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**GJMR-G Classification :** NLMC Code: QW 70



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# Increased TNF Serum Levels are Related to Highly Aggressive Behavior in Male Swiss Webster Mice

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## 1. INTRODUCTION

In the field of animal behavior, various ethological studies on aggression have related the ontogenetic characteristics and the phylogenetic adaptability of this type of behavior (Yerkes 1913; Blanchard et al. 1988; Pellis & Pellis 1988). Since the 1960s, the behavior of mice (and other animal models) has been studied systematically in the laboratory environment (McKinney 1989); however, there is still no consensus regarding the causes of aggression in laboratory animals. There is a complex network of factors involving genetics, biochemistry, physiology and neuroanatomy that contribute to the occurrence of aggressive episodes among individuals of a particular group (Edwards et al. 1993, Liebenauer & Slotnick 1996, Brodtkin et al. 2003, Bray & Cotton 2003, Birger et al. 2003, Gleason et al. 2009).

Aggressive experimental models have been obtained through behavioral, pharmacological and

genetic manipulations (Brodtkin et al. 2002, Coccaro et al. 1997). However, such models are questioned for the following reasons: a) a small number of animals (often only two) is used, ignoring interactions with other individuals; b) aggression is induced directly, primarily by inducing aggressive territorial behavioral (e.g., test resident/intruder); c) only two hierarchical positions, the dominant (the aggressor) and the subordinate (the attacked), are determined; and d) the aggressive behavior is evaluated only for a short period of time (e.g., 5 minutes/test), thus preventing the assessment of the behavioral characteristics, such as those related to depression (depression-like) or anxiety (anxiety-like) disorders, inherent to each individual before the test, particularly during sexual maturation (Cryan & Holmes 2005).

The involvement of the immune response in aggressive animal behavior has not been well elucidated. There are interconnections between the genesis of psychiatric disorders and inflammation (Krishnadas & Cavanagh 2012). Most of this knowledge has arisen from an attempt to link specific illnesses (Major Depressive Disorder - MDD) with 'stress' biology, and the data raise the possibility of an 'initial common pathway' in which immune/inflammatory biomarkers and stress combine to cause changes in brain structure and function (Raison & Miller 2011). Consistent with the centrality of inflammation in this model system, in separate studies, elevated levels of interferon alpha (IFN- $\alpha$ ), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- $\alpha$ ) have been shown to correlate with depressive symptoms (Prather et al 2009, Raison et al. 2010). In recent years, several groups have reported that a positive relationship exists between proinflammatory cytokines and aggression/hostility (Zalcman & Siegel 2006). For example, Suarez and colleagues showed in healthy male subjects that the scores on the Buss-Perry Aggression Questionnaire, which measures hostility, anger, and aggression, were associated with the increased production of TNF- $\alpha$  (Suarez et al. 2002). A recent study identified a potent role for this cytokine in the regulation of aggressive and anxiety-related behaviors, and the data suggest that

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TNF receptor (TNF-R1 and TNF-R2) signaling tonically modulates activity in specific brain regions (Patel et al. 2010).

The main objective of our study was to evaluate the production of pro- and anti-inflammatory cytokines in highly aggressive mice in the laboratory environment. We believe it is essential to monitor the behavioral characteristics of each individual mouse from the time of weaning. In this context, we grouped the animals before sexual maturity assessed the activity profile of each animal using the tail suspension test (TST). The mice were regrouped based on the activity categories in adulthood. Thus, we developed and analyzed a model of spontaneous aggressiveness (MSA). The serum levels of TNF, IL-6 and IL-10 were determined and were found to be correlated with depression-like and anxiety-like disorders and the pattern of aggressive behavior.

## II. MATERIAL AND METHODS

### a) Mice

Male albino Swiss mice (3 weeks old) were maintained in our animal facilities at the Division of Animal Experimentation of the Cell Biology Laboratory, Instituto Oswaldo Cruz (SEA/LBC - IOC). They were adapted to the environment for one week in ventilated racks, and the temperature, humidity and photoperiod were controlled according to the standard environmental regulations. The animals were maintained under stable conditions of temperature and light, with a 12-h light/dark cycle, and both food and water were available *ad libitum*. Routine cleaning was performed twice per week. The procedures were performed under license number LW-5/12 of the Ethics Committee for the Use of Animals (CEUA/FIOCRUZ).

