

## BRIEF COMMUNICATION

## Erythropoietin dampens injury-induced microglial motility

Miso Mitkovski<sup>1,8</sup>, Liane Dahm<sup>2,8</sup>, Ralf Heinrich<sup>3</sup>, Mathieu Monnheimer<sup>2</sup>, Simone Gerhart<sup>2</sup>, Judith Stegmüller<sup>4,5</sup>, Uwe-Karsten Hanisch<sup>6</sup>, Klaus-Armin Nave<sup>5,7</sup> and Hannelore Ehrenreich<sup>2,5</sup>

Traumatic brain injury causes progressive brain atrophy and cognitive decline. Surprisingly, an early treatment with erythropoietin (EPO) prevents these consequences of secondary neurodegeneration, but the mechanisms have remained obscure. Here we show by advanced imaging and innovative analytical tools that recombinant human EPO, a clinically established and neuroprotective growth factor, dampens microglial activity, as visualized also *in vivo* by a strongly attenuated injury-induced cellular motility.

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## INTRODUCTION

Microglia are essential mediators of innate immunity in brain providing constant surveillance and protection *via* rapid reaction to homeostatic disturbances. Enduring microglia (over)activation, however, may foster neurodegeneration.<sup>1–4</sup> Recently, we reported a *bilateral* increase in activated microglia in the brain 24 hours after a small, standardized, *unilateral* parietal cortical cryolesion in juvenile mice, which persisted for at least 1 year and was associated with progressive global brain atrophy and cognitive decline. Intriguingly, early treatment with recombinant human erythropoietin (EPO) prevented this increase as well as the downstream neurodegenerative consequences.<sup>5,6</sup>

Erythropoietin has been recognized for nearly two decades in both preclinical and clinical studies as a potent neuroprotective and neuroregenerative growth factor with a multifaceted, hematopoiesis-independent action profile.<sup>6</sup> The present paper has been designed to test the hypothesis that EPO might modulate microglial motility.

## MATERIALS AND METHODS

Materials and methods with associated references as well as four videos with legends are provided in the Supplementary information on the JCBFM website.

## RESULTS AND DISCUSSION

We first tested *in vivo* the hypothesis that EPO might modulate microglial motility. Using *CX<sub>3</sub>CR1<sup>+/EGFP</sup>* mice, we performed our standardized right parietal cortical cryolesion and injected EPO or placebo intraperitoneally immediately thereafter. At 24 hours later, we compared *in vivo* microglial process protrusion before ('basal') and upon laser-induced microinjury in the contralateral (left)

parietal cortex. Microglia respond strongly with goal-directed process movement to laser injury (Supplementary video 1).<sup>7,8</sup> This intense response was dampened under EPO compared with placebo. In contrast, basal movements were similar between treatment groups (Figures 1A–C).

Next, we wondered whether the effect of systemically injected EPO on microglia would be reproducible in a less complex system, i.e., primary murine microglia-astrocyte mixed cultures, and thus be clearly independent of hematopoiesis. We developed an *in vitro* method of quantifying microglia movements upon laser-induced microinjury. To first characterize the activity state of the cultures under our experimental conditions, immunocytochemistry was performed with EPO or placebo, again added 24 hours before the planned time point of injury. Under placebo conditions, mainly ramified microglia in surveilling state (94% with only weak Iba1 staining) were seen. ATP, added for 2 hours as *in vitro* lesion equivalent<sup>7,9,10</sup> provoked strong Iba1 and L-citrulline staining (Figures 1D–H). Expectedly, the L-citrulline signal, readout of inducible nitric oxide formation, was reduced under EPO (Figure 1I).<sup>11</sup> Both microglial process motility (Figures 1J and 1K; Supplementary video 2) and cell migration (Figure 1L; Supplementary video 4), observed upon *in vitro* laser microinjury in this coculture, were attenuated by EPO.

Multiple cell types in the brain are known to express EPOR (EPO receptor) and may contribute to the measured EPO effect on microglial motility. To prove a direct effect of EPO on microglia, we further reduced complexity by using pure microglia cultures ( $\geq 99\%$ ). This allowed us to quantify the effect of EPO on microglial migration in the Boyden chamber under the influence of ATP (again as injury model). The EPOR mRNA expression declined from very prominent expression at seeding throughout the 4-hour experiment, likely related to the settling of the initially highly stressed cells (Figure 2A). Again, EPO acted on microglial

<sup>1</sup>Light Microscopy Facility, Max Planck Institute of Experimental Medicine, Göttingen, Germany; <sup>2</sup>Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen, Germany; <sup>3</sup>Department of Cellular Neurobiology, Institute of Zoology, Georg-August-University, Göttingen, Germany; <sup>4</sup>Cellular and Molecular Neurobiology Group, Max Planck Institute of Experimental Medicine, Göttingen, Germany; <sup>5</sup>Center for Nanoscale Microscopy and Molecular Physiology of the Brain (CNMPB), Göttingen, Germany; <sup>6</sup>Institute of Neuropathology, Georg-August-University, Göttingen, Germany and <sup>7</sup>Department of Neurogenetics, Max Planck Institute of Experimental Medicine, Göttingen, Germany. Correspondence: Professor H Ehrenreich, Clinical Neuroscience, Max Planck Institute of Experimental Medicine, Göttingen 37075, Germany. E-mail: ehrenreich@em.mpg.de

<sup>8</sup>These authors contributed equally to this work.

