4）脊髄損傷後の機能評価

マーモセットの脊髄損傷後の機能評価は既にわれわれが報告した方法で行った(Iwanami et al, 2005)。いずれの損傷程度でも損傷直後は重度の四肢麻痺を呈するが、１５ｇと１７ｇ損傷群では上肢の機能を反映するBar-grip test, cage-climbing test, MIKY scoreでは早期に改善がみられたが、四肢の機能を反映する３次元自発運動量は２週以降に徐々に改善した。一方、２０ｇ損傷群では損傷後早期は完全麻痺を呈し、２週後に軽度の上肢の機能改善がMIKY scoreでみられるが、他の評価ではいずれも回復はみられなかった。その後４週以降に徐々に改善がみられたが、１０週の時点でも重度の麻痺を呈していた。特筆すべきは損傷後早期に残存する髄鞘が観察できれば、早期に完全麻痺を呈していても、その後機能的な改善が期待できる点である。

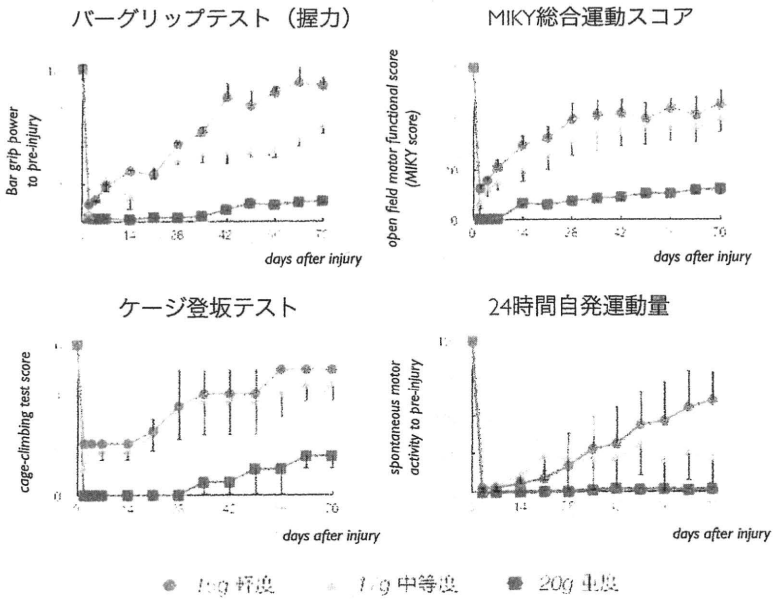

図4　運動機能評価

D．考察

MRIは非侵襲的に生体内部構造を観察できる方法であるが、従来のT1/T2強調像のみから脊髄損傷の重症度や機能的予後を正確に予測することは困難であった。脊髄損傷による軸索の断裂や神経細胞死、それに続いて起こる脱髄、空洞やグリア瘢痕形成等の変

化のうち、残余髄鞘面積が運動機能と相関があることを既に我々は報告した（Iwanami et al. JNR 2005）。今回の検討より、１）髄鞘可視化技術であるMyelin mapによって動物を生かしたままで脊髄内の髄鞘の変化を定量的に評価できる、２）脊髄損傷後の比較的早期に残余髄鞘を定量的に捉えることにより、機能的予後を早期に予測することできる可能性が示唆され、今後の脊髄再生医療における脊髄損傷後の重症度判定あるいは種々の治療法の効果判定における強力な評価法となりうると考えている。

E．結論

髄鞘可視化技術であるMyelin mapにより、脊髄損傷後の早期に機能的予後の予測が可能になる可能性が示唆された。

F．健康危険情報

特になし

G．研究発表

1. 論文発表

なし

2. 学会発表

口頭発表

in vivo QSIを用いた霊長類脊髄損傷モデルにおける損傷強度別経時的Myelin map
許斐 恒彦, 藤吉 兼浩, 疋島 啓吾, 岡野 栄之, 戸山 芳昭, 中村 雅也
第25回日本整形外科学会基礎学術集会, 京都, 日本　2010年10月14日

ポスター発表

Myelin map after graded spinal cord injury in adult common marmosets
Tsunehiko Konomi, Kanehiro Fujiyoshi, Keigo Hikishima, Fumika Toyoda, Yuichiro Takahashi, Satoshi Nori, Akimasa Yasuda, Yoshiaki Toyama, Masaya Nakamura, Hideyuki Okano
40th Annual meeting Neuroscience 2010, San Diego, CA, USA　2010年11月15日

H．知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（難治性疾患克服研究事業）
分担研究報告書

「肝細胞増殖因子による筋委縮性側索硬化症に対する新規治療法の開発」
－臨床試験実施に向けての研究開発－
1）カニクイザルによるGLP毒性試験、2）治験薬製造、3）プロトコール作成

研究分担者：　安達　喜一（クリングルファーマ株式会社 事業開発部長）
共同研究者：　阿部　哲士（クリングルファーマ株式会社 医薬開発部長）
福田　一弘（クリングルファーマ株式会社 研究開発部長）
井上　逸男（クリングルファーマ株式会社 品質保証部長）

研究要旨

筋萎縮性側索硬化症（ALS）を対象とする肝細胞増殖因子（HGF）の臨床試験実施に向けて、下記3項目を達成した。1）カニクイザルによるGLP毒性試験を行い、脊髄腔内投与によるHGFの安全性を確認した。2）脊髄腔内投与用の製剤化検討を行い、第Ⅰ相臨床試験で用いる治験薬を製造した。3）東北大学病院および東北大学TRセンターと協議を重ね、第Ⅰ相臨床試験のプロトコールを作成した。