### b) Model of spontaneous aggressiveness (MSA)

The mice were separated into 10 groups (A1 to A10) of 10 mice each, and the mice in each group were individually identified (c1 to c10) (Scheme 1) (50 mice/assay). Three behavioral assessments—an ethological study, the TST and the Motor/Exploratory activity test (described below)—were used to assess all animals once per week between the 4th and 8th weeks of life. During the 10th week, the animals were regrouped into low, medium and high mobility groups, without directly induced aggression, based on the mobility profile defined by the TST. The same behavioral assessments were performed once per week between the 12th and 16th weeks of life.

### c) Behavioral Analysis

#### i. Ethological analysis

We recorded (top view) each group (4th, 6th, 8th, 12th, 14th and 16th weeks) for 60 continuous minutes using a Canon Power Shot SX20 IS® (Lake Success, New York, USA). A total of 3600 minutes of video was recorded, and the most representative

changes were recorded by photography. From these movies, we determined the variables to be evaluated in the ethogram: a) the pattern of aggressive behavior (PBA), including bites, wounds and injuries to an animal caused by fights among individuals in each group, and b) the qualitative and quantitative PBA intensities, determined by a scoring system. The scores were as follows: 0 (zero): the absence and/or presence of vocalizations and persecution, with no signs of bites or lesions on the animal's body; 1+: the presence of a low number of aggressive events, with or without sexual characteristics (attempted intercourse between individuals) and with the presence of small bites or injuries anywhere on the body; 2+: the presence of a low number of aggressive events without bites with sexual characteristics and discrete marks on the tail, back or scrotum; 3+: the presence of a moderate number of aggressive events and the observation of injuries and mild lesions on the tail, back and scrotum of the animals; and 4+: a high frequency (or intensity) of aggressive events (with or without specificity between individuals) and the presence of marked lesions and injuries on the tail, back and scrotum. In some cases, injuries of varying intensities were observed on other parts of the body, such as the chest, abdomen and forelegs (defensive injuries) (Oliveira 2012).

#### ii. Tail suspension test (TST)

Steru and collaborators (1985) introduced this methodology, which allows the determination of an animal's mobility in response to a stressful situation. This technique was developed and used for the preclinical evaluation of the efficacy of antidepressants and anxiolytics (Steru et al. 1985). In the TST, the animal is held vertically by the tail (final third) in a circular structure so that its muzzle is approximately twenty inches from the floor (Steru et al. 1985). We performed this test during the 4th, 6th and 8th weeks of life and after regrouping (12th, 14th and 16th weeks). We estimated the length of time that the animal remained motionless (immobile) during the five-minute test. Immobility was characterized by the absence of torsional movements, rotation and attempts to lift the body. We defined three categories based on the duration of immobility related to the animal's physical reaction to the stress challenge: a) hypoactive (Hypo) (low mobility or depression-like behavior), with a duration of 104 to 150 seconds, b) normal (Norm) (medium mobility), with a duration between 51 and 103 seconds, and c) hyperactive (Hyper) (high mobility or anxiety-like behavior), with a duration of 0 to 50 seconds (Oliveira 2012).

#### iii. Measurement of cytokine levels

TNF, IL-6 and IL-10 were detected in serum samples of mice using the BD CBA Mouse Soluble Protein Flex Set, according to the manufacturer instructions. The samples were acquired in a

FACScalibur flow cytometer (Becton Dickinson, USA) and data analysis were performed using The CBA analysis FCAP software (Becton Dickinson, USA).

#### iv. *Statistical Analysis*

The Mann-Whitney non-parametric test was used to compare the two groups (SPSS software, version 8.0). Values correspond to the means  $\pm$  standard deviations of three independent experiments.

### III. RESULTS

Our results clearly demonstrate the effectiveness of the MSA in the analyzing of aggressive behavior in Swiss Webster mice. The intensity of aggression was directly related to the profile for the TST (Fig. 1). During the weeks studied, we did not observe alterations in the individual results of the TST for the Hyper and Norm categories, but there were individual modifications in the Hypo group. The individual TST profile of this group consisted of 50% low, 40% high and 10% median mobility mice (Fig. 1A) during the 16th week of life. Regarding the ethogram results, regrouped animals with low mobility (Hypo) showed PBAs with 2+ relative scores (Fig. 1B). The animals with median mobility (Norm) had scores of 0/1+, with the lowest level of aggressive behavior (Fig. 1C). These data were the opposite of those for the high mobility (Hyper) group, which exhibited increased aggressive behavior, with relative PBA scores of 4+ (Fig. 1D). We also observed that the injuries from fights occurred in specific regions and had specific physical characteristics. Injuries to the front legs, chest and muzzle regions were the result of individual confrontations and defensive actions (Fig. 1E). It is important to note that during the grouping between the 4th and 8th weeks of life, no PBA was observed in any group studied (score = 0).

We measured the production of pro- and anti-inflammatory cytokines in various groups of animals (Fig. 2). Increases in the mean TNF serum levels (Fig. 2A) were observed in the Hypo (13.3 pg/ml) and Hyper (19.6 pg/ml) groups. Norm mice had very low levels of TNF (3.5 pg/ml). Furthermore, there was a greater difference in the values between individuals. The average deviations of the groups were as follows: Hypo:  $13.3 \pm 11.0$ ; Norm:  $3.5 \pm 0.31$ ; and Hyper:  $19.6 \pm 13.8$ . The high value of the deviation was related to the individual PBA scores (Table 1). The occurrence of moderate or high-frequency (or intensity) aggressive events (with or without specificity between individuals) and the presence of marked lesions and injuries to the tail, back and scrotum were directly linked to the high levels of TNF (e.g., HypoC1: 34.2/4+; NormC7: 4.8/0; and HyperC3: 40.8/4+; TNF level/PBA score). In addition, the increase in the group's average value (Hypo - 13.3/2; Norm 3.5/0 and Hyper - 19.6/3, respectively; TNF level/PBA score) was directly related to the increase in the PBA score. The IL-6 level did not

differ significantly between groups (Fig. 2B). However, there was a subtle trend of higher levels for the Hypo and Hyper groups: Hypo:  $12.0 \pm 0.5$ ; Norm:  $11.5 \pm 0.5$ ; and Hyper:  $12.7 \pm 0.5$  pg/ml. In addition, there were no significant differences in the IL-10 between the animals with different mobility profiles (Fig. 2C). However, there was a non-significant increase in the level of this cytokine in the Hypo group ( $6.3 \pm 0.1$  pg/ml) relative to the others (Norm:  $5.6 \pm 0.1$  pg/ml and Hyper:  $5.5 \pm 0.1$ ).

### IV. DISCUSSION

We question the relevance of experimental models in which aggressive events are triggered by pharmacological or genetic manipulations for the study of aggression in mice (Steimer 2011, Oliveira 2012). Three relevant characteristics of the MSA are as follows: (i) individual/social behaviors are evaluated, and the same animal is monitored from the time of weaning through adulthood; ii) animals are separated into categories based on mobility (or activity), and the individuals most likely to be aggressive are selected; and iii) group formation, interactions between individuals and the hierarchy structure can be observed. In the standard methodologies, a pair of animals is used, in which case it is possible to identify only the aggressor/attacked relationship or the hierarchy defined as dominant/submissive at the time of the observation (5 minutes) (Ensminger & Crowley 2007).

Our results demonstrate that in the Norm and Hyper groups, the TST mobility profile remained constant. Furthermore, it was clearly observed that male Swiss Webster mice with anxiety-like behaviors are highly aggressive when regrouped in adulthood. Hypotheses regarding territorialism, disputes over females and access to food as the causes of such behavior do not satisfactorily explain the intensity of aggression (Benton et al. 1980, Kareen & Barnard 1986, Drickamer 2001, Weidt et al. 2008). The factors associated with the anxiety state impair the ability to establish a linear hierarchy (Oliveira 2012). Thus, the maintenance of leadership (or dominance) is imposed by increasing the number and severity of aggression behaviors. Furthermore, the severity of the lesions appears to be related to the individual's defensive ability. Animals with defensive behaviors had bites located on the front legs, chest and muzzle.