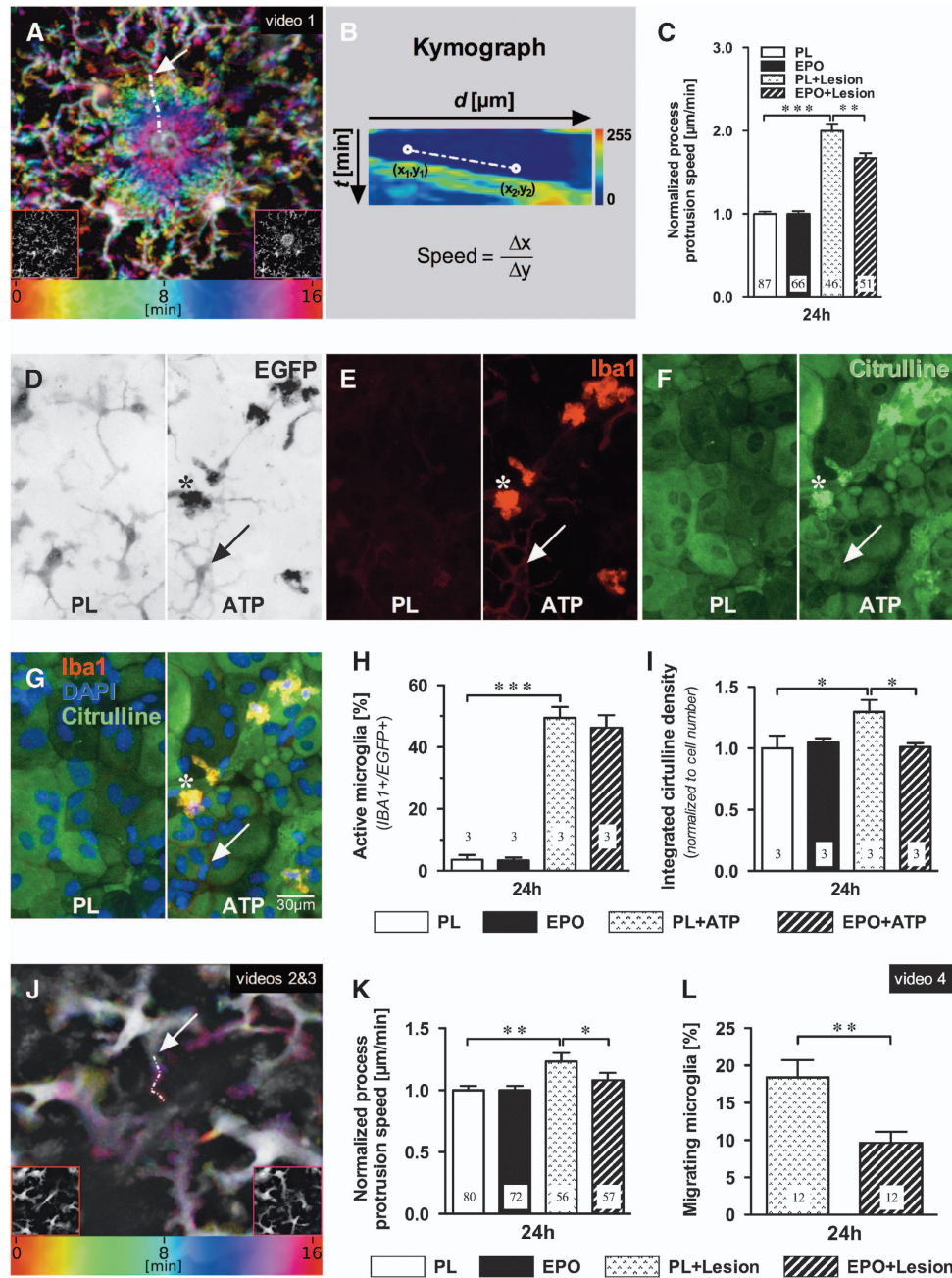
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