. With the evidence of a beneficial effect of immunization on the long-term
143 memory in healthy mice described above, we decided to evaluate the effect of the
144 same immune stimuli in mice with behavioral alterations resulting from non-CM.

145 The behavioral tests were performed 77 days after Chloroquine (CQ) treatment of
146 infected mice. *Pb*A-infected and treated mice did not present total mobility reduction in
147 the training session of OFT (Extended data, Fig.1b) when compared to non-infected
148 mice. However, infected-immunized (Pool, T1 or T2) animals showed a trend towards
149 a reduction of locomotion in OFT, when compared to infected mice (Extended data,
150 Fig.1b). This trend becomes a significant reduction when locomotory activity of
151 infected-immunized mice (Pool and T2) is matched to that of healthy ones (Extended
152 data, Fig.1b).

153 As expected, there was no object preference in the NORT training session since all
154 mice explored similarly (for a mean of 25 seconds - data not show) both familiar objects
155 (Fig.1b, Extended data, Fig.1d). Control, infected and infected-immunized groups
156 (Pool and T2, but not T1) of mice presented normal behavior with a significant
157 decreased locomotion in the test session as compared to the training session of the
158 OFT (Extended data, Fig. 1b). Consistently, non-CM recovered mice presented long-
159 term recognition memory sequelae expressed by similar exploration of the FO and NO
160 in NORT. As hypothesized, this impairment disappeared after the use of the Pool and
161 T2 strategies of immunization (Fig.1d, Extended data, Fig.1f), pointing to the foreseen
162 beneficial effect of anti-inflammatory T2 immune responses reversing cognitive deficit
163 associated to malaria.

164 **_P. berghei_ ANKA infection of mice induces an anxiety-like behavior that is**
165 **reverted by the T2 immunization strategy**
166 The distances travelled in the periphery and in the center of the OFT are inversely
167 related. Since the latter was decreased in *Pb*A infected recovered mice (Fig.1f) and no
168 OFT1 locomotion change occurred, one can understand this effect as the expression
169 of an anxiety-like behavior (Extended data, Fig.2b). This anxiety-like behavior was also
170 detected in the light-dark task, since the infection also reduced the time spent in the
171 light zone of the apparatus. More importantly, this decrease was reversed by Pool and
172 T2 immunization (Fig.1h).

173 This is in accordance with the increased distance travelled in the periphery of the OFT
174 (data not show). This anxiety-like phenotype was, effectively, confirmed by the
175 reduction of time spent in the light zone of light-dark task. This decrease was reversed
176 by the immunization with Pool and T2 strategies (Fig.1h).
177
178 **Immunization procedures and non-complicated _P. berghei_ ANKA malaria do**
179 **elicit specific immune responses**
180 Pool and T2 immunized mice showed increased spleen weight and total number of
181 splenocytes (Extended data, Fig. 2a,b). The specific immune responses triggered by
182 the immunogens used were evaluated and the effectiveness of the stimuli was
183 confirmed (Extended data, Fig.3a,b,c,d). We also evaluated the frequencies of B and
184 T cells, as well as the T cell activation status in the spleen, at the end of the cognitive

task evaluation. Mice immunized with either the Pool or the T2 strategies (Pool and T2 groups) presented similar patterns of modulation of different immune components analyzed. We observed, in both groups, an increase in the frequency of B cells, CD4 and CD8 T cells with central memory phenotype and CD4 T cells with regulatory function when compared to non-immunized animals (Extended data Fig.4a,b,c,d). A reduction in the frequency of splenic CD8 T cells was also recorded mice immunized with the T2 stimuli, when compared to the control group (Extended data, Fig.5a). Besides, mice immunized with the Pool and T2 strategies presented increased serum levels of IL-4 cytokine, TNFα and IFNγ (Extended data, Fig.6a,b,d).

Alterations in the immune response profile were also evaluated late (77 days following treatment) after *Pb*A infection. *Pb*A-infected and treated mice showed higher frequency of B cells, CD4 and CD8 T cells and increase in the frequency of CD4 and CD8 T cells with naïve and central memory phenotype (Extended data, Fig.4a,b,c,e,f; Fig 5a,b). The frequency of regulatory CD4 T cells, however, was similar between infected and control mice (Extended data Fig.4d). Also, at the time they were immunologically evaluated, infected and treated animals did not present detectable levels of serum cytokines (Extended data, Fig.6a,b,c,d) and only showed an altered shade color, attributable to the hemozoin pigment of the erythrocytic cycle of the *Plasmodium* parasite, with no more difference in the weight and total number of splenocytes in relation to the control group (Extended data, Fig.2a,b,c). It is clear, therefore, that alterations of the immune system are triggered by plasmodium, even though the infection is treated and contained rapidly, and that some of them can be observed several weeks after parasite elimination.

As observed in healthy immunized animals, immunization of *Pb*A-infected and treated mice with Pool or T2 strategies induced splenomegaly (Extended data, Fig2a,b,c), humoral immune response to the vaccine stimuli (Extended Data, Fig.3a,b,c,d) and patterns of splenic B cells, CD4 and CD8 T cells and of their different function subsets comparable for the two stimuli (Extended data, Fig.4a,c,d,e; Fig.5a,b). The marked splenomegaly recorded after immunization could partially explain the reduced total locomotion in OFT (Extended data, Fig.2a,b) and harmed conclusions on the effect of immunization on anxiety-like phenotype in this behavioral task (Fig.1e,f).

It is important to highlight that, in general, the object exploratory pattern in NORT training test was not modified in any feature considering the most rigorous aspects reported in NORT protocol[50], which sustain our interpretation about the beneficial effect of anti-inflammatory T2 immune responses reversing cognitive deficit associated to malaria.

Taken together, our data point to a positive influence of immune responses induced by T2 stimuli on the long-term memory in healthy mice, confirm our previous demonstration of neurocognitive behavioral dysfunction after a single episode of non-cerebral malaria, and indicate a recovering effect of this deficit exerted by T2 immunization procedures.

DISCUSSION

The existence of lymphocyte neuropeptide receptors[51] and cytokine receptors in neurons[52] are commonplace notions that have paved the anatomical and functional basis of the "classical neuroimmunology". On the other hand, as the immune and nervous systems are arrangements with cognitive functions and plastic properties, it is reasonable to assume that learning in one cognitive system may influence the performance of the other.

Different studies have shown that stimulation of the immune system may exert different effects on neurocognitive function depending on the nature and balance of the immune inputs[6,51-52] and the cumulated knowledge points to the notion that, while anti-inflammatory immune responses are responsible for the proper performance of the brain[51,53], pro-inflammatory responses can impair cerebral function and neurocognitive activity[6].

Stimulus such as physical exercise, reading and music are mentioned cognitive enhancement strategy[56-59]. Here, we describe, for the first time, beneficial immunomodulatory effects improving cognition in healthy mice. Our findings show a marked positive effect of immune stimuli specifically triggering type-2 immune response by improving long-term memory of normal adult mice, as verified by the new object recognition task (NORT), a robust and most used behavioral tasks for the analysis of the parameter in rodents[50].

We have previously reported cognitive-behavioral impairment as late sequelae of a single uncomplicated malaria episode using a MC experimental model and treating animals before the appearance of any neurological sign[60]. In our concept, the model is illustrative and adequate for the study of the, by far, most common presentation of malaria; ie: the non-cerebral *Plasmodium falciparum* malaria[61]. In fact, both the experimental model and the human situation correspond to conditions in which we have a *Plasmodium* parasite capable of causing - and a host susceptible of developing - cerebral malaria that is, however, prevented by timely treatment.

As hypothesized, we were able to observe a positive effect of immune stimuli reversing cognitive-behavioral impairment associated with non-complicated malaria (nCM) in the prototype described above. The results show that mice with nCM, treated at D4 of *Pb*A infection and immunized with T2 and Pool strategies for 60 days from D11 on, did not present the long-term memory sequelae - expressed by similar exploration of the FO and NO in NORT - as non-immunized infected mice did. Besides, we reported anxious-like behavior reversal in the light-dark task, also considered a reference tool for specifically analyzing anxiety[62-63], after immunization of infected and treated mice.

These results confirm our conceived idea of an effect of immune stimuli on brain function and downstream consequences reflected in improvement of the cognitive-behavioral performance. It is know that inflammatory stimuli may cause microgial activation and increase of cytokine T1 profile such as TNF-α and IL1-β reported as evil for cognitive function[64] while cytokine T2 profile are beneficial cognitive enhancers such as IL-4 referred to as cytokine to remember[35]. It has also been shown that LPS can cause the basal ganglia to malfunction but the dosages applied to promote this effect are higher than that used in the present study[65].