Regarding the cytokine production, our results suggest that mice with anxiety-like behaviors and highly aggressive mice have higher serum levels of TNF and a tendency to have lower levels of IL-10. We emphasize that in mice with depression-like behaviors, increases in the mean TNF and IL-6 levels were also observed in aggressive animals. Raison & Miller (2011) and Krishnadas & Cavanagh (2012) provided data that support two opposing explanations for this phenomenon: first, that individuals with increased

inflammation comprise a physiologically discrete depressive/anxiety subtype, and second, that because inflammatory pathways are fully integrated into larger mind-body systems that have evolved to cope with environmental danger, it may be the case that even low/higher levels of inflammatory stimulation may be depressogenic/anxiogenic in individuals with vulnerability patterns in the nonimmune elements of these larger systems (Raison & Miller 2011, Krishnadas & Cavanagh 2012),.

Furthermore, we believe that the TNF/PBA correlation in animals is related to secondary infection by microorganisms after injury, resulting in increased levels of bacterial endotoxin and/or lipopolysaccharide (LPS), which is a potent hypothalamic-pituitary-adrenal (HPA) activator and elicits marked sickness behavior (Dantzer et al. 1998, Yirmiya 1996, Dantzer 2001). When LPS is systemically administered, Toll-like receptor-4 (TLR-4) on immune cells is activated, inducing the release of IL-1b along with a cascade of other cytokines, such as IL-6 and TNF (Laflamme & Rivest 2001, Kosnan et al. 2002, Rivest 2003). These cytokines are thought to influence central neuronal processes, thus promoting behavioral changes (Dantzer 2001, Kosnan et al. 2002, Kelley et al. 2003), including changes in the level of aggressiveness.

## V. CONCLUSIONS

We believe that the cause of aggression is related to a complex and integrated network of genetic, behavioral, neurological and biochemical factors. In Swiss Webster mice, increased aggressive behavior is most likely related to maternal care disruptions, an anxiety-like profile and neuroendocrine deregulation. Our results suggest that TNF production is directly related to highly aggressive behavior in mice, especially in mice with the Hyper mobility profile. Moreover, due to the severity of the lesions promoted by bites and fights, there is a significant increase in the levels of cytokines, which clearly compromises the animal's welfare and the reliability of the obtained data.

