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Research Report

Characterization of Fyn signaling on the age-dependent immuno-modulation on traumatic rats

*Sheng Xiao, Jun Wang, Jianwei Jiang, Xiaoding Cao, Gencheng Wu, Hui Zhao**

Department of Integrative Medicine and Neurobiology, National Key Lab of Medical Neurobiology, Institute of Brain Research Sciences, Fudan University, 138# Yixueyuan Rd. Box 291, Shanghai, 200032, PR China

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ABSTRACT

Traumatic stress is well characterized to develop immuno-depression in our previous report. Here, we provide evidence that adult and aged rats showed similar decrease in lymphocyte proliferation and natural killer (NK) cell activity. However, compared with beginning recovering from traumatic stress after 3 day and fully recovered by 7 day in adult rats, aged rats begin the recovery phage later than 3 day and do not fully recovered by 7 day. In parallel, Fyn expression in cerebral cortex was augmented with the highest level at 3 day of trauma in both age groups of rats, although aged rats exhibited lower level than the younger cohorts. Immune consequences were consequently modified by intracerebroventricular (icv) injection of Fyn antibody or recombinant adenovirus expressing active Fyn. Finally, the increase in Fyn expression was converged on ERK1/2 (extracellular signal regulated kinase 1/2) activation. Taken together, the data indicated that immunological processes in response to traumatic stress was age dependent, Fyn-ERK1/2 signal pathway was required to convey the recovery signals.

1. Introduction

It is well defined that severe stress induced by surgical trauma resulted in immuno-suppression, such as decreased splenocyte proliferation to concanavalin A (Du et al., 1998), depressed antigen production (Makarenkova et al., 2006), impaired microbial activity (Jori et al., 2006), decreased natural killer cell activity (Huang et al., 2002) and reduced production of a number of cytokines (Zhao et al., 2001; Bartfai et al., 2007). It was also reported in adult rats that traumatic stress induced immuno-depression reached the highest effects at 1 day of trauma, which began recovering after 3 day and fully recovered by 7 day of stimulation (Du et al., 1998).

Whereas, the question is whether these host priorities is normally adaptive in aged subjects? Aging was recently proposed to prime immune cells and exhibited a persistent neuro-inflammatory state (Streit, 2006). Also, aged mice exhibited acute bouts of impairment in the self-care behaviors, which ultimately leads to exaggerated responses than the younger cohorts, for example, the discordant responses to lypopolysacharride (LPS) injection (Chen et al., 2008) or morphine tolerance (Wang et al., 2005). Based on our previous finding, the disturbance of central responses by the traumatic stress might represent a key element in the changes in the immune function. Therefore, it can be reasoned that certain age-dependent brain responses may strongly favor the presence of discordant immunological behavior between adult and aged subjects.

Fyn, a member of the Src-family protein tyrosine kinase is highly expressed in the central nervous system (Goto et al.,

* *Corresponding author*. Fax: +86 21 54237023.
E-mail address: mayzhao5375@yahoo.com (H. Zhao).

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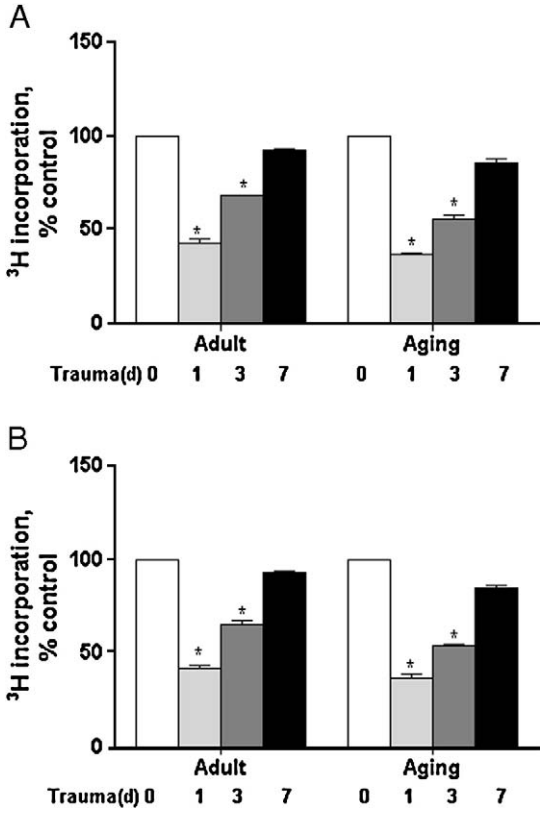