The inedited data reported here may correspond to a new paradigm and promising alternative for the design of memory improvement strategies in homeostasis and cognitive function impairment, including those associated to malaria and reflected as poor school performance in hundreds of thousands of children in the world. In fact, the relevance of maintaining an updated vaccination calendar is usually claimed for the sustenance of efficient policies for prophylaxis of "immune-preventable" diseases, through individual and collective protection against infections and prevention of epidemics. Our data may suggest that a new thought on the importance of vaccination may arise from its potential use as a strategy for boosting cognition function in health and recovering cognitive dysfunctions in infectious and chronic diseases and ageing. This new perceptiveness of the vaccination benefits could constitute a new weapon to fight the growing anti-vaccine movement in the world.

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AUTHOR CONTRIBUTIONS

LPS was responsible for the realization of all experiments (including infection and treatment; immune stimulation, and conduction, observation and data collection/systematisation of cognitive tests and immune response analyses in mice), helped in the analysis and interpretation of tests and drafted the manuscript. FLRG followed all the stages of the experiment, realization of experiments, discussed the protocols and the project, was in charge of the analysis and discussion of immune response data and helped in drafting the manuscript. RFA and TMS helped in systematization of data concerning behavioral tests and analyzed and interpreted the cognitive data. GW proposed the statistical analyzes of the data and was responsible for it. DOS discussed the project since its conception and helped in designing the experiments. CTDR is responsible for conception and design of the study, and helped in data analysis, interpretation and drafting and finalizing the manuscript together with LPSV and FLRG. All authors read, reviewed and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

METHODS

Mice and Parasite. The “Instituto de Ciência e Tecnologia em Biomodelos” of the “Fundação Oswaldo Cruz” (ICTB- Fiocruz, Brazil) granted seven week-old C57BL/6 female mice weighing 20-25 g. Mice were conditioned housed in racks with an air filtration system in a room maintained at 25°C and light/dark cycles of 12 hours in cages containing five animals with free acquisition to food and water. All procedures were carried out in accordance with animal welfare approved by the Ethical Committee on the Use of Laboratory Animals of “Instituto Oswaldo Cruz” under CEUA-IOC: L-010/2015 concession. *Plasmodium berghei* ANKA (*Pb*A) infections were carried out using a stable transfected strain of *Pb*A expressing a green fluorescent protein (*Pb*A-GFP) generated as described previously[1].

Infection and treatment of experimental groups. C57BL/6 mice were infected intraperitoneally (ip) with 150 µl of *Pb*A-infected red blood cells, duly cryopreserved and thawed, being called "mice-passage." Five days after infection, the total blood was collected, adjusted to 1 x10^6 parasitized erythrocytes in 100 µl of PBS and injected in the peritoneum of C57BL/6 mice from the experimental groups. Parasitemia was monitored by flow cytometry, based on the percentage of GFP^+ erythrocytes. In this experimental model, the evolution to and establishment of cerebral malaria (CM) occur between the fifth and sixth day of infection[2]. In this study, mice were treated on the fourth day of infection (mean parasitaemia 2.5%) with 25 mg/kg of chloroquine (CQ) by gavage for seven days[3], before any clinical signs of CM. All groups were similarly manipulated on procedures. Experiments carried out with groups of uninfected mice treated with CQ or not (control group received PBS) have previously shown, that the CQ treatment did not influence the performance in behavioral tasks and anxiety phenotype[4].

Experimental Description. C57BL/6 mice were divided into groups of *Pb*A infected and Control animals (no infected) and both were treated with chloroquine (CQ) for seven days from the fourth day of infection. Thirteen days after treatment, mice from respective groups were subdivided into Non-Immunized and Immunized groups (Fig. 1). The following vaccines and antigens were used for immunization: Diphtheria and Tetanus toxoids (dT) vaccine for adults, Influenza vaccine, *Plasmodium falciparum* Merozoite Surface Protein 3 (pfMSP-3), White chicken egg ovalbumin (OVA) and Lipopolysaccharide of *Escherichia coli* (ecLPS). Three different immunization strategies were performed: a combination of all antigens and vaccines described above (from here now, called Pool); a combination of antigens and vaccines (Influenza vaccine and ecLPS) that trigger, preferentially, a type 1 pattern of immune response (from here now, denominated T1); and a combination of antigens and vaccines (dT vaccine, pfMSP-3 and OVA) that trigger, preferentially, a type 2 pattern of immune response (from here now, called T2). The groups of mice were denominated: Control (Non-infected/Non-Immunized); Pool, T1 and T2 (Non-infected/Immunized Pool, T1 or T2 of the immunization strategy); Inf (Infected/Non-immunized); and Inf-Pool, T1 or T2 (Infected/Immunized Pool, T1 or T2 of the immunogens inoculation strategy). Subsequently, mice behavioral performance was assessed by Open Field Test (OFT), Novel Object Recognition Test (NORT) and Light-Dark (L/D) (Fig. 2). About 300 mice were used for these experimental strategies in five consecutive sessions.

578 **Immune system stimuli.** The immune stimulus was initiated thirteen days after the
579 end of CQ treatment, being performed in the course of the following sixty days (Fig. 2).
580 Antigens and/or vaccines were administered by different routes, in different regions of
581 the animals's body (Table 1). The doses administrated were defined based on dose-
582 response protocols available in the literature capable of stimulating the murine immune
583 system without imparting risk of death to mice immunized[5-12].
584
585 **Immunization with Plasmodium falciparum Merozoite Surface Protein 3 (pfMSP-**
586 **3).** The mice were challenged with 10 µg of pfMSP-3 recombinant (in collaboration with
587 Clinical Trials of Malaria Vaccines (Vac4All Initiative), Paris, France) adsorbed on 70%
588 adjuvant solution MONTANIDE™ ISA 50 V2 W / O (SEPPIC. Air Liquide - Healthcare),
589 in 100 µl of PBS. Three subcutaneous injection were performed at the tail region with
590 a twenty-day interval between immunizations[9-10] (Fig. 2, Table 1).
591
592 **Immunization with Tetanus- Diphtheria and Influenza Vaccines.** The vaccines
593 used in this study were: bacterial double [(Tetanus-Diphtheria (dT): Biological E
594 Limited - BE, Telangana - India, Lot. 34005815. In collaboration with the Division of
595 Health Surveillance - CAP 3.1 of the "Fundação Oswaldo Cruz", Rio de Janeiro, Brazil]
596 and Trivalent Influenza (granted by the Technological Development and Production
597 Division of "Instituto Butantan, São Paulo, Brazil, Lot. 160034).
598
599 Mice received 100 µl (1/5 of the human dose) of dT and Influenza vaccines by
600 subcutaneous (dorsal region) and intramuscular (left quadriceps region), routes
601 respectively (Table 1). Three inoculations with a twenty-day interval between
602 immunizations were performed[5-6] (Figure 2).
603
604 **Immunization with Ovalbumin (allergen).** Mice received 50 µg of white chicken egg
605 ovalbumin (SIGMA-ALDERICH, Cod. A5503-50g) adsorbed onto aluminum hydroxide
606 [Al (OH) 3] in a final volume of 200 µl per animal in three inoculations. The first
607 inoculation was performed at the dorsal region by subcutaneous injection and the
608 following (second and third inoculation) by ip route (Table 1) with seven days between
609 them[11] (Fig. 2).
610
611 **Immunization with Lipopolysaccharide from *Escherichia coli (ecLPS)*.** Mice were
612 challenged with 0.1 mg/kg of ecLPS O111: B4 (SIGMA-ALDERICH, L2630-10MG, Lot
613 025M4040V12140701) diluted in phosphate-buffered saline (PBS). Two ip inoculations
614 were performed (Table 1) with a range of ten days between immunizations[12] (Fig. 2).
615
616 **Evaluation of the immunization efficacy.** Mice were randomly selected and
617 sacrificed for withdrawal of whole blood via cardiac puncture following stimulation of
618 the immune system (twenty days after the last immunization with pfMSP-3 and dT and
619 Influenza vaccines; six days after the last inoculation of Ovalbumin and two days after
620 the last dose of ecLPS), serum samples were preserved at -70 ° C. Total IgG against
621 *pf*MSP-3, Tetanus-Diphtheria toxoids (dT) and Influenza vaccine; the Th1, Th2 and
622 Th17 cytokine profile; lymphocyte subpopulations spleen; and intradermal test for the
623 response against Ovalbumin were evaluated.
624
625 The antibody response against *pf*MSP-3 recombinant and Influenza vaccine were
626 determinate by conventional Enzyme-Liked immunosorbent Assay – ELISA[9-10], and