## VI. ACKNOWLEDGMENTS

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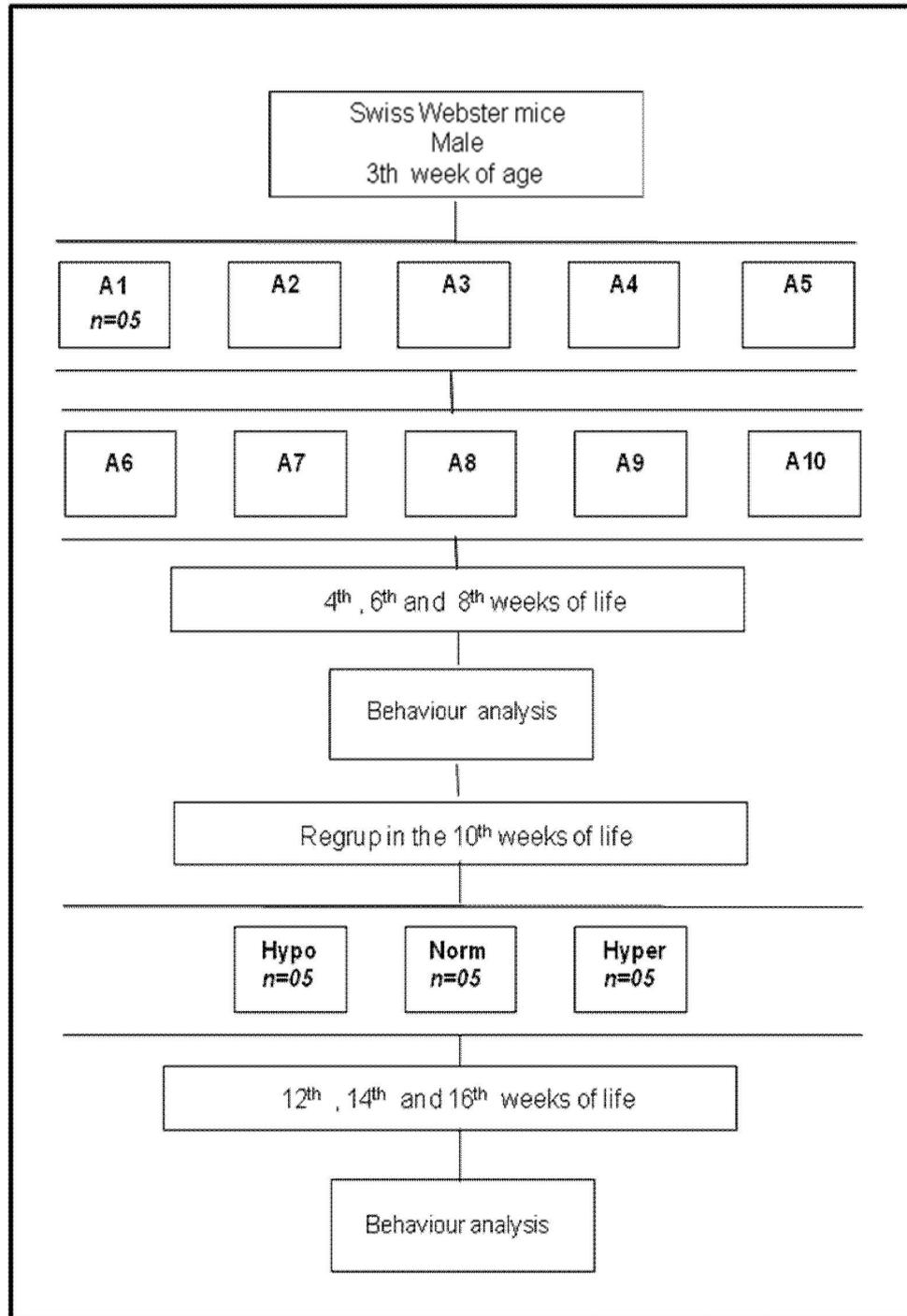