Fig. 1 – Discordant immuno-depression induced by traumatic stress in adult and aged rats. Rats were undergone traumatic stress, 1, 3 and 7 days later spleen cells were isolated, lymphocyte proliferation (A) and NK cell activity (B) were determined by [^{3}H]thymidine incorporation as described in Experimental procedures. Results are presented as means ± SD (n = 5). $^*p < 0.05$ *vs* Control.

2008). Substantial evidence suggested that Fyn plays an important role in building neuronal networks and determining the pattern of behavior (Seiwa et al., 2007). Rather, Fyn-deficient mice have severe defects in cortical structure (Garg et al., 2007), and consequently, with a progressive maladaptive to the age-associated remodeling neuronal circuits (Fukazawa et al., 2006). Src-family protein tyrosine kinases are believed to be involved in the modification of ERK1/2, which may be transmitting and aggravating various information from the cell surface to the nucleus and promoting an array of cellular responses including senescent-related alterations (Godeny and Sayeski, 2006; Xing et al., 2008). We therefore propose that Fyn and subsequent ERK1/2 activation, may preferentially involve in the traumatic stress induced immune consequences.

2. Results

2.1. *Discordant immuno-depression induced by traumatic stress in adult and aged rats*

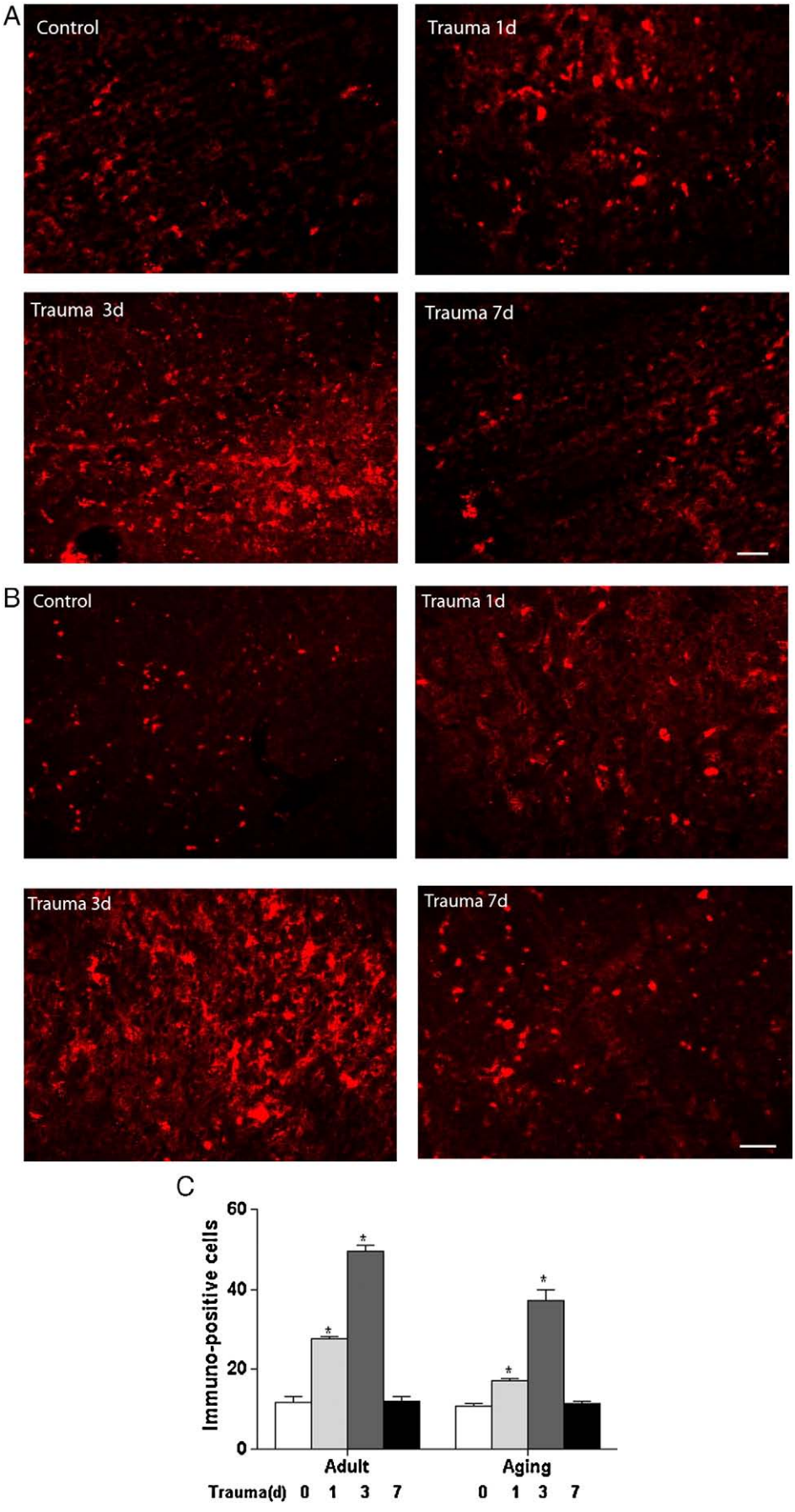
Age-dependent alterations in response to several stresses have been reported, but little is known about the traumatic stress. Here, we performed traumatic stress in 1-year-and 3-week-old rats, which represented adult and aged rats respectively based on the previous report (Wang et al., 2005). As shown in Fig. 1, both adult and aged rats demonstrated the highest decrease in lymphocyte proliferation and NK cell activity after 1 day of trauma, which was 43.0±1.22, 42.0±1.01% control in adult rats and 36.5±0.67, 36.8±1.24% control in aged rats respectively. In 3 day of trauma, immuno-suppression began to improve, with lower level in aged rats (55.6±1.32, 54.0±0.40% control) compared with the adult ones (67.9±0.14, 65.3±1.08% control). By 7 day of trauma, fully recovery from the immuno-depression could be detected on adult rats, with lymphocyte proliferation was 92.1±0.51% control and NK cell activity was 92.9±0.37% control but not on aged subjects (85.4±1.13, 84.8±0.67% control respectively). Since frontal cortex was reasoned to implicate in traumatic stress induced neuro-immune modulation (Zhao et al., 2002b), then the difference in this region may preferentially accounted for the diverse response observed in adult and aged rats.

2.2. *Fyn expression in cerebral cortex in traumatic adult and aged rats*

The possibility that observed immune activity changes were correlated with Fyn expression in frontal cortex was examined. The traumatic stress was undergone in both age groups of rats, Fyn immuno-positive cells were detected by immnohistochemistry. Immunoreactivity for Fyn was analyzed and semi-quantified in three randomly chosen areas under micrography (100×) (Fig. 2). The distribution of Fyn immunoreactivity appeared strong in individual preparations, the number of Fyn immunopositive cells were 11.7±0.73 and 10.8±0.31 in control group of adult and aged rats, which were substantially increased to 27.6±0.38 and 17.0±0.46 respectively in two age groups of rats after 1 day of traumatic stress. Time course analysis showed that Fyn immunoreactivity was persistently increased and reached the peak at 3 day of trauma. Similarly, adult rats had a higher amplitude (49.5±0.77) than the aged subjects (37.2±1.45). In contrast, there was no apparent change after 7 day of trauma compared with the respective control group, Fyn immunopositive cell numbers were 12.1±0.5 in adult rats and 11.5±0.18 in aged rats. Based on the above observation, we therefore proposed that 3 days after the traumatic stress might be the best timing for the immune response modification.

2.3. *Fyn modification in the cortical region and the subsequent immune consequences*

Since immuno-suppression began to recover in 3 day of trauma, Fyn expression in the cortical region reached its peak at the same time, this time point was then chosen for the remaining study. As demonstrated in Fig. 3, icv injection of Fyn antibody, lymphocyte proliferation and NK cell activity were attenuated in adult (46.4±0.42, 43.6±0.07% control) and aged rats (35.5±0.60, 32.6±0.48% control), with statistical significance compared with the vehicle treatment. In contrast, recombinant adenovirus expressing active Fyn resulted in the improvement in immuno-depression. In adult rats, lymphocyte proliferation and NK cell activity were increased to 88.2±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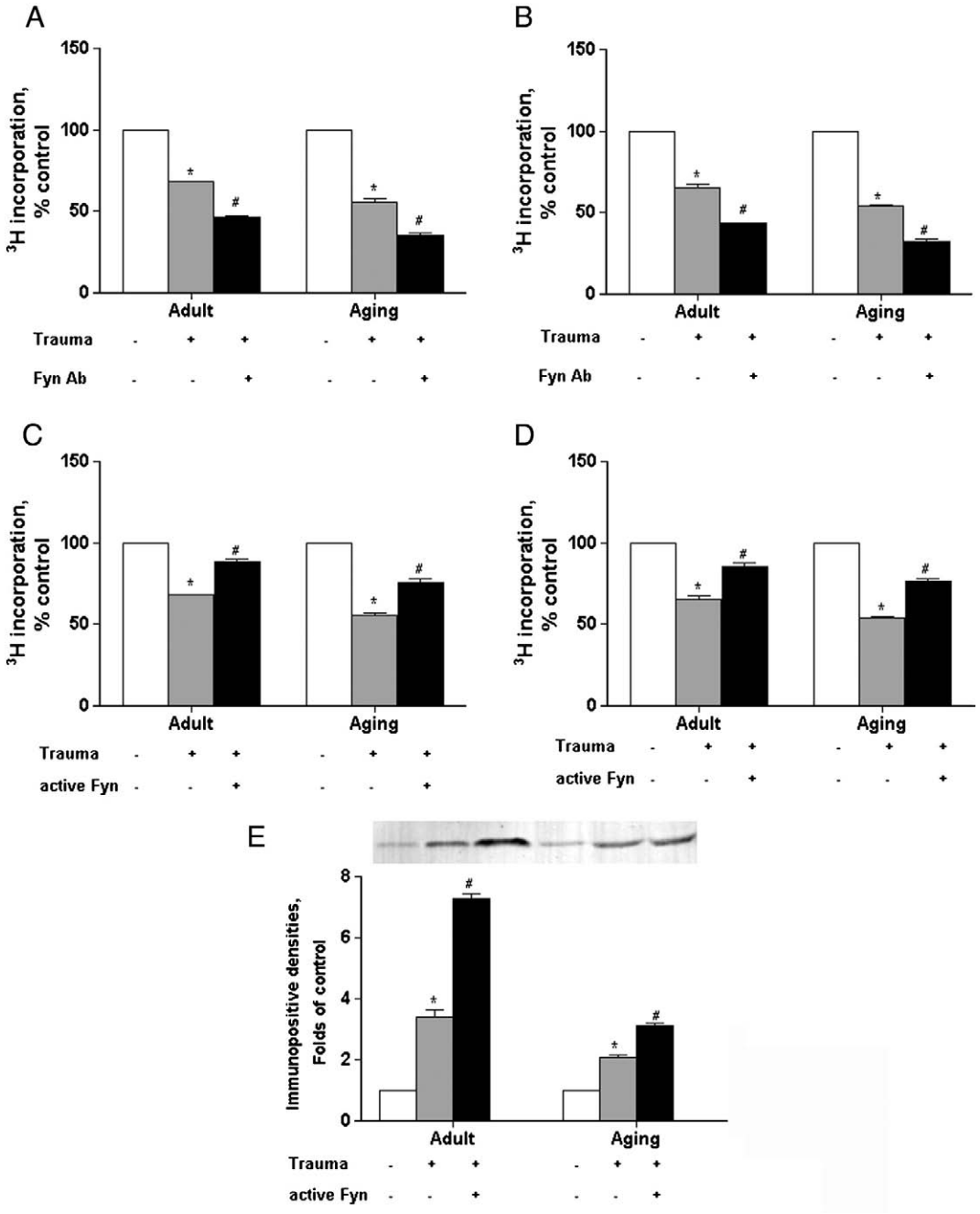

Fig. 3 – Immune consequences by Fyn modification in traumatic adult and aged rats. Two age groups of rat were divided into 4 subgroups: Control, Trauma 3 day, Trauma 3 day+icv injection of Fyn antibody (24 h), Trauma 3 day+icv injection of active Fyn (72 h) ($n=5$). Spleen cells were isolated, lymphocyte proliferation (A, C) and NK cell activity (B, D) were determined by [^{3}H] thymidine incorporation. Quantification for Fyn expression in frontal cortex was determined by Western Blot (E). Results are presented as means ± SD ($n=5$). $^*p<0.05$ *vs* Control, $^\#p<0.05$ *vs* Trauma 3 day.

0.82 and 85.6 ± 1.29% control respectively by Fyn up-regulation 2.1 folds. Without exception, aged rats exhibited similar change but with lower level than the adult subjects, 1.5 folds of Fyn up-regulation led to increased in lymphocyte proliferation and NK cell activity (75.9 ± 1.0 and 76.7 ± 0.70% control).

2.4. *Association of Fyn expression and ERK1/2 activation and the role on the immuno-suppression*

Src kinase and ERK1/2 signaling pathway have been shown to participate in a diverse array of cellular programs. ERK1/2

Fig. 2 – Fyn immuno-reactivity in frontal cortex of adult and aged rats. Cross section of frontal cortex of adult (A) and aged rats (B) were immunostained for anti-Fyn antibody, and the density of Fyn immuno-positive cells were semi-quantified in three randomly chosen areas (C). Results are presented as means ± SD ($n=5$). $^*p<0.05$ *vs* Control. Scale bars, 50 μm.

could be activated by phosphorylation on threonine and tyrosine residues, and once activated, is able to phosphorylate other downstream kinases and transcription factors. Accordingly, ERK1/2 activation was detected by its phosphorylation state. This was assayed by Western Blot analysis. As shown in Fig. 4, pERK1/2 expression level was increased following traumatic stress, and lasted longer than 7 days with highest level at day 3. The resultant ERK1/2 activation also displayed differential effect between adults (1.85±0.03, 3.49±0.03 and 2.43±0.06 folds of control) and aged rats (1.41±0.05, 2.18±0.07 and 1.79±0.03 folds of control).

At 3 days of the trauma, we found that icv injection of Fyn antibody caused marked decrease in pERK1/2 expression level, which were 2.22±0.06 and 1.40±0.07 folds of control in adult and aged rats respectively. When treatment with recombinant adenovirus expressing active Fyn, pERK1/2 expression level was transformed into increase, they were 4.46±0.11 and 3.69±0.06 folds of control in adult and aged rats. Additionally, lymphocyte proliferation and NK cell activity were reduced to 50.2±1.47 and 53.2±0.82% control in adult rats, 40.0±2.07 and 43.4±1.56% control in aged rats respectively when treatment with PD98059 (100 mM), a ERK inhibitor. This down-regulation was also found when ERK phosphorylation was blocked by U0126 (50 mM), suggesting that ERK activation was implicated in traumatic stress induced neuro-immune modulation. Interestingly, improvement in lymphocyte proliferation and NK cell activity was then attenuated by concurrently application of Fyn and PD98059, they were 70.7±1.18 and 66.4±1.63, 59.9±1.62 and 58.4±0.63% control in adult and aged rats respectively.

3. Discussion

Aging was recently proposed to prime microglial cells and provoke of inflammatory cytokines production. For example, myosin heavy chain class II, a marker for activated microglia, was reported to increase in the healthy aged brain (Moore et al., 2007). LPS administration resulted in an exaggerated inflammatory cytokine response in the aged brain (Perreau et al., 2007). Our previous study indicated that microglial cells

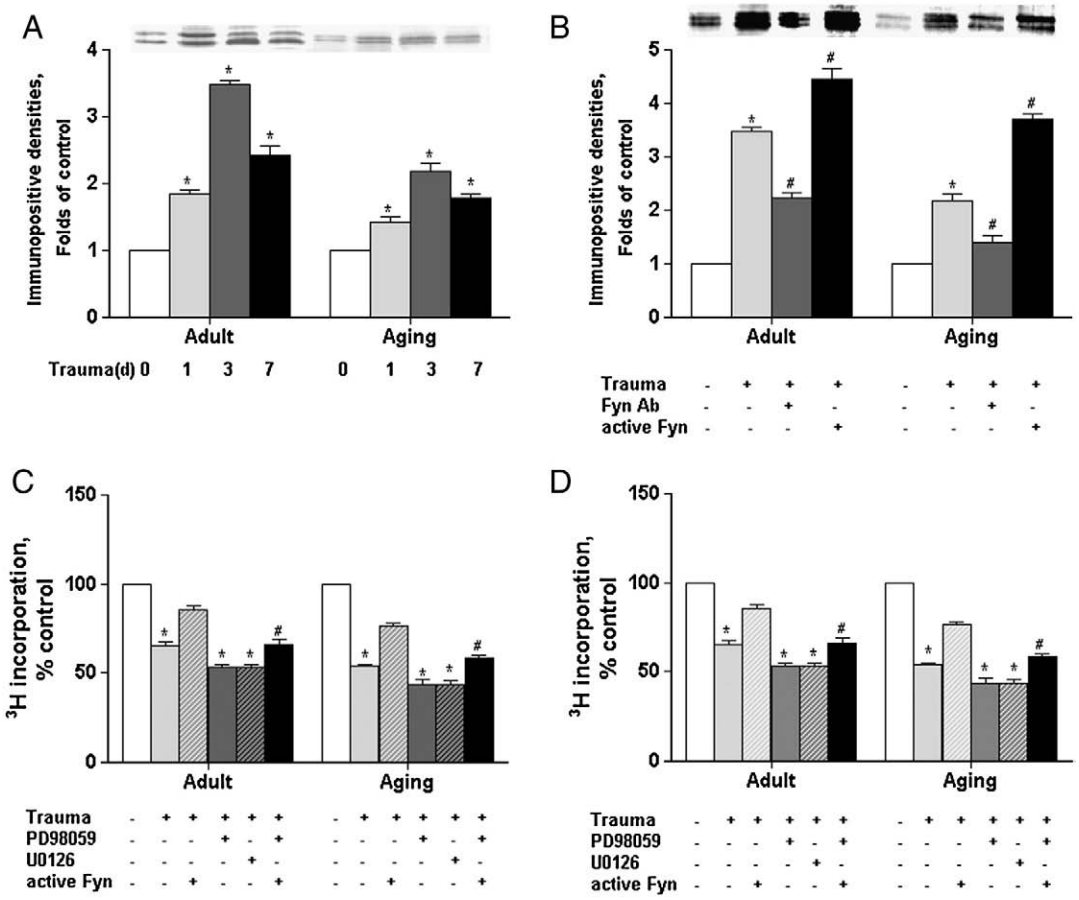

Fig. 4 – Association of ERK1/2 activation with Fyn and the effect on the traumatic stress induced immuno-suppresion. Two age groups of rat were divided into 6 subgroups: Control, Trauma1, 3 and 7 day, Trauma 3 day+icv injection of Fyn antibody (24 h), Trauma 3 day+icv injection of active Fyn (72 h) ($n=5$). ERK1/2 activation in frontal cortex was quantified, (A) control, 1, 3, and 7 day of trauma (B) 3 day of trauma with Fyn modification. After icv treatment with ERK inhibitors, PD98059 (100 mM), U0126 (50 mM) or co-application of PD98059 with active Fyn, lymphocyte proliferation (C) and NK cell activity (D) were analyzed by [^{3}H]thymidine incorporation. Results are presented as means±SD ($n=5$). $^*p<0.05$ *vs* Control, $^\#p<0.05$ *vs* Trauma+active Fyn.

and pro-inflammatory cytokines were mainly contributed in the traumatic stress induced immuno-suppression (Zhao et al., 2002a,b). Then, the intriguing issue is whether this effect is age-dependent?

In the present study, two age groups of rats were selected according to the recent study on the morphine tolerance (Wang et al., 2005), in which 3 week-and 1-year old rats represented adult and aged subjects respectively. As a result, traumatic stress could induce immuno-depression, as demonstrated the decrease in lymphocyte proliferation and NK cell activity. In adult rats, immuno-depression reach the peak effect at the day 1, then begin to improve at day 3 and fully recovered by day 7. However, aged rats exhibited exaggerated immune consequences and longer duration than the younger cohorts. The present result was in agreement with the report that aged subjects exhibited higher amplitude of response to LPS and extended function recovery (Chen et al., 2008).

As previous report, frontal cortex was critical for processing and regulating the traumatic stress induced neuro-immune modulation (Zhao et al., 2002b), wherein some defined molecules like Fyn, one member of Src family kinases, was presumably implicated in senescent-associated functional changes, and might be the basis for the diversity in immune response which was considered to be derived from the reorganization of host priorities and maladaptive in aged subjects.

In support of this consensus, we examined Fyn immunoreactivity in the cortical region following the traumatic stress. It was displayed that Fyn immunoreactivity began to increase at 1 day, reached the peak at 3 day, and returned to normal level at 7 day of trauma in adult rats. Aged rats exhibited similarly change pattern, although with lower amplitude. They could not return to the control level until 10 day of trauma (data not shown). Therefore, the parallel changes in Fyn expression in cortical region and immune consequences indicated that the improvement of immuno-suppression and the diversity response to the traumatic stress between aged rats and younger cohorts could be attributed to the Fyn expression level.

We found that the treatment with Fyn antibody caused a deteriorated immuno-suppression, with aged rats exhibited robust response than the adult subjects. Furthermore, both age groups of rats became resistant to traumatic stress when Fyn was overexpressed. Then, the requirement of Fyn for neuro-immune modulation was strengthened, and several possible pathways are assumed to reside on cortical region. For example, Fyn is known to bind opioid receptors (data not shown). Also, there may be interplay between Fyn and CRH (corticotropin-releasing hormone)-IL-1β (interleukin-1β) circuit, the interaction between Fyn and these molecules produces signals for controlling neuroimmune activities.

We should also stress that Fyn belongs to Src-family protein tyrosine kinases, which has been shown to be dispensable for the early activation on several signal pathways like T cell receptor, IL-2R, and CD2 (Maksumova et al., 2005). Also, Fyn is highly expressed in the central nervous system, and serves as control switches in a variety of signal transduction pathways governing essential cell processes including cell growth, division, differentiation, survival, and death of eukaryotic cells (Miyamoto et al., 2008). Besides that, the transactivating of Src-family protein tyrosine kinases and ERK could ultimately leads to altered neuronal activity, including age-related processes (Li et al., 2008).

Finally, we observed that phosphorylation of ERK (Davis, 1993; Grewal et al., 1999) was increased after traumatic stress and that the time course of activation matched the emergence of enhanced Fyn sensitivity to traumatic stimuli. As expected, aged rats also displayed the lower activation than the adult ones. Besides that, Fyn modification resulted in the change in ERK activation. The rescue of immuno-suppression by Fyn could adversely affected by ERK inactivation. Thus, these observations allow speculation that Fyn may converge on ERK signal pathway, there is congruence between these signal pathway and functional recovery of traumatic stress induced immuno-suppression. Also, the study supported the notion that Fyn and ERK are integral part, which could produce prominent modulation on aging process.

4. Experimental procedures

4.1. *Recombinant adenoviruses*

Recombinant adenovirus expressing rat cDNAs for Fyn in constitutively active (Tyr-528/Phe) form was constructed by inserting the corresponding cDNAs into the adenoviral shuttle vector pDE1sp1A (Microbix Biosystems, Inc. Canada) and homologous recombination with the backbone vector PJM17. Adenovirus was purified by $CsCl_2$ gradients and PD-10 Sephadex chromatography.

4.2. *Traumatic animal model*

3-week and 1-year-old of male SD rats (Animal center of Fudan University Shanghai Medical College) were used in the present study. Dorsomyotomy and exploratory laparotomy were performed on the rats under anesthesia (sodium pentobarbital 35 mg/kg, i.p.) as the model of traumatic stress. No post operative infection occurred. Tissue samples were taken 1, 3, and 7 days after operation. All protocols were approved by the Committee on Research Animal Care of Fudan University, and the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals were observed.

4.3. *Intracerebroventricular injection of drugs*

Implantation of the cannula was performed stereotaxically under anesthesia, the stainless steel guide cannula (0.5 mm in diameter) with an inserted cannula (0.25 mm in diameter) was implanted into the right lateral ventricle (posterior 0.5, lateral 1.5, horizontal 4.5) and fixed on the skull with dental cement. Fyn antibody (10 μg,) or recombinant adenovirus (5×10^9 plaque-forming units (pfu)) dissolved in sterilized PBS was injected over 10 s via the cannula at a volume of 10 μl. Rats from the control group were injected with vehicle or empty virus. At the end of each experiment, the position of the cannula was assessed by histological examination. Only data collected from experiments in which correct insertion of the cannula was verified are reported. Animals were killed 24 h

after Fyn antibody injection, or 72 h after recombinant adenovirus injection.

4.4. *Immunal assay*

For lymphocyte proliferation, spleens were pressed through stainless steel mesh and red blood cells were lysed by treatment with NH4Cl solution. Then cell suspension of 1×10^7 cells/ml in a final volume of 200 μl of complete tissue culture medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine) was seeded in triplicate in a U-bottom 96-well plate in the presence and absence of concanavalin A (Con A, 1 mg/L). Plates were incubated at 37°C in a 5% CO2. After 48 h, cultures were labeled with 0.5 μCi of [^{3}H]thymidine (Amersham Biosciences, Buckinghamshire, UK) and after 24 h, cells were harvested using a cell harvester. Samples were counted in a liquid scintillation counter. Proliferation results are presented as the mean cpm ± SD of triplicate cultures.

For natural killer cell cytotoxicity, firstly suspensions of YAC-1 lymphoma cells with a concentration of 2×10^5/ml at a final volume of 100 μl were targeted with 0.5 μCi of [^{3}H] thymidine and incubated at 37°C, 5% CO2 for 6 h. Then spleens were homogenized and the resultant cell suspensions were pooled in the presence and absence of Con A and seeded in triplicate with the effector: target ratios of 50:1 for 16 h. Cytotoxic activity results were determined as follows:

$$\text{Percent response} = [(\text{counts in tested well} - \text{counts in spontaneous response well})/(\text{counts in maximum response well} - \text{counts in spontaneous response well})] \times 100$$

4.5. *Immunohistochemical analysis*

Rats were anesthetized with sodium pentobarbital (35 mg/kb, i.p.) and perfused transcardially with fixative (4% paraformaldehyde). Coronal brain sections (25 μm) were obtained using a cryostat. For fluorescent based detection, frozen sections were subjected to immunostaining with anti-Fyn at 1:100 and secondary antibody at 1:200. The data derived from each group were analyzed by Leika Q500IW image analysis system. Frontal cortex was chosen for analysis and immuno-positive cells were semi-quantified under photomicrography.

4.6. *Western blotting*

Frontal cortex was sonicated with about seven volumes of protein extraction buffer containing (20 mM HEPES (pH 7.5), 10 mM potassium chloride, 1.5 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid, 1 mM EGTA, 0.7% protease inhibitor cocktail, 1% phosphatase inhibitor cocktail). The sonicated sample was centrifuged at 10,000 g for 15 min at 4 °C and the supernatant was used for analysis. Lowry assays was employed to determine the protein concentration 5 μg of protein was resolved in SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane (PVDF, Amersham). The membrane was probed with anti-Fyn (1:100) and anti-pERK1/2 (1:400) and subsequent alkaline phosphatase conjugated secondary antibody (1:5000). The bands were detected by ECF substrate (GE healthcare) and scanned in the storm 860 imaging system, whose intensity was quantified and analyzed with the ImageQuant software.

4.7. *Statistical analysis*

Results are represented as mean ± SD of 3–5 experiments. Immunal activity alteration within animals was assessed by repeated-measures analyses of variance (ANOVAs). Differences in individual parameters were analyzed using separate repeated-measures ANOVAs.

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