627 the antibody response against dT vaccine was determined by Toxin Binding Inhibition
628 – ToBI[13].
629
630 **Evaluation of the immune response triggered by immunization.** Cytokine
631 production triggered by the Pool, T1 and T2 stimuli was evaluated in the serum of the
632 different mice groups. Cytokine profile was performed by Cytometric Bead Array
633 Th1/Th2/Th17 (CBA - BD Biosciences (cat# 560484). The data were collected and
634 analyzed using FacsCANTO flow cytometer (BD Biosciences) and FACScomp (BD),
635 respectively. Spleens from the animals of all groups were removed and homogenized
636 with a syringe plunger above 70-µm strainer, and red blood cells were eliminated by
637 using ACK lysing buffer (Sigma). The cell suspension was counted and
638 immunollabeled. For staining, cells were incubated with anti-Fc III/II (CD16/32)
639 receptor Ab (2.4G2), followed by surface staining with various combination of the
640 following antibodies for 30 min at 4°C in the dark: anti-CD8 (PE-Cy7, 53-6.7 clone, BD
641 Pharmingen), anti-CD3 (PerCPCy 5.5, 145-2c11 clone, BD Pharmingen), anti-CD4
642 (APC-H7, GK1.5 clone, BD Pharmingen), anti-B220 (APC, RA3-6B2 clone, BD
643 Pharmingen), anti-CD62L (BB515, MEL14 clone, BD Horizon), APC anti-CD44 (APC,
644 IM7 clone, BD Pharmingen) and Foxp3 (Alexa Flour, 647, R16715, BD Pharmingen).
645 Acquisition and data analyze by flow cytometry using BD FACSCanto™ and FlowJo
646 ®.
647
648 **Behavioral analysis.** Mice behavioral performance was assessed according to the
649 experimental schedule presented in Fig. 2. To note, mice was submitted to different
650 behavioral paradigms to evaluate exploratory and locomotor activity, cognitive abilities,
651 and parameters involved in anxiety-like behavioral at days twenty one, seven and three
652 days after *pf*MSP-3, Tetanus-Diphtheria and Influenza vaccine; Ovalbumin; LPS
653 inoculation, respectively, and seventy seven days after CQ treatment. The same co-
654 hort of mice were evaluated on OFT, NORT, Y-maze and L/D, respectively (Fig. 2). All
655 experiments were performed with incandescent light source of 200 lux of intensity in
656 the evening period. Animals were acclimatized in the experimental room at least for 2
657 hours before the experimental sessions. Behavioral performance of mice was recorded
658 by AnyMaze® software (Stoelting Co., Wood Dale, IL, USA) that captures the videos
659 and interpret exclusively the mice open field performance and only the locomotor
660 activity in the novel object recognition training and test sessions. A trained blind-to-
661 treatment researcher evaluate others behavioral parameters of each mouse
662 individually by video analysis. In all behavioral tests mice were individually placed on
663 the apparatus and monitored by a video camera (positioned above and at 90 ° of the
664 equipment) connected to a monitor to obtain images and data analyze. The apparatus
665 were properly cleaned with 70% alcohol and dried after each test.
666
667 ***Open Field Task (OFT).*** To address the effect of immune responses on locomotion
668 and on long-term habituation, mice were submitted to the open field task (OFT) with a
669 training and a test session 24 hours apart, as described elsewhere[4]. In each OFT
670 session [training (OFT1) and test, (OFT2)] mice was individually submitted to freely
671 explore a gray acrylic square box in (50 × 50 × 50 cm, length × width × height) for 10
672 minutes. In the training session it was evaluated the short-term habituation to novelty
673 by decreasing the distance travelled during the 1st to 3rd minute, since mice typically
674 exhibit less exploratory behavior during the first few minutes of testing in a familiar
675 open field arena; the locomotor activity (the distance travelled during the last 6 minutes

of the test); and anxiety-like behavior by the time and distance travelled in the center zone. In the test session (OFT2), we evaluate the first 3rd and the last 7th minutes total distance travelled. Finally, it was analyzed the long-term habituation to novelty, characterized by a significant decrease in the total distance travelled comparing the first 3 minutes of the OFT test (OFT2) with the OFT training (OFT1) sessions.

Novel Object Recognition Task (NORT).
To evaluate the recognition memory, the NORT was carried out in the same OFT apparatus, 24 hours after the OFT2 session[4]. NORT starts with a training session, in which mice are exposed to two familiar objects: FO1 and FO2. On the training session, a similar exploratory activity (of approximately 20 seconds) of mice for both familiar objects familiar, is expected[15]. The test session is performed 24 hours later. Mice are exposed to a New Object (NO) and to one of the previously exposed Familiar Objects (FO1 or FO2). NORT is based on the tendency of mice to differentiate a novel object (NO) from a familiar object (FO), thus exploring more the NO in detriment of the FO[4,15].

Animals were placed in the periphery of the apparatus with the FO and allowed to explore them 10 minutes. To the NORT test session, 24 h after the training session, the animals were placed back in the arena with one of the FO and the NO to measure the long-term recognition memory. The time spent exploring the objects were again evaluated for 10 min by a trained blind-to-treatment observer, and an exploration was only considered when the animal's nose or mouth was in contact with the objects. During both the training and the test phase, objects are located in opposite and symmetrical corners of the arena. In the training session both objects are novelty and time explored on both should be similar. Differently, a successful test session is indicated by a longer time spent on NO. The time exploration was expressed as percentage of time exploring each object. Animals that recognized the NO as such explored it for more than 50% of the total time.

Light/Dark Task. The light / dark task was performed as described by Almeida and collaborators[16] with minor modification to measure anxiety behavior-like phenotype[17]. The apparatus consist in a rectangular box (50 x 30 x 30 cm, height × length × width) in acrylic with two different sides (colored white and black), separated by a wall (5x5cm) with an opening at the level of the base of the apparatus joined both sides of the apparatus. A white 100W lamp, placed 60cm above the center of the apparatus, illuminated the white side of the apparatus, while the black side was kept closed without illumination. The mice were individually placed in the light compartment for free exploration of the apparatus for 5 minutes. The following behavioral parameters were analyzed: the time spent in the light compartment and the number of transitions between the compartments (light and dark).

Statistical analysis. All statistical analyses were performed using a statistical software package (Prism 5.0, GraphPad). The data were extracted from the AnyMaze® software. To analyze OFT and Light / Dark task were used absolute data. The time in each object in NORT was transformed into a percentage, from which the delta was extracted based on the subtraction: OF1 - OF2 (training session) and NO - FO (test session). The two-way ANOVA with Bonferroni correct were used to analyze OFT. The Student t-test with Mann-Whitney correction were used for to analyze the groups in

NORT, light-dark task and immune response. Data are presented as mean ± standard error. P <0.05 was considered statistically significant.

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LEGENDS FOR FIGURES AND TABLE

Fig. 1. **Behavioral analysis of healthy and *Pb*A-infected mice**. In the New Object Recognition Task (NORT), mice explored similarly the familiar objects in the training session: **a)** in healthy mice and **b)** in infected mice. In the test session, **c)** control (non-infected non-immunized mice) group showed increased new object exploration, as expected. Mice immunized with the Pool and T2 Ag showed increased new object exploration as compared to the Control and the T1 groups and **d)** infected mice showed decreased new object exploration in relation to the mice of the Control and Infected-Immunized groups. In the OFT1, **e)** all groups of healthy immunized mice walked less in the center of the arena when compared to the control group mice and **f),** the infected mice showed a decrease in the distance travelled in the center of the OFT1, as compared to the non-infected mice group. No difference was observed in the light / dark task between **g)** healthy mice groups. In contrast, **h**) *Pb*A-infected mice groups presented anxiety-like behavioral which was attenuated after immunization with the Pool and T2 immune strategies. Healthy mice groups (Control, n = 6; Pool, n = 8; T1, n = 8 and Control, n = 10; Pool, n = 10; T2, n = 10) and infected mice groups (Control, n = 6; Inf, n = 8; Inf-Pool, n = 6; Inf-T1, n = 6 and Control, n = 25; Inf, n = 17; Inf-Pool, n = 20; Inf-T2, n = 18). Data are mean and s.e.m. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; Unpaired t-test without and with Mann-Whitney test was used. For the NORT and OFT analysis, data are representative of five (Control, Pool and Inf-Pool groups), one (T1, T2 and Inf-T1 groups) and two (Inf-T2 group) independent experiments; and for the light-dark analysis, data are representative of four (Control, Pool and Inf-Pool groups), one (T1, T2 and Inf-T1 groups) and two (Inf-T2 group) independent experiments.