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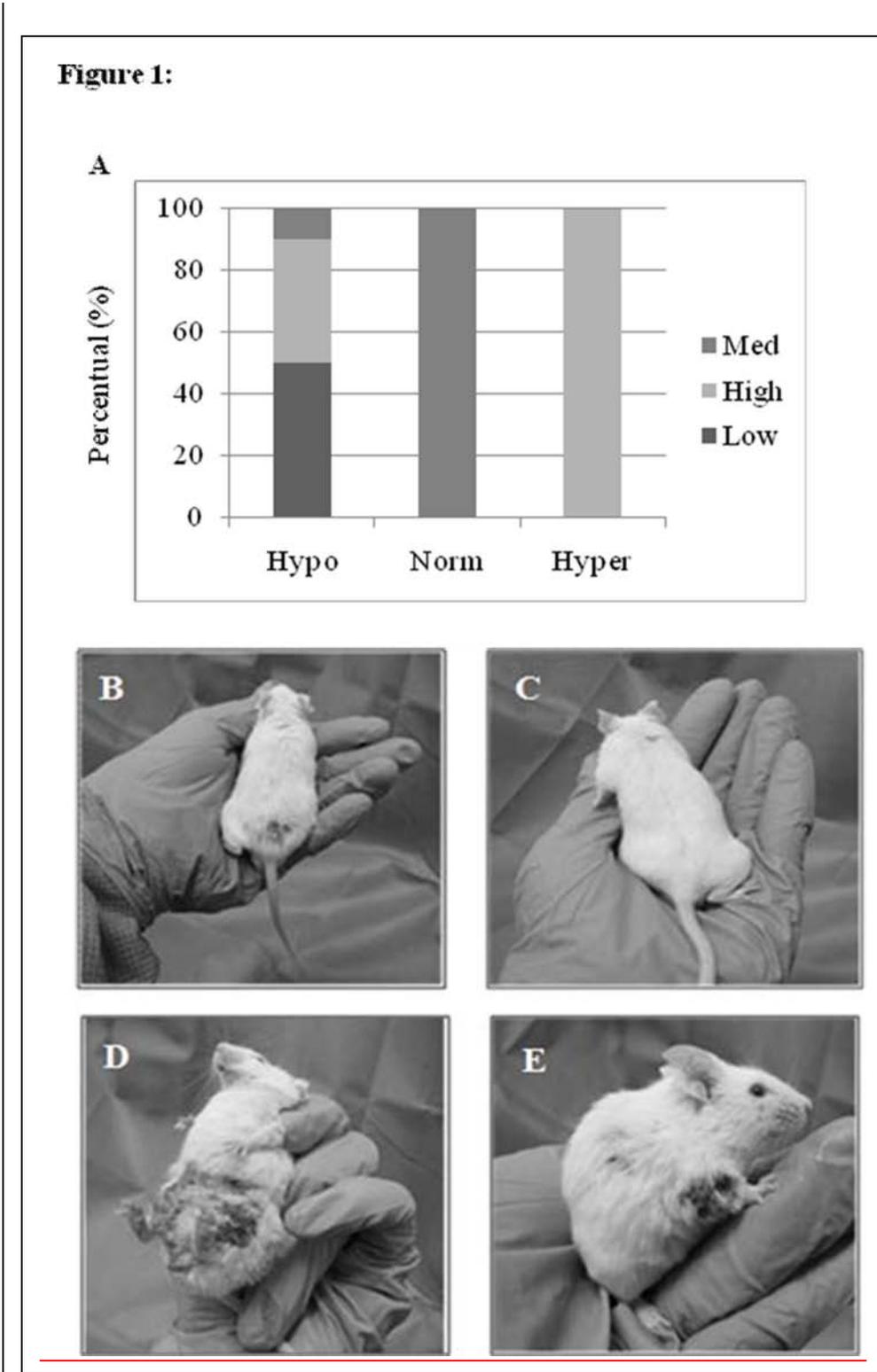
LEGENDS TO FIGURES