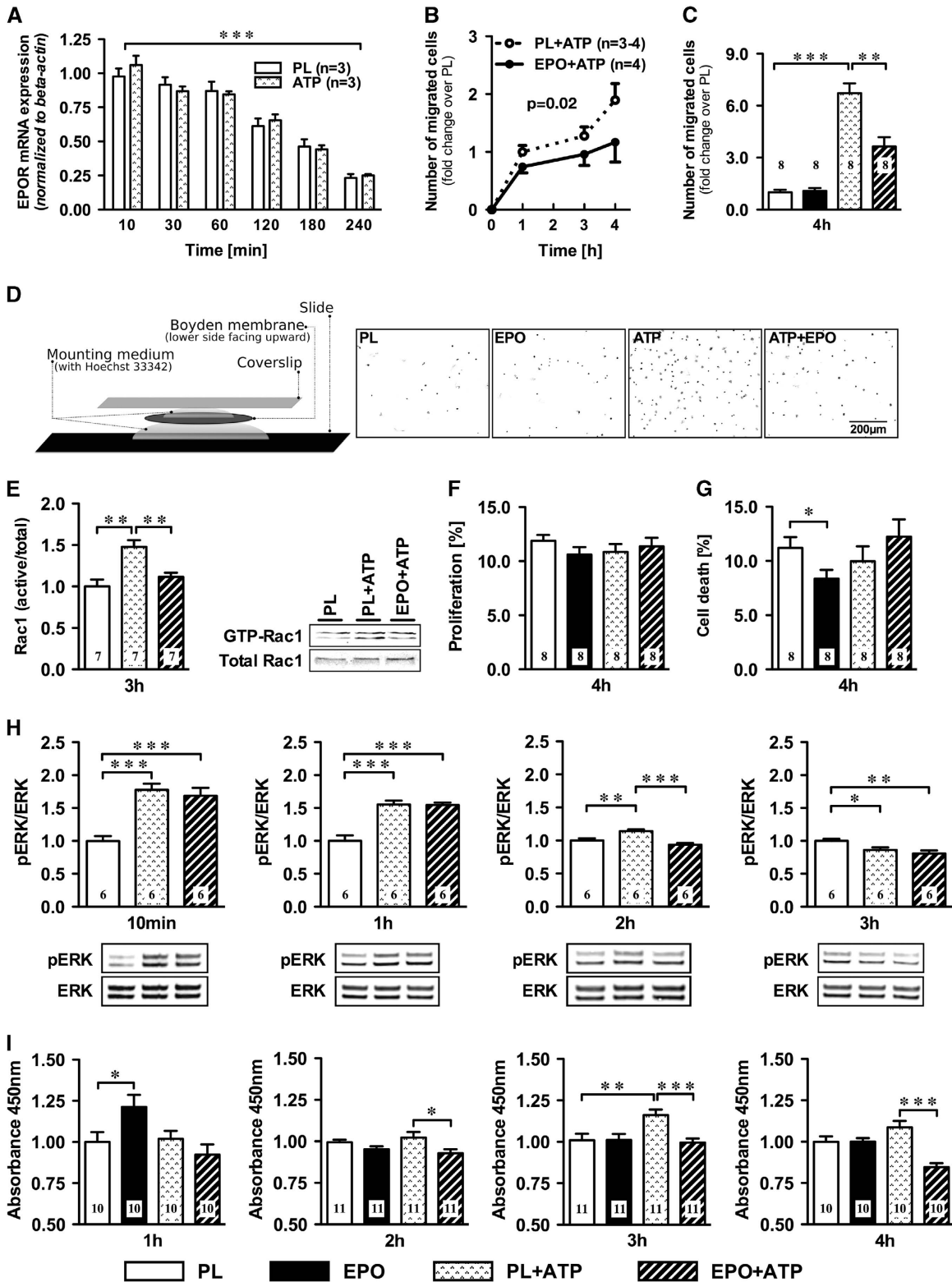
motility, diminishing ATP-stimulated migration by up to 50% (Figures 2B–D).