Extended data, Fig. 1. **Behavioral analysis of healthy and infected mice**. In the open field task (OFT), all groups showed a reduction in the distance travelled in the test session (OF2) when compared to the training session (OF1), **a)** healthy mice groups (Control, n = 6; Pool, n = 8; T1, n = 8 and Control, n = 10; Pool, n = 10; T2) and **b)** infected mice groups (Control, n = 6; Inf, n = 8; Inf-Pool, n = 6; Inf-T1, n = 6 and Control, n = 25; Inf, n = 17; Inf-Pool, n = 20; Inf-T2, n = 18). The (Pool and T1) immunized mice groups **a)** walked less on OFT1 when compared to non-immunized mice and the T2 immunized group showed a tendency (P = 0.0524) towards a decrease in the distance travelled. The same was observed in the infected and Pool and T2 immunized mice groups, **b)** but only when compared to non-infected / non-immunized mice group. Data are representative of five (Pool and Inf-Pool groups), one (T1, T2 and Inf-T1 groups) and two (Inf-T2 group) independent experiments. In the new object recognition task (NORT), all groups of mice explored similarly the familiar objects in the training session: **c)** healthy mice groups and **d)** Infected mice group. **e)** The control group (non-infected non-immunized mice) showed and increased new object exploration, as expected, and the Pool group showed a still increased new object exploration as compared to the Control group. The infected mice **f)** showed a decreased exploration of the new object, when compared to the control group mice; differently from the infected and Pool Immunized mice. Healthy mice group (Control, n = 22 ; Pool, n = 21) and infected mice group (Control, n = 22; Inf, n = 20; Inf-Pool, n = 21). Data are representative of two independent experiments for all groups. Data are mean and s.e.m. ***P < 0.001; **P < 0.01; *P < 0.05; Two-way ANOVA (a, b) and Unpaired t-test (c, d, e, f) was used.

Extended data, Fig. 2. **Spleen weight and size, and total number of spleen cells**. Immunized mice showed increases in the **a)** spleen weight and **b)** total number of splenocytes. **c)** Representative photograph of all studied groups of mice. Groups of infected mice showed a dark color attributed to hemozoin, even two and a half months after infection. All groups of immunized mice showed increased spleen sizes. Samples were collected after the behavioral evaluation. Five mice per group were analyzed. Data are mean and s.e.m. **P < 0.01; *P < 0.05; Unpaired t-test with Mann-Whitney test was used.

Extended data, Fig. 3. **Immune response against dT and influenza vaccines; pfMSP-3 and OVA**. Specific immune responses were triggered by : **a)** dT (n = 7 mice per group); **b)** Influenza vaccine (Control, n = 6; Pool, n = 6; T1, n = 3; Inf, n = 6; Inf-Pool, n = 6; Inf-T1, n = 3); **c)** *pf*MSP-3 (n = 5 mice per group) and **e)** OVA (Control, Pool and T2, n = 5 mice per group; Inf, Inf-Pool and Inf-T2, n = 4 mice per group), confirming the effectiveness of the immune stimuli. Mice were randomly chosen from the experiments, according to the type 1 and 2 immunization strategies, for the analysis of the immune responses. Samples were collected before the behavioral evaluation for analysis of responses against dT and influenza vaccines and were collected after behavior tests for the study of other parameters. Data are mean and s.e.m. **P < 0.01; *P < 0.05; Unpaired t-test with Mann-Whitney test was used.

Extended data, Fig. 4. **Flow cytometry analysis of B and T cell subpopulations in the spleen.** Healthy or infected and treated mice were immunized, or not, with Pool or T2 strategy and the populations of interest analyzed at the end of the cognitive behavioral tasks. **a)** representative gating strategy to identify the populations of B cells (B220+), CD4 T cells (CD3+CD4+), CD8 T cells (CD3+CD8+) and T regulatory cells (CD3+CD4+CD25+Foxp3+); frequency of **b)** B cells; **c)** CD4 T cells and **d)** CD8 T cells per spleen and e**)** frequency of Treg cells among the CD4 T cells. Five mice were randomly chosen per group, after behavior tests, for the analysis. Data are mean and s.e.m. $^{**}P < 0.01$; $^{*}P < 0.05$; Unpaired t-test with Mann-Whitney test was used.

Extended data, Fig. 5. **Analysis of different subsets of splenic CD4 and CD8 T cells.** Healthy or infected and treated mice were immunized, or not, with Pool or T2 strategy and populations of interest analyzed at the end of the cognitive behavioral tasks: **a)** representative gating strategy to identify the Naive (gate: 1; $CD44^{-}$ $CD64L^{+}$); Effector/Effector Memory (gate: 2; $CD44^{+}CD64L^{-}$) and Central Memory (gate: 3; $CD44^{+}CD64L^{-}$) subpopulations of CD4 and CD8 T cells; frequency of **b)** Naïve; **c)** Effector/Effector Memory and **d)** Central Memory subsets of CD4 T cells; frequency of **e)** Naïve; **f)** Effector/Effector Memory and **g)** Central Memory subsets of CD8 T cells. Five mice were randomly chosen per group, after behavior tests, for the analysis. Data are mean and s.e.m. $^{**}P < 0.01$; $^{*}P < 0.05$; Unpaired t-test with Mann-Whitney test was used.

Extended data, Fig. 6. Serum cytokine profile analysis. Mice immunized with Pool and T2 strategies presented increased serum levels of TNFα, IFNγ and IL-4 cytokine (**a, b, d**). IL-6 levels were only increased in **c)** infected-Pool and T1 immunized mice groups (Control, n = 5; Pool, n = 5; T1, n = 3; Inf, n = 5; Inf-Pool, n = 5; Inf-T1, n = 3 and Control, n = 3; Pool, n = 4; T2, n = 5; Inf, n = 5; Inf-Pool, n = 5; Inf-T2, n = 5). Data are representative of three (Control, Pool, Inf and Inf-Pool groups) and one (T1, T2, Inf-T1 and Inf-T2 groups) independent experiments. Samples were collected before (Control, Pool, Inf-Pool and Inf-T1) and after (Control, Pool, T2, Inf, Inf-Pool and Inf-T2) the behavioral evaluation. Data are mean and s.e.m. $^{**}P < 0.01$; $^{*}P < 0.05$; Unpaired t-test with Mann-Whitney test was used.

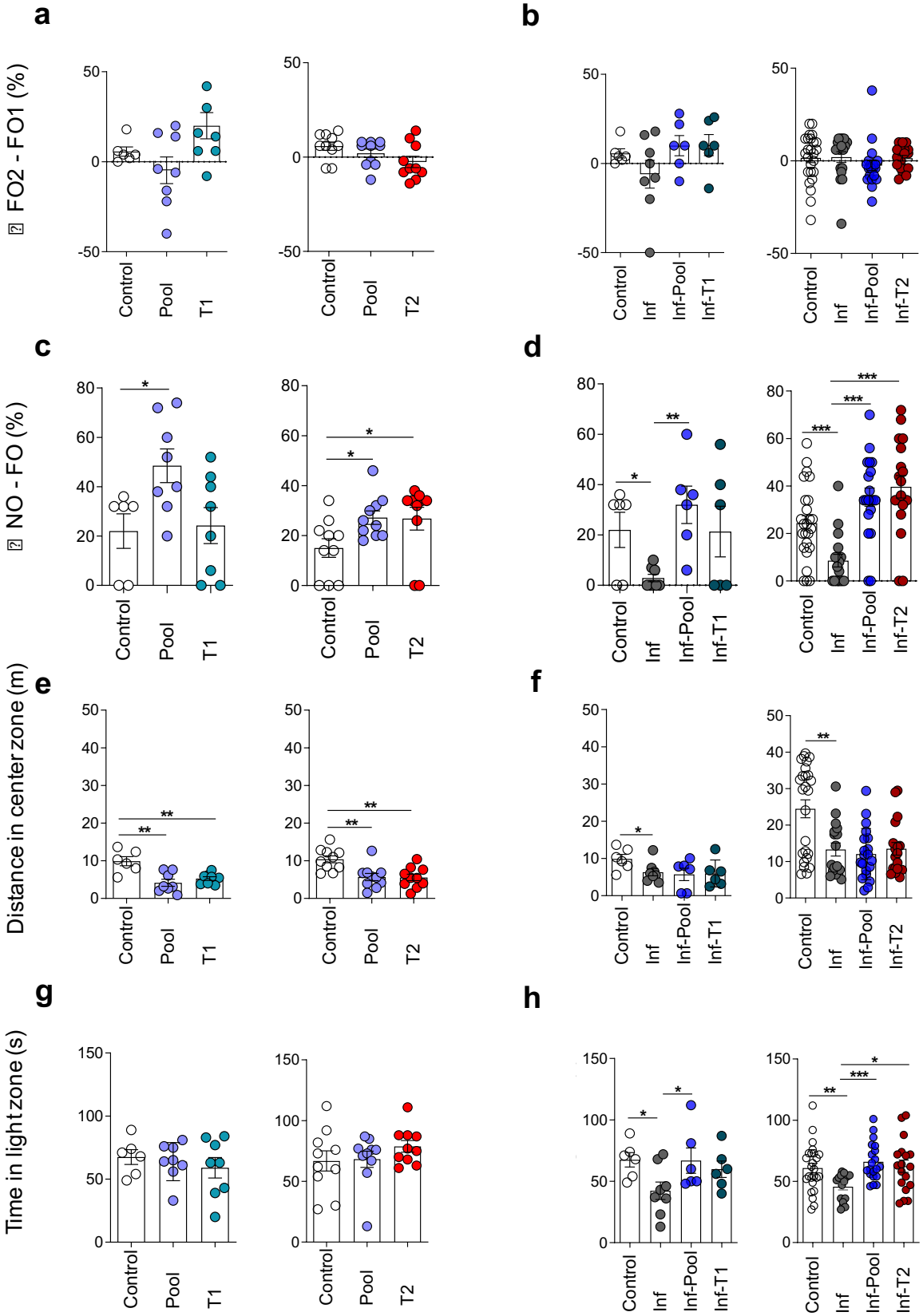
Fig. 1 Material & Methods. **Experimental draw**. Groups of mice, infected or not with *Plasmodium berghei* ANKA (*Pb*A), were treated with chloroquine (25 mg / kg) for seven days via gavage. After 14 days, they were subdivided into groups of immunized (B and D) or non-immunized (A and D) mice, according to the immunization strategies P (Pool), T1 (type 1 stimuli) and T2 (stimuli of type 2). Subsequently, mice were evaluated in behavioral tasks for locomotivity, memory and anxiety phenotype.

Fig. 2 Material & Methods. **Flow chart of immune stimuli and behavioral assessment**. After infection and treatment with CQ, mice were categorized as immunized or non-immunized, according to the immunization (P, T1 and T2)

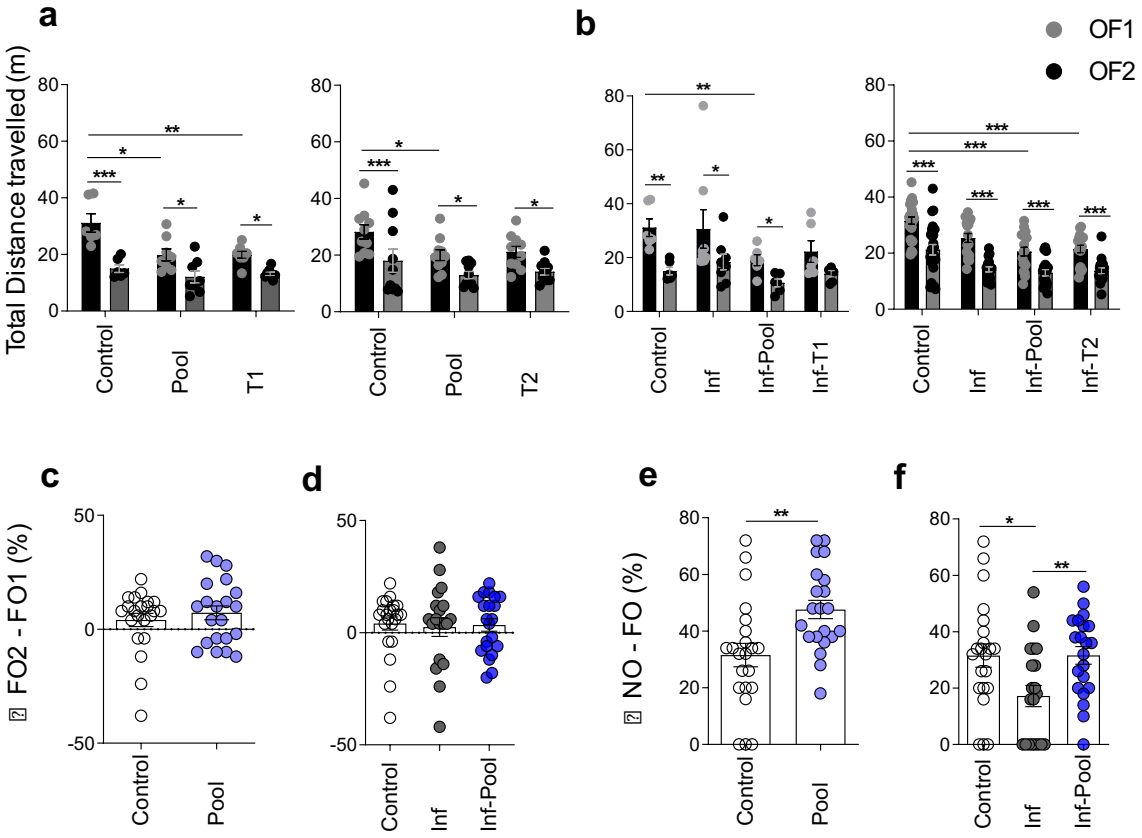
strategies used. The dT and Influenza vaccines and pfMSP-3 were inoculated at the same day, with three doses in different immunization pathways, with a 20-day interval between inoculations. OVA was inoculated after the third dose of dT and Influenza vaccines and pfMSP-3 protein was administered, three doses with a seven-day interval between the inoculations. The ecLPS was inoculated after the second dose of OVA, being two doses with an interval of ten days between them. Assessment of performance on behavioral tasks started 77 days after chloroquine treatment; 22 days after last inoculation of pfMSP-3 and dT and influenza vaccines; 7 days after last inoculation of Ovalbumin and 3 days after last inoculation with ecLPS. The open field was performed to measure locomotivity, habituation memory and anxiety phenotype, being performed in two sessions [training (OF1) and test (OF2)]. Then, the new object recognition task was performed to measure long-term memory, also in two sessions at consecutive days (training and testing). Finally, the anxious behavior phenotype was specifically evaluated by the light-dark test, performed in an unique session.

Table 1. Immune stimulus inoculation strategy: route, region, concentration, volume and number of injections of immunogens.