A．研究目的

肝細胞増殖因子（HGF）の筋萎縮性側索硬化症（ALS）に対する第Ⅰ相臨床試験を開始するためには、1）カニクイザルによるGLP毒性試験、2）治験薬製造、3）プロトコール作成を行う必要がある。本年度は、上記3項目とそれに付帯する臨床試験の準備作業を行った。

B．研究方法

1）カニクイザルによるGLP毒性試験

HGF組換え蛋白質（3用量）をカニクイザル（各群雄4匹）の脊髄腔内に4週間持続投与したときの毒性変化を調べるとともに、4週間の休薬期間を設けてその回復性について検討した。また，そのときの全身的曝露についても評価した。対照群には、脊髄腔内投与用溶媒を被験物質と同様の方法で投与した。本試験は株式会社新日本科学に委託しGLP基準で実施した。

2）治験薬製造

HGF組換え蛋白質の製剤（液剤）化の試作検討を行い、既存の髄注製剤の使用実績を超えない範囲で賦形剤の組成を決定した。第Ⅰ相臨床試験に使用する注射液剤（開発コード：KP-100IT）と投与濃度に希釈するための希釈液を、東洋紡績株式会社に委託しGMP基準で製造した。また、KP-100ITの試作品を用いて予備安定性試験を開始した。

3）プロトコール作成

東北大学病院および東北大学TRセンターと協議を重ね、かつ開発業務受託機関（CRO）であるシミック株式会社のレビューを受けながら、第Ⅰ相臨床試験のプロトコールを作成した。

C．研究結果

1）カニクイザルによるGLP毒性試験

投与および休薬期間中、いずれの例においても死亡はみられなかった。また、

一般状態、一般症状および神経行動学的機能観察では、いずれの群においてもHGFに起因すると考えられる変化は認められなかった。病理組織学的検査では、臨床用量の60倍を超える高用量群においても毒性所見は認められなかった。以上の結果より、脊髄腔内投与によるHGFの安全性を確認し無毒性量を決定した。

2) 治験薬製造

第Ⅰ相臨床試験に使用するKP-100ITと希釈液をGMP基準で製造した。製造工程において異常は認められず、製造標準書どおりに製造することができた。製造した注射液剤と希釈液について規格試験を実施したところ、すべての規格に適合し、出荷可否判定において合格と判定された。

3) プロトコール作成

第Ⅰ相臨床試験の目的は、KP-100ITを脊髄腔内注射した時の安全性および認容性を評価すること、併せて髄液および末梢血中の薬物動態を検討することである。第Ⅰ相臨床試験ではALS患者を被験者とし、KP-100ITの単回投与と反復投与を行う。試験のデータは独立した効果安全性評価委員会で評価を受け、投与量・用法の妥当性、治験の継続などに関して助言を得る体制を構築する。なお、2010年12月10日に（独）医薬品医療機器総合機構と事前面談を行い、プロトコールの概要について相談した。

D. 考察

HGFの臨床試験実施に向けて、1) カニクイザルによるGLP毒性試験、2) 治験薬製造、3) プロトコール作成を滞りなく遂行することができた。今後は、治験実施機関である東北大学病院や東北大学TRセンターとさらに連携を深め、またCROのシミック株式会社と協調しながら第Ⅰ相臨床試験の準備を加速する必要がある。

E. 結論

HGFのALSに対する第Ⅰ相臨床試験を開始するための要件である3項目、1) カニクイザルによるGLP毒性試験、2) 治験薬製造、3) プロトコール作成を達成した。今後、第Ⅰ相臨床試験の準備を加速し、早期の試験開始を目指す。

F. 健康危険情報

特記なし。

G. 研究発表

1. 論文発表

なし。

2. 学会発表

なし。

H. 知的財産権の出願・登録状況

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

研究成果の刊行に関する一覧

研究成果の刊行に関する一覧

原著論文

著者	論文タイトル	掲載誌名	巻 頁	出版年
Mukaino M, Nakamura M, Yamada O, Okada S, Morikawa S, Renault-Mihara F, Iwanami A, Ikegami T, Ohsugi Y, Tsuji O, Katoh H, Matsuzaki Y, Toyama Y, Liu M, Okano H.	Anti-IL-6-receptor antibody promotes repair of spinal cord injury by inducing microglia-dominant inflammation.	Exp Neurol	224(2) 403-414	2010
Tsuji O, Miura K, Okada Y, Fujiyoshi K, Mukaino M, Nagoshi N, Kitamura K, Kumagai G,Nishino M,Tomisato S,Higashi H, Nagai T, Katoh H, Kohda K, Matsuzaki Y, Yuzaki M, Ikeda E, Toyama Y, Nakamura M, Yamanaka S, Okano H.	Therapeutic potential of appropriately evaluated 'safe' induced pluripotent stem cells for spinal cord injury.	Pro Nat Acad Sci USA	13;107(28) 12704-12709	2010
Takahashi Y, Tsuji O, Kumagai G, Miyauchi HC, Okano HJ, Miyawaki A,Toyama Y, Okano H, Nakamura M	Comparative study of methods for administering neural stem/progenitor cells to treat spinal cord injury in mice.	Cell Transplant		in press
Nagoshi N, Shibata S, Hamanoue M, Mabuchi Y, Matsuzaki Y, Toyama Y,Nakamura M, Okano H	Schwann Cell Plasticity After Spinal Cord Injury Shown by Neural Crest Lineage.	Glia		in press
Shang J., Deguchi K., Yamashita T., Ohta Y., Zhang H., Morimoto N., Liu N., Zhang X., Tian F., Matsuura T., Funakoshi H., Nakamura T., and Abe K	Anti-apoptotic and anti-autophagic effects of GDNF and HGF after transient MCAO in Rats.	J. Neurosci. Res	88(10) :2197-206	2010
Funakoshi H., and Nakamura T	Hepatocyte growth factor (HGF): Neurotrophic functions and therