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A peripheral immune response to remembering trauma contributes to the maintenance of fear memory in mice

Matthew B. Young[a], Leonard L. Howell[a], Lauren Hopkins[b], Cassandra Moshfegh[b], Zhe Yu[b], Lauren Clubb[b], Jessica Seidenberg[b], Jeanie Park[e,f], Adam P. Swiercz[b], Paul J. Marvar[b,c,d,*]

[a] Division of Neuropharmacology and Neurological Disease, Yerkes National Primate Research Center, Emory University, Atlanta GA, USA
[b] Department of Pharmacology and Physiology, George Washington University, Washington DC, USA
[c] Department of Psychiatry and Behavioral Sciences, George Washington University, Washington DC, USA
[d] GW Institute for Neuroscience, George Washington University, Washington DC, USA
[e] Renal Division, Department of Medicine, Emory University School of Medicine, Atlanta, GA, USA
[f] USA Research Service Line, Atlanta VA Medical Center, Decatur, GA, USA

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ABSTRACT

Alterations in peripheral immune markers are observed in individuals with post-traumatic stress disorder (PTSD). PTSD is characterized in part by impaired extinction of fear memory for a traumatic experience. We hypothesized that fear memory extinction is regulated by immune signaling stimulated when fear memory is retrieved. The relationship between fear memory and the peripheral immune response was tested using auditory Pavlovian fear conditioning in mice. Memory for the association was quantified by the amount of conditioned freezing exhibited in response to the conditioned stimulus (CS), extinction and time-dependent changes in circulating inflammatory cytokines. Brief extinction training with 12 CS rapidly and acutely increased circulating levels of the cytokine interleukin-6 (IL-6), downstream IL-6 signaling, other IL-6 related pro-inflammatory cytokines. Transgenic manipulations or neutralizing antibodies that inhibit IL-6 activity did not affect conditioned freezing during the acquisition of fear conditioning or extinction but significantly reduced conditioned freezing 24 h after extinction training with 12 CS. Conversely, conditioned freezing after extinction training was unchanged by IL-6 inhibition when 40 CS were used during the extinction training session. In addition to effectively diminishing conditioned freezing, extinction training with 40 CS also diminished the subsequent IL-6 response to the CS. These data demonstrate that IL-6 released following fear memory retrieval contributes to the maintenance of that fear memory and that this effect is extinction dependent. These findings extend the current understanding for the role of the immune system in PTSD and suggest that IL-6 and other IL-6 related pro-inflammatory cytokines may contribute to the persistence of fear memory in PTSD where fear memory extinction is impaired.

1. Introduction

The fear response to a memory is diminished or 'extinguished' by neurobiological processes that are initiated when the fear response repeatedly fails to predict an actual threat (Milad and Quirk, 2012). Persistent and powerful fear responses to remembered trauma in post-traumatic stress disorder (PTSD) are hypothesized to result from impairments in extinction processes that regulate fear memory maintenance (Rothbaum and Davis, 2003). Although the maintenance of a retrieved fear memory can be disrupted in animal models by targeting neurobiological processes known to mediate extinction, translation of such approaches in humans does not reliably disrupt the maintenance of powerful fear responses in PTSD (Singewald et al., 2015). Characterizing alternative biological substrates that influence fear memory extinction may provide better targets for improving fear extinction in PTSD.

Individuals with PTSD exhibit a range of altered peripheral immune markers at baseline, in response to psychological stressors, or in response to immune challenges (Breen et al., 2015; Michopoulos et al., 2016). Baseline immune alterations include elevated peripheral serum levels of pro-inflammatory cytokines (Gill et al., 2009; Tursich et al., 2014), increased *in vitro* cytokine release from peripheral blood

* Corresponding author at: Department of Pharmacology and Physiology, George Washington University, Washington DC, USA.
E-mail addresses: mbyoung@emory.edu (M.B. Young), lhowell@emory.edu (L.L. Howell), leh@gwu.edu (L. Hopkins), cmoshfegh@gwmail.gwu.edu (C. Moshfegh), zyu@email.gwu.edu (Z. Yu), lclubb18@students.claremontmckenna.edu (L. Clubb), jseidenberg@email.wm.edu (J. Seidenberg), adampaul@gwmail.gwu.edu (A.P. Swiercz), pmarvar@gwu.edu (P.J. Marvar).

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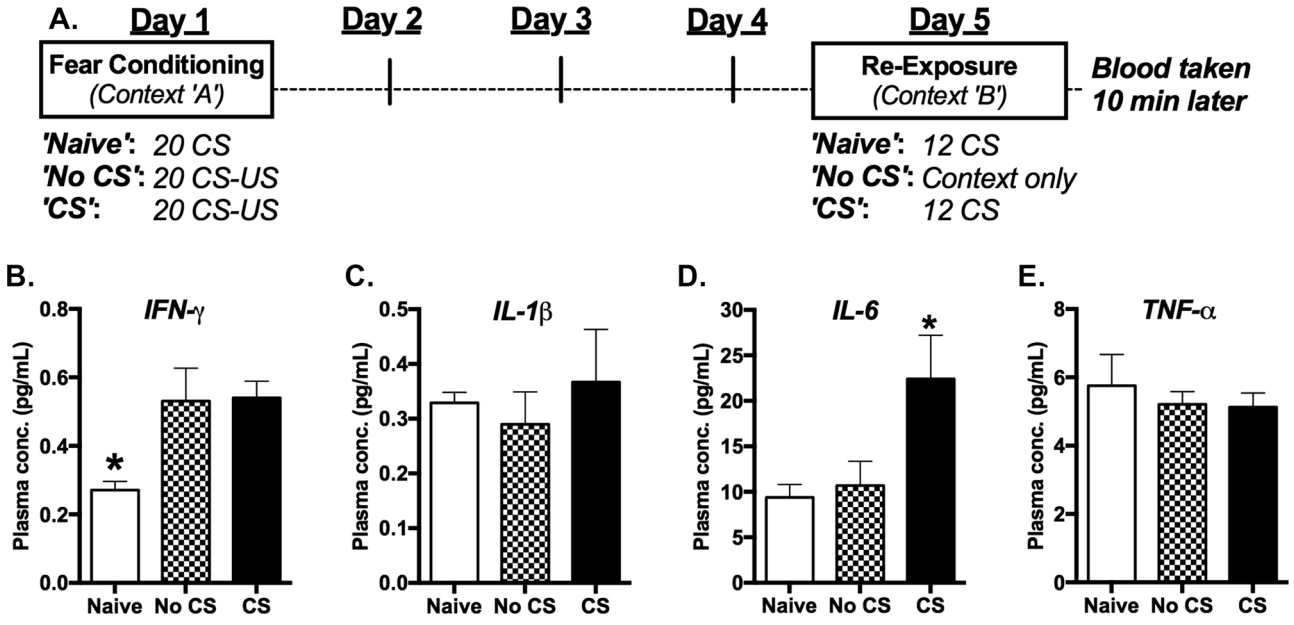

Fig. 1. Rapid effect of fear memory retrieval on plasma cytokine levels. **a** Study design. **b–e** Plasma levels of IFN-γ, IL-1β IL-6 and TNF-α 10 min after mice were removed from the re-exposure apparatus. "Naïve" mice were not fear-conditioned, but were re-exposed to the CS. "No CS" mice were fear conditioned, but were not re-exposed to the CS while in Context B. "CS" mice were both fear conditioned and re-exposed to the CS. (n = 7–8/group) *P < 0.05.

mononuclear cells (Gola et al., 2013), and altered proportion or activity of T lymphocytes (Jergović et al., 2014; Lemieux et al., 2008). In addition to baseline alterations, PTSD has been associated with heightened immune responses to psychological stressors (Newton et al., 2014) and a greater likelihood of developing an autoimmune disorder (O'Donovan et al., 2015). Despite known physiological consequences, the relevance of altered immune signaling to the lasting psychological symptoms of PTSD has not been determined.