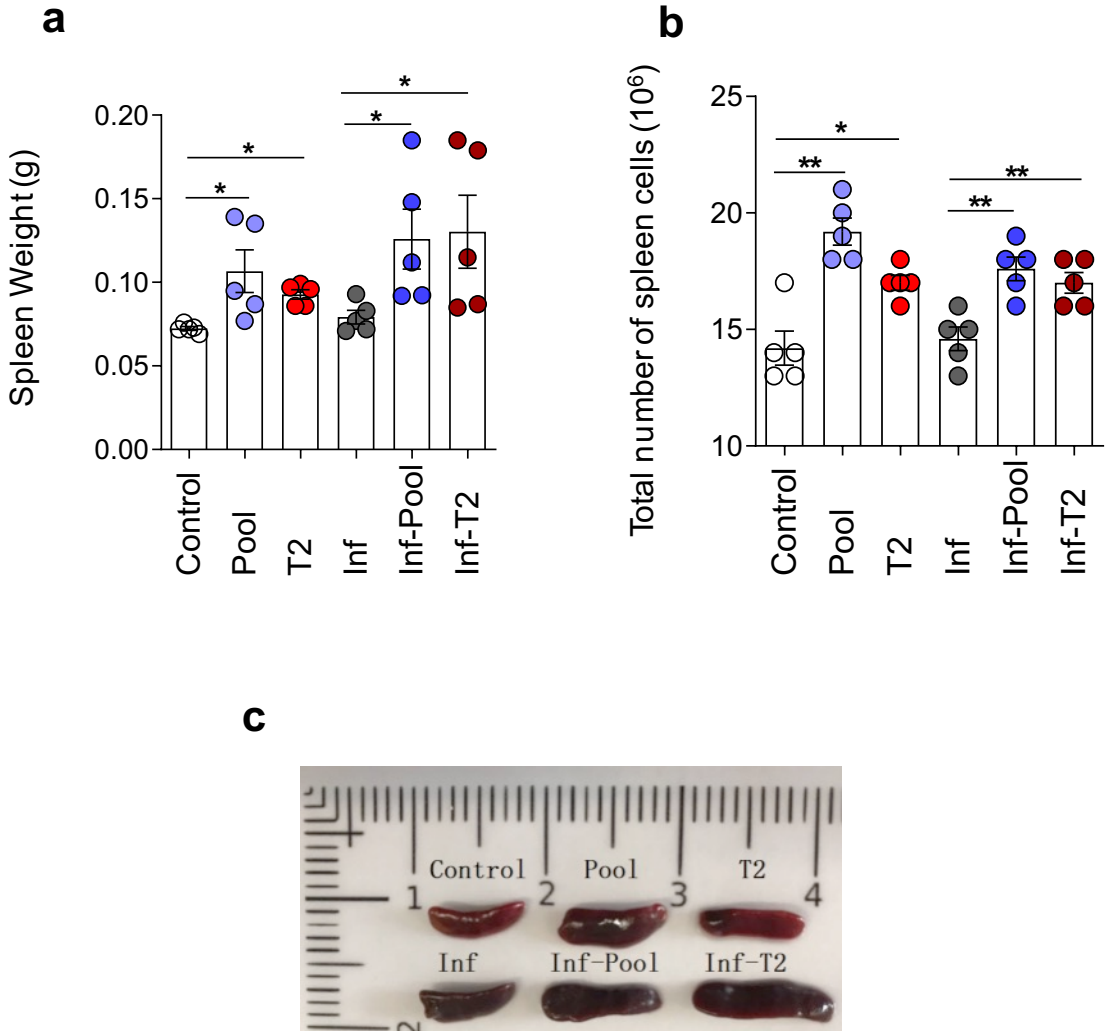
Figure 1


a
b
c
d
e
f
g
h
FO2 - FO1 (%)
NO - FO (%)
Distance in center zone (m)
Time in light zone (s)
Control
Pool
T1
T2
Inf
Inf-Pool
Inf-T1
Inf-T2

Extended data, Figure 1



Extended data, Figure 2



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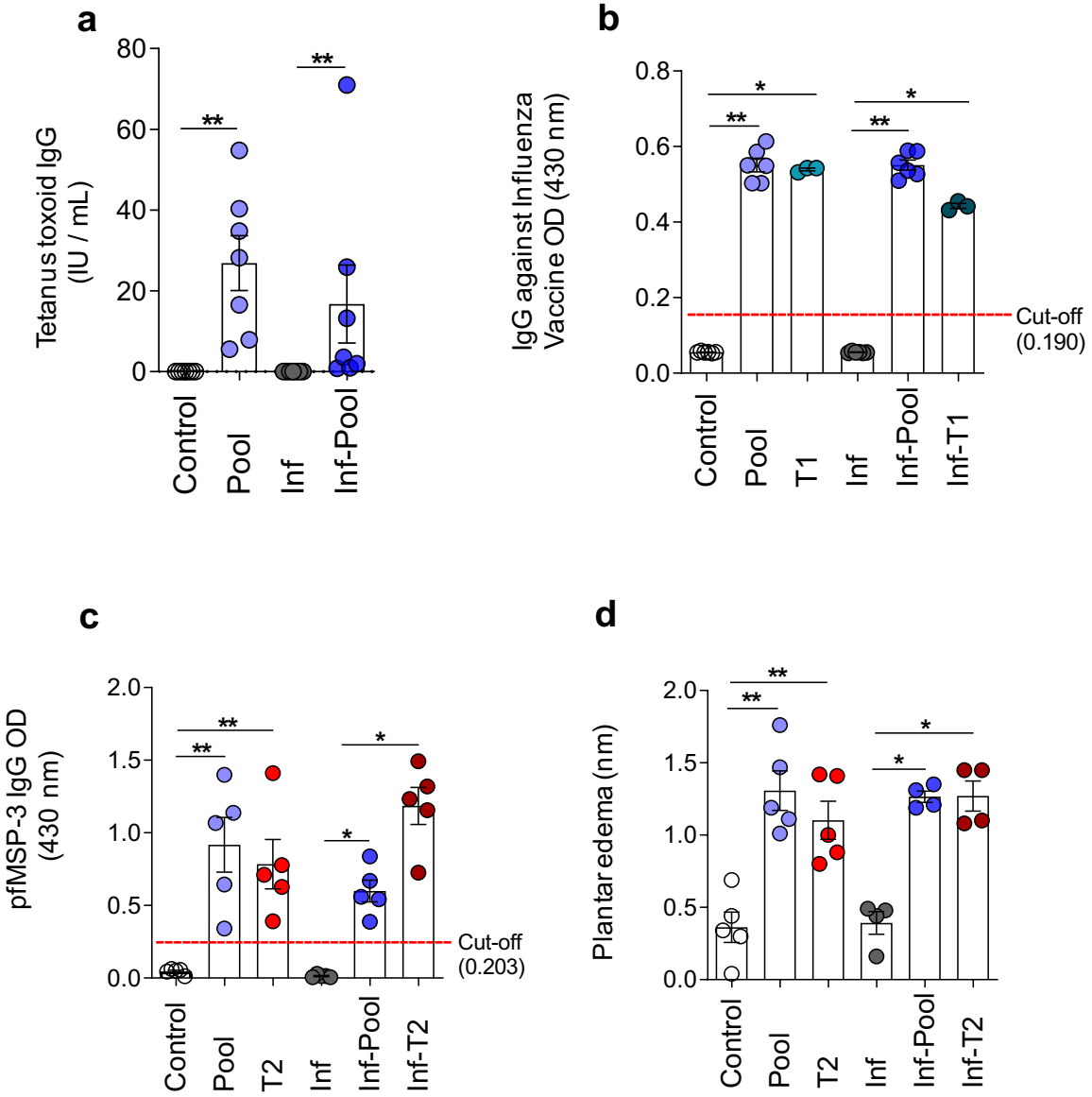

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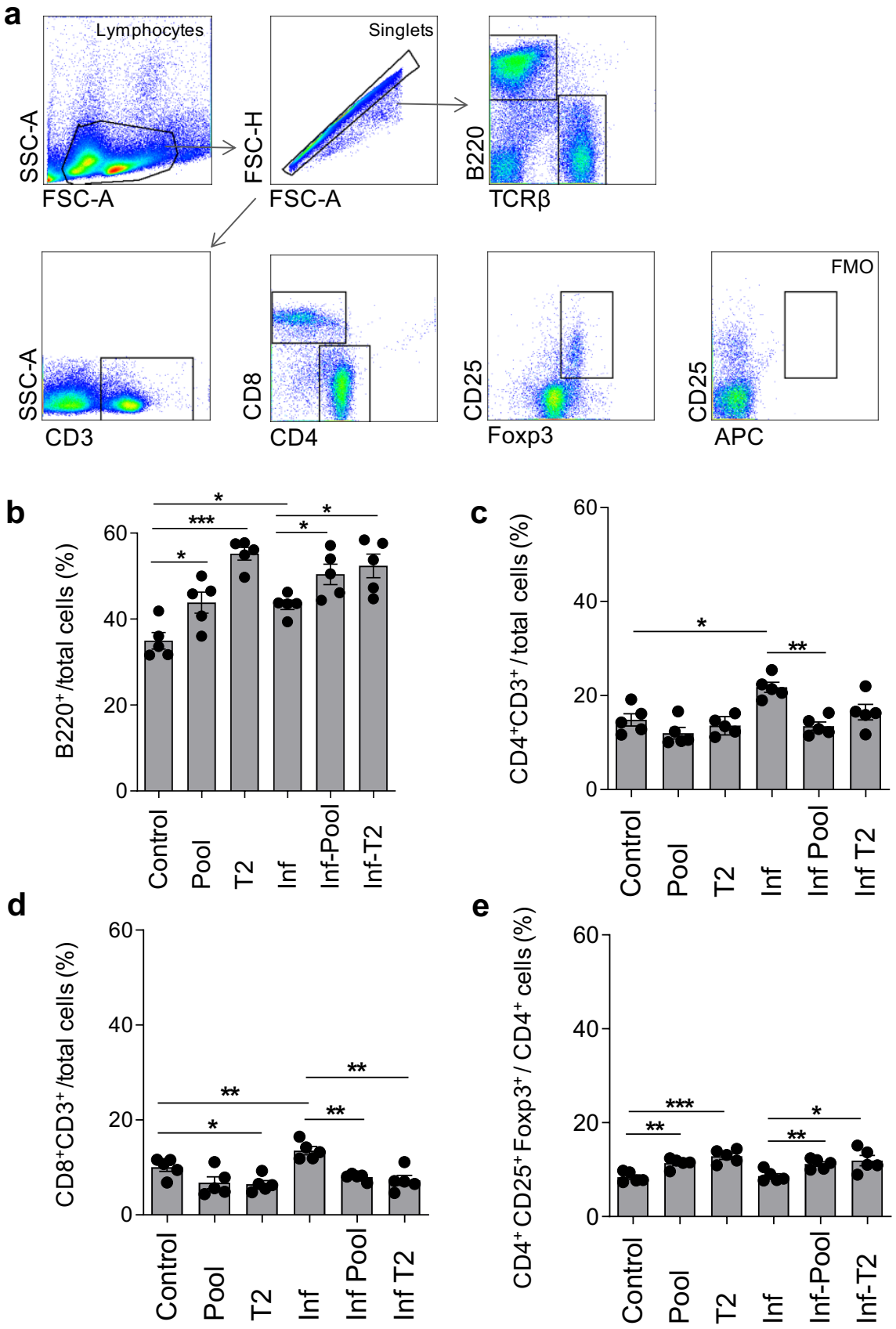