We next searched for first hints regarding molecular mediators of this striking motility reduction. Taking into consideration that

active microglial Rac1 is associated with numerous neurodegenerative diseases,<sup>12</sup> we determined the activation state of this Rho GTPase 3 hours after seeding, the time point at which the migration curves under EPO versus placebo start dissociating



**Figure 1.** Erythropoietin (EPO) effects on cortical microglia *in vivo* and on microglia in mixed cultures. ***In vivo***: (A) Microglial processes converge onto a central laser lesion (colors represent time). Lower left and right insets illustrate pre- and post-laser lesion views (see Supplementary Video 1). (B) Process movements were traced (arrow in A shows exemplary path) and sampled to create kymographs, whose slope corresponds to the process protrusion speed. (C) Laser lesion-induced process protrusion was higher than basal motility, but reduced by 24 hours EPO pretreatment (5 IU/g intraperitoneally), compared with placebo (PL). Numbers of analyzed processes from 4 to 5 independent experiments each are given in the bars. Mean  $\pm$  s.e.m. presented, \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  (two-tailed *t*-test). ***In vitro***: (D to G) Representative images of surveilling (placebo, PL) and ATP-stimulated microglia: EGFP fluorescence (D, inverse signal), Iba1 (E), L-citrulline (F), overlay plus DAPI (G); arrows denote a weakly Iba1-stained, L-citrulline-negative ramified microglia; stars designate a strongly Iba1+/L-citrulline+ cell. (H) Increase in strongly Iba1+/EGFP+ microglia under ATP (300  $\mu\text{mol}/\text{L}$ , 2 hours) and (I) reduction of ATP-stimulated L-citrulline by EPO pretreatment (3 IU/mL, 24 hours); numbers of independent experiments are given in the bars. (J) Microglia send processes to a central laser lesion (colors represent time). Insets correspond to pre- and post-laser lesion time points. (K) Kymograph-derived speed measurements (arrow in J shows exemplary path, see Supplementary videos 2 and 3) indicate increased process protrusion rate upon laser lesion, which was reduced under EPO (3 IU/mL, 24 hours). (L) Under EPO, a lower percentage of microglia migrates toward the laser lesion (Supplementary video 4). Numbers in bars refer to analyzed processes (K) or independent cultures (L). Mean  $\pm$  s.e.m. presented, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  (one- or two-tailed *t*-tests).



**Figure 2.** Erythropoietin (EPO) effects on pure microglia cultures. (A) EPO receptor (EPOR) mRNA levels decrease over time, independent of ATP. (B) Increasing number of ATP stimulated microglia (expressed in fold change of the placebo condition) passing through Boyden chamber membranes after 1, 3, and 4 hours, with EPO-treated cells showing less migration (area under the curve  $P=0.02$ ), (C) most obvious at 4 hours. (D) Boyden membrane slide mount with representative fields-of-view of Boyden chamber membranes of the Hoechst-33342-stained nuclei (inverted) at 4 hours of placebo (PL) or EPO-pretreated microglia  $\pm$  ATP exposure. (E) EPO pretreatment reduces ATP-stimulated Rac1 GTPase activation at 3 hours; representative western blots. (F) Proliferation or (G) cell death in these cultures. (H) MAPK signaling, investigated by monitoring pERK/ERK after 10 minutes, 1 hour, 2 hours, and 3 hours of 300  $\mu$ mol/L ATP and 3 IU/mL EPO or PL, shows accelerated inactivation by EPO. (I) EPO decreased microglial water soluble tetrazolium-1 (WST-1) conversion starting with the 2-hour ATP stimulus. Numbers of experiments are given in the bars. Mean  $\pm$  s.e.m. presented, \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  (two-tailed t-test).

(Figure 2B). Rac1 signaling is a critical component in the formation of lamellipodia, one of the earliest structural events in microglia motility.<sup>13,14</sup> ATP led to the expected increase in active/total Rac1,<sup>15</sup> which was attenuated by EPO (Figure 2E). Erythropoietin did not influence proliferation of microglia in this context but slightly reduced cell death in the basal (nonATP) condition (Figures 2F and 2G).

Since EPO was shown to inhibit overactivation of MAPK in astrocytes,<sup>16</sup> and MAPK signaling in turn is associated with Rac1 activity,<sup>12,17</sup> we determined the pERK/ERK ratio over time. Under EPO, the inactivation of the ERK pathway after ATP stimulation was accelerated, leading to a reduced pERK/ERK ratio at the 2-hour time point (Figure 2H). This acceleration could be a consequence of EPO-induced Rac1 inhibition, or indicate a mediator role of ERK for the reduced Rac1 activity under EPO.<sup>12,17</sup> We also noted that EPO decreased ATP-stimulated WST-1 (water soluble tetrazolium-1) conversion in microglia (Figure 2I), suggesting mitigated metabolic activity and lower superoxide production under EPO in conditions of injury.<sup>18</sup>

At three different levels of experimental complexity, ranging from *in vivo* to *in vitro*, we showed the capacity of EPO to reduce motility as an important feature of microglial (over)reaction to damage. This mechanism may underlie in part the beneficial effects observed for EPO in neurodegenerative diseases.

#### AUTHOR CONTRIBUTIONS

MM executed all microscopy data analyses, including the development of novel imaging and analytical approaches. LD, with the help of MaM and SG, performed all cell culture work including confocal microscopy. MM conducted the TPLSM experiments. HE and KAN developed study concept and design, supported in respective parts by MM, LD, UKH, RH, and JS. HE, MM, and LD wrote the manuscript, had full access to all data in the study and take responsibility for data integrity and accuracy of analyses. All authors have seen and approved the manuscript.

#### DISCLOSURE/CONFLICT OF INTEREST

HE holds user patents for EPO in stroke, schizophrenia, and multiple sclerosis. The remaining authors state no conflict of interest.

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