Reduced neuroinflammation via astrocytes and neutrophils promotes regeneration after spinal cord injury in neonatal mice

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Disclosures: No Disclosure

INTRODUCTION: Neonatal spinal cord injury (SCI) shows better functional outcomes than adult SCI[1]. Although the regenerative capability in the neonatal spinal cord may have cues in the treatment of adult SCI, the mechanism underlying neonatal spinal cord regeneration after SCI is unclear. We previously reported age-dependent variation in the pathogenesis of inflammation following SCI. Therefore, we explored differences in the pathogenesis of inflammation after SCI between neonatal and adult mice and their effect on axon regeneration and functional outcome.

METHODS: All experimental procedures were conducted in compliance with animal protocols approved by the Committee of Ethics on Animal Experimentation in the Faculty on Medicine, Kyushu University (A22-238-0) We used two-day-old and 8-week-old mice as a model of neonatal and adult SCI, respectively. Using wild-type C57/BL6N mice and Aldh1l1-EGFP transgenic mice (astrocyte-reporter line), we established spinal cord crush injury model by performing fully compression for 2 seconds with forceps (width: 100 μm) to the spinal cord of Th 10 segmental level. The spinal cord specimen and blood samples were examined by immunohistochemistry, flowcytometry, and quantitative PCR analysis. Statistical analyses to compare the means of data between two groups were performed using unpaired *t* tests. For analyses of the repeated measures and multiple group comparisons, a two-way repeated-measures ANOVA with the Tukey–Kramer post-hoc test was performed. All tests were two-tailed, and *P* values of < 0.05 were considered statistically significant. All analyses were conducted using the JMP PRO 16.0.0 software program.

RESULTS: Flow cytometry on the blood sample revealed that the expression of a chemokine receptor (CXCR2) and an adhesion molecule (β2 integrin) was significantly lower in neonatal circulating neutrophils than in adult neutrophils which initiates neuroinflammation after SCI (Fig. 1A). Furthermore, flow cytometry on the spinal cord of Aldh1l1-EGFP mice revealed that neonatal astrocytes secrete significantly lower levels of chemokines to recruit circulating neutrophils (e.g., Cxcl1 and Cxcl2) after SCI in comparison to adults (Fig. 1B). Strikingly, these neonate-specific cellular properties seemed to be associated with no neutrophil infiltration into the injured spinal cord (Fig. 1C), followed by significantly lower expression of inflammatory cytokines (Il-1β, Il-6 and Tnf-α) after SCI in the spinal cords of neonates than in those of adults (Fig. 1D). Consequently, significantly fewer apoptotic neurons (Fig. 1E) and greater axonal regeneration (Fig. 1F) were observed in neonates in comparison to adults, which led to a marked recovery of locomotor function as assessed by BMS system at 28 day after SCI (Neonate scored 6.5 whereas adult 2.5, n=6, $P<0.0001$).

DISCUSSION: Neonatal mice with SCI exhibited axon regeneration in the context of extremely low inflammation that is characterized by a lack of neutrophil infiltration. Since neutrophil-specific CXCR2-depleted adult mice with experimental autoimmune encephalomyelitis showed the infiltration of neutrophils into the spinal cord[2], neutrophil-free inflammation seen in this study could not be solely caused by the reduced expression of CXCR2. This fact emphasizes the significance of lower expression of β2 integrin which forms the adhesion molecules LFA-1 and MAC-1 when combined with CD11a and CD11b to undergo neutrophil infiltration into the spinal cord[3]. Taken together, the neutrophil-free inflammation in the neonates of this study was due to the simultaneous lower expression of CXCR2 and β2 integrin, in addition to lack of astrocyte derived chemokine release. Considering the fact that neutrophil infiltration has been observed—to some extent—after CNS injury in 9- and 10-day-old mice[4], the 2-day-old mice with SCI used in this study are an extremely optimal model for analyzing the mechanisms of successful suppression of neutrophil infiltration and subsequent inflammation.

SIGNIFICANCE/CLINICAL RELEVANCE: Our findings provide deeper insight into the role of astrocytes and neutrophils in neuroinflammation and axon regeneration in neonatal mice Reproducing neonatal-like reduced inflammation in the adult spinal cord may lead to vigorous axon regeneration with favorable functional recovery.

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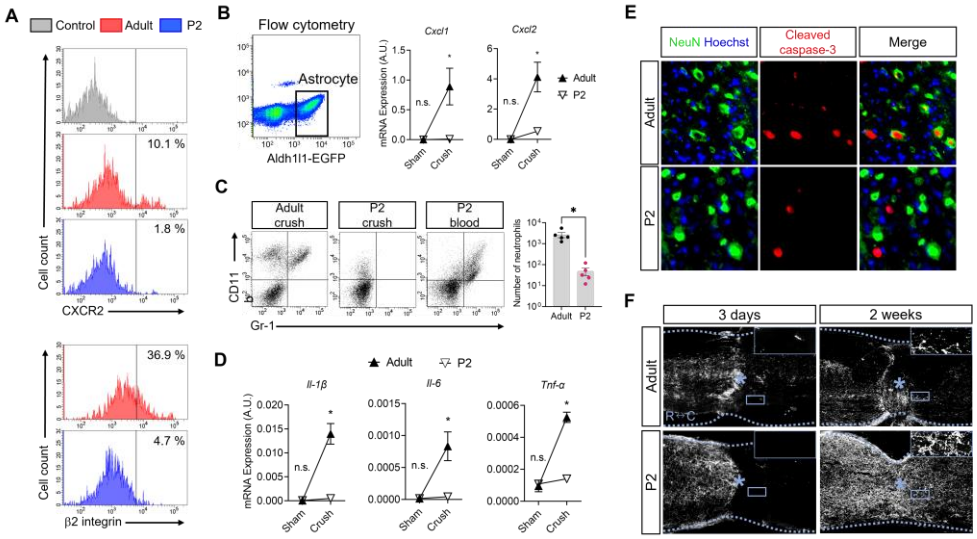

Figure 1: **(A)** Histograms from flow cytometry showing the expression level of CXCR2 and β2 integrin in circulating neutrophils of neonates and adults. **(B)** A gating strategy for sorting EGFP$^+$ astrocytes in the spinal cord of Aldh1l1-EGFP mice (left). mRNA expression levels of Cxcl1 and Cxcl2 at 12 h after crush injury (right, n=6). **(C)** Flow cytometry of the spinal cord.at 12 h after SCI and the number of neutrophils per $1.0*10^7$ events detected by flow cytometry (n=5). **(D)** mRNA expression levels of Il-1β, Il-6, and Tnf-α at 12 h after SCI (n=8). **(E)** Iintranuclear cleaved caspase-3 with NeuN$^+$ neurons in the adult spinal cord but not in neonates. **(F)** 5-HT$^+$ axons in the neonatal and adult spinal cord at 3 days and 2 weeks after SCI. Asterisks; epicenter of the lesion. $^*P<0.05$