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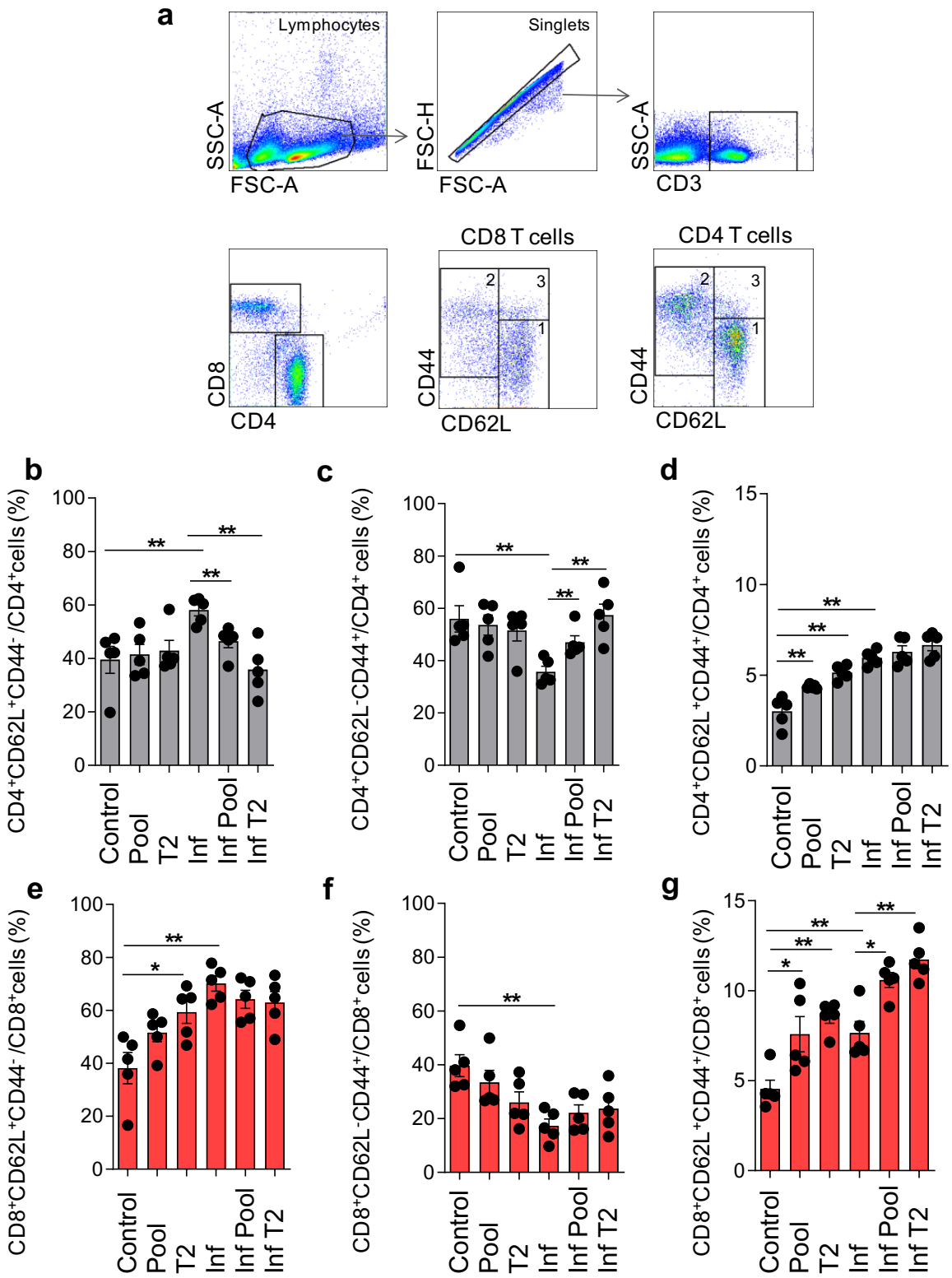