*Scheme 1 : Structure and development of the MSA:* Three-week-old male Swiss Webster mice were separated into 10 groups (A1 to A10) with 10 animals per group. During the 4th, 6th and 8th weeks, each animal was monitored by an ethological analysis and assessed using the TST. During the 10th week, the animals were regrouped (10 mice/cage) into the following categories based on the TST mobility profile: Hypo (low), Norm (medium) and Hyper (high mobility). The behavioral tests (ethological analysis and TST) were repeated in the 12th, 14th and 16th weeks. In the 16th week, the animals were euthanized, and blood samples were collected for the quantification of cytokines

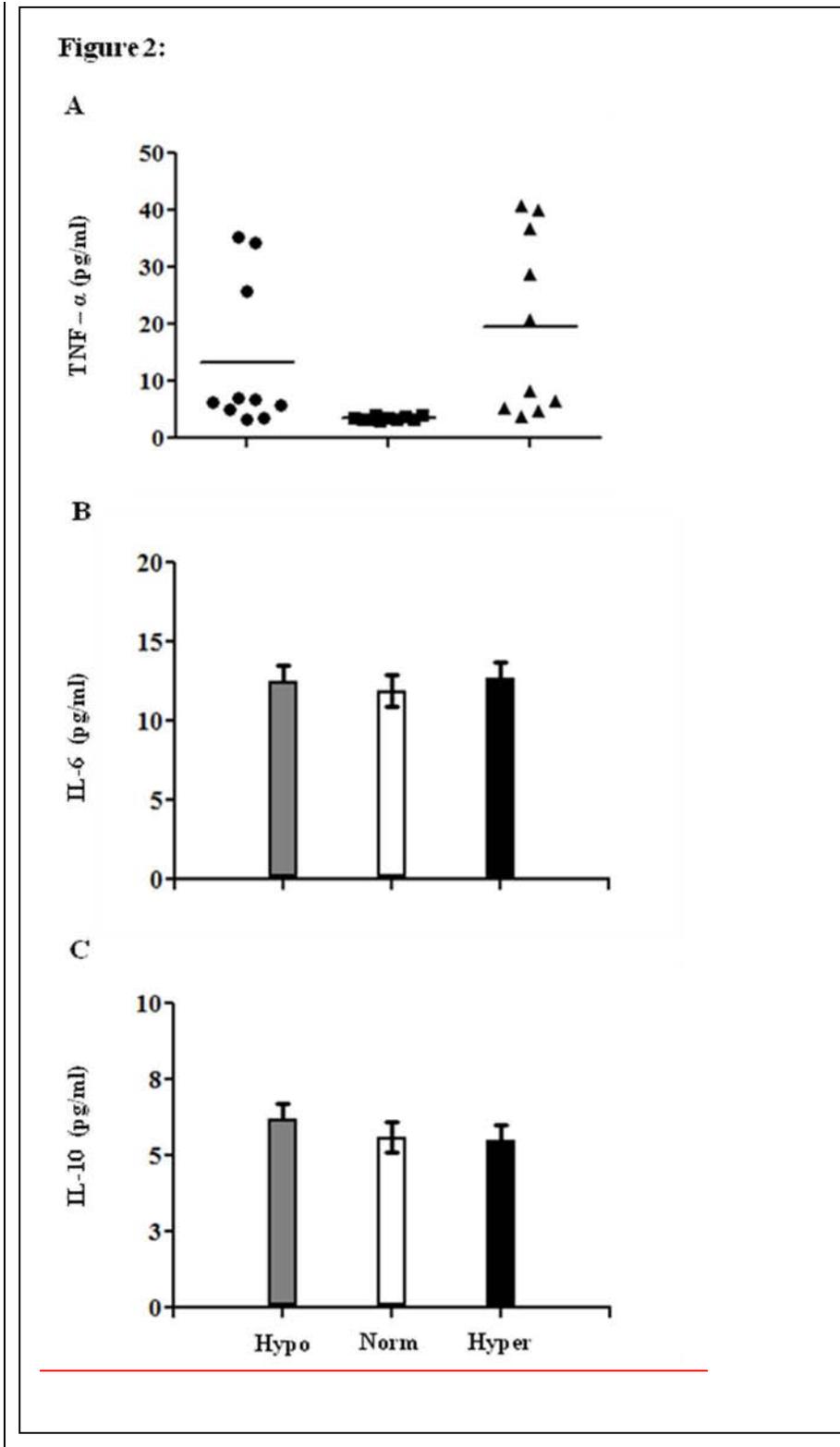
**Scheme 1:**



*Figure 1* : Categorization based on the TST results and the PBA score: (A) During the 10th week, the animals were regrouped based on the mobility profile defined by the TST: Low-*Hypo* (dark gray), Medium-*Norm* (gray), and High-*Hyper* (light brow). At the 16th week, the *Hypo* group consisted of 50% animals with low mobility, 40% animals with medium mobility and 10% animals with high mobility. The injuries associated with the different PBA scores are illustrated: (B) *Hypo* animal with a score of 2+; (C) *Norm* animal with a score of 1+ and (D) *Hyper* animal with a score of 4+ (D). Figure 3E shows injuries sustained during defense against aggression



**Figure 2 : Cytokine production:** When the mice were 16 weeks of age, the pro- and anti-inflammatory profiles of the humoral immune response were evaluated. (A) The TNF levels (pg/ml) in Hypo (circle), Norm (square) and Hyper (triangle) mice. The horizontal line indicates the average value for each group. The IL-6 (pg/ml) (B) and IL-10 (pg/ml) (C) were measured in the Hypo (gray bar), Norm (white bar) and Hyper (black bar) groups, and no significant differences were observed between the groups. Values correspond to the means  $\pm$  standard deviations of three independent experiments



*Table 1* : Correlation between TNF levels and PBA score

Mice	TNF- $\alpha$ (pg/ml)	PBA (+)	Mice	TNF- $\alpha$ (pg/ml)	PBA (+)	Mice	TNF- $\alpha$ (pg/ml)	PBA (+)
HypoC1	34.2	4	NormC1	4.2	0	HyperC1	8.2	2
HypoC2	25.9	3	NormC2	4.5	1	HyperC2	36.9	3
HypoC3	35.3	3	NormC3	3.9	0	HyperC3	40.8	4
HypoC4	5.0	0	NormC4	4.0	0	HyperC4	20.9	3
HypoC5	6.9	1	NormC5	5.2	0	HyperC5	28.7	3
HypoC6	6.2	1	NormC6	6.2	1	HyperC6	40.1	4
HypoC7	5.9	1	NormC7	4.8	0	HyperC7	5.3	1
HypoC8	3.5	0	NormC8	5.9	0	HyperC8	6.6	2
HypoC9	3.3	0	NormC9	5.5	0	HyperC9	3.8	2
HypoC10	7.0	2	NormC10	4.0	0	HyperC10	4.7	0
AVG	13.3	2.0	AVG	3.5	0	AVG	19.6	3.0

*AVG: Average number/group*