Studies in rodents using Pavlovian fear conditioning support a role for the immune system in the formation and maintenance of fear memory. Impaired fear memory maintenance is observed in mice lacking peripheral T lymphocytes (Clark et al., 2016). Broad anti-inflammatory treatments prior to extinction training enhance contextual fear extinction and reverse fear conditioning-induced hippocampal levels of TNF-alpha (Yu et al., 2017). Conversely, extinction acquisition is impaired by direct administration of the cytokines interferon-α or interleukin-6 (IL-6) to the amygdala prior to extinction training (Bi et al., 2016; Hao et al., 2014). Despite previous observations using exogenous immune manipulations the role of endogenous peripheral immune responses in fear memory processes has not been well studied.

We hypothesized that alterations in immune signaling triggered by fear memory retrieval serves the maintenance of that memory. Using a mouse model of Pavlovian fear conditioning, we report that peripheral IL-6 is a key signaling mechanisms involved in a labile immune response to fear memory that also contributes to fear memory extinction. These observations provide new insights regarding the role of the immune system in modulating the maintenance of fear memory.

2. Materials and methods

2.1. Animals

C57BL/6J and IL-6-deficient mice (IL-$6^{-/-}$) were from Jackson Laboratory (Bar Harbor, ME, USA) and subsequently bred in-house at the Yerkes National Primate Research Center at Emory University or George Washington University. Mice were group housed in ventilated cages and maintained on *ad libitum* food and water. Lights turned on at 7:00 A.M. and turned off at 9:00 P.M. All experiments were performed between 9:00 A.M. and 5:00 P.M. in 10–16 week-old male mice. Studies were in accordance with NIH guidelines and all procedures were approved by the IACUC at Emory and George Washington University.

2.2. Experimental compounds

Mouse IL-6 monoclonal antibody (IL6Ab; clone MP5-20F3) and Normal Rat IgG (ControlAb) was obtained from R&D Systems (Minneapolis, MN, USA). All antibodies were diluted in sterile 0.9% saline and injected intraperitoneally once per day (4 μg, 0.2 mL). This concentration was used based on literature demonstrating tolerability and minimal effects on motor behavior following repeated dosing (Hodes et al., 2014).

2.3. Behavioral approaches

Fear conditioning, extinction training and memory maintenance testing took place over five days. Two days prior to conditioning animals were habituated to the conditioning apparatus (Med-Associates; Georgia, VT, USA) in a brightly-lit room for 20 min. On the conditioning day animals were trained to 20 pairings of an auditory CS (6 kHz, 75–80 dB) and an US-shock (0.7 mA, 2–4 s). The CS varied 15–30 s before co-terminating with the US and the inter-trial interval varied 45–90s. This unpredictable conditioning protocol was adapted from other unpredictable conditioning paradigms (Moberg and Curtin, 2009; Walker et al., 2003) was used to model significant psychological and physical trauma that precedes the development of PTSD. In total, the conditioning phase lasted 27 min "Naïve" animals were placed in the conditioning apparatus and exposed to an identical regimen of CS, but without the US. During both habituation and conditioning the behavioral apparatus was cleaned with 70% ethanol. In antibody experiments mice were treated with 4 μg of either IL6Ab or ControlAb in 200 uL of saline beginning the day after conditioning and ending on the day of re-exposure. Four days after conditioning all animals were placed in a novel testing chamber with a black Plexiglas floor, cleaned with 5% Roccal-D (Pfizer; New York, NY, USA) and in a different room lit only by red light. "Naïve" animals and half of the fear-conditioned animals ('*CS*' group) were exposed to the CS-tone. The remaining half of fear-conditioned animals ('*No CS*') were placed in the testing apparatus without re-exposure to the CS (Fig. 1A). For experiments investigating