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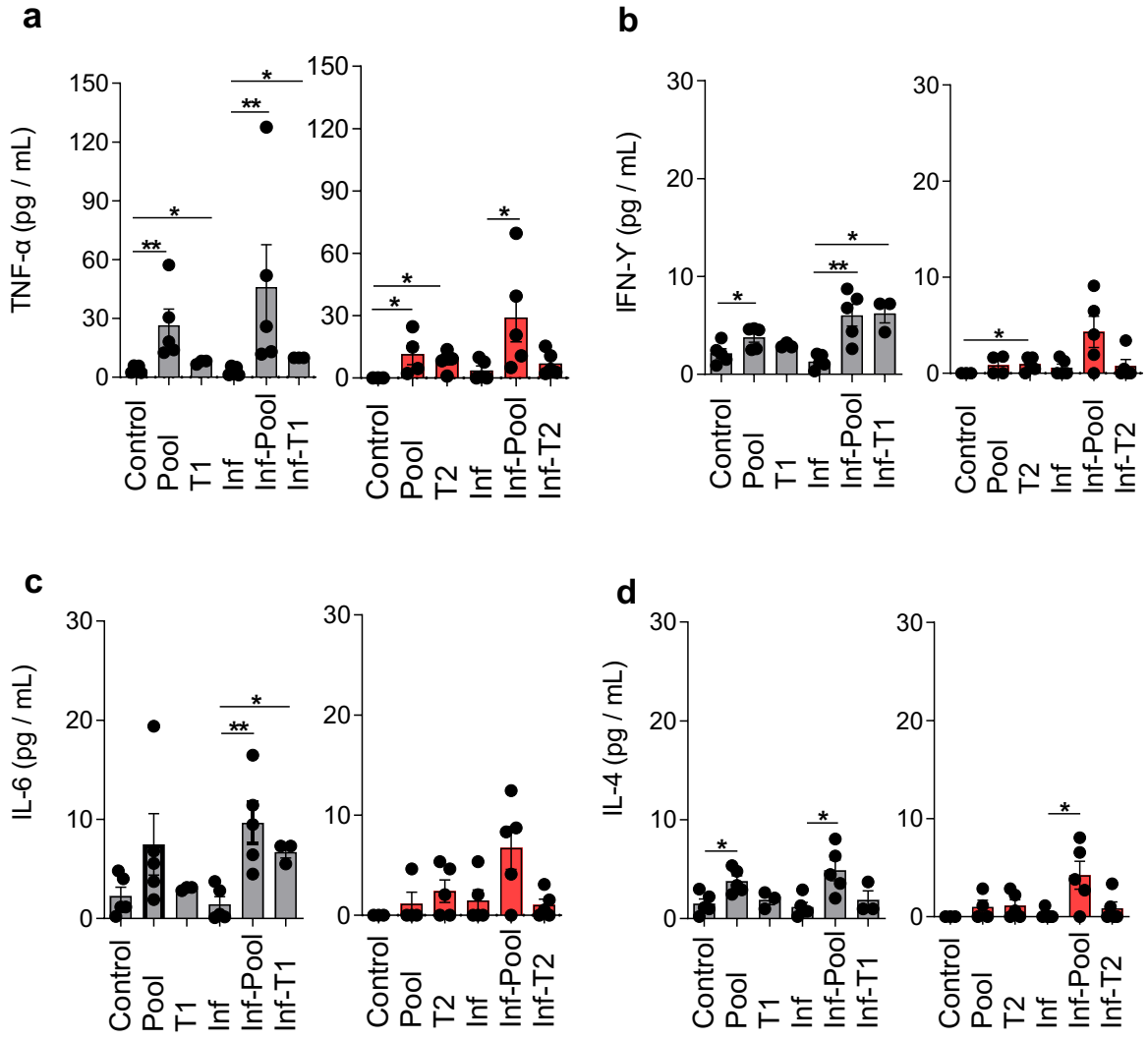
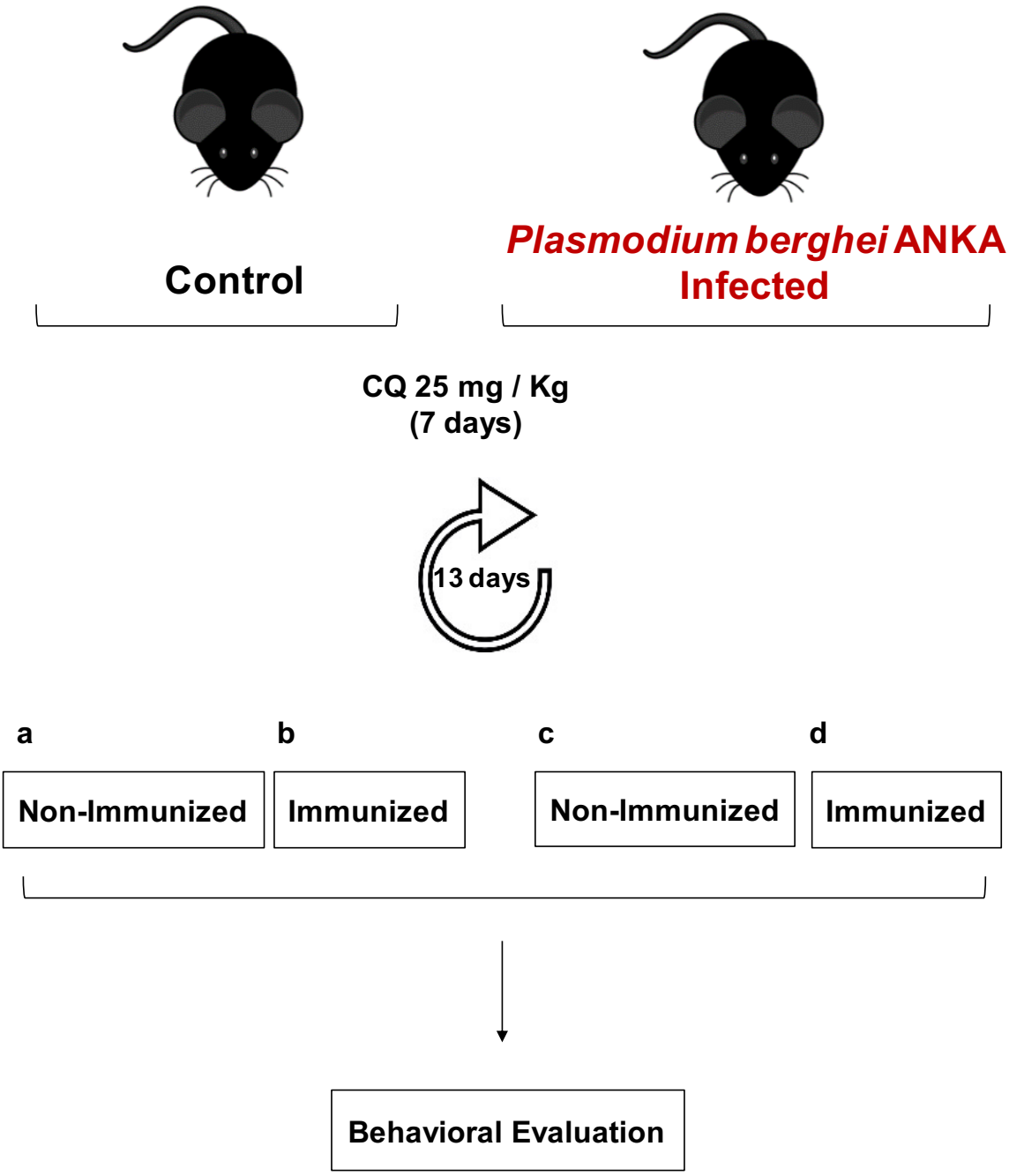
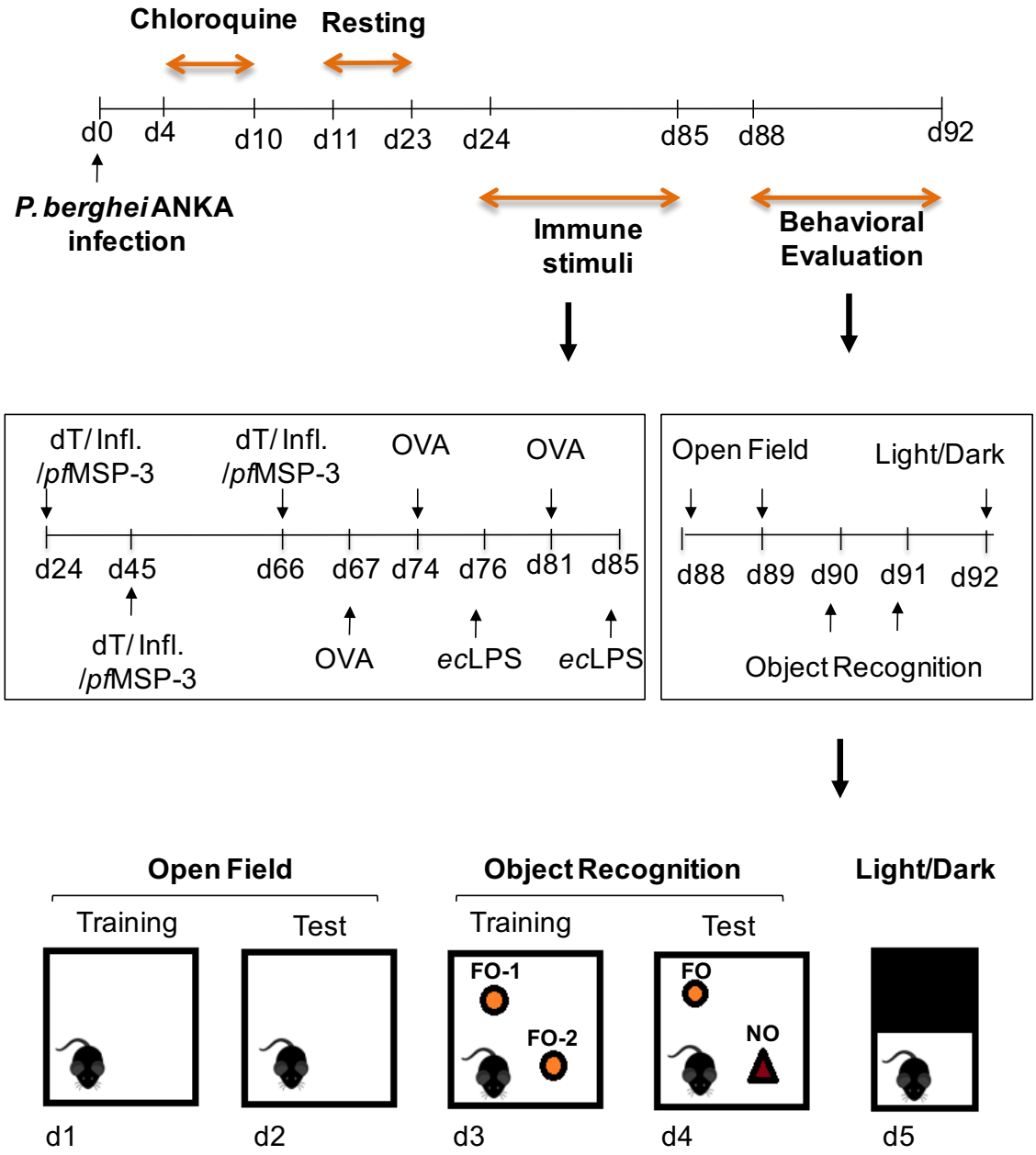


Material & Methods, Figure 1



Material & Methods, Figure 2



Material & Methods, Table I

Immune Stimuli	Route	Region	Concentration	Volume	Inoculation
dT vaccine	Subcutaneous	Back	1/5 human dose	100 µl	3
Influenza vaccine	Intramuscular	Quadriceps	1/5 human dose	100 µl	3
pfMSP-3	Subcutaneous	Base Tail	10 µg/mice	100 µl	3
ecLPS	Intraperitoneal	Abdomen	0,1 mg/kg	100 µl	2
Ovalbumin	Subcutaneous Intraperitoneal	Back and Abdomen	50 µg	200 µl	1 s.b.c. 2 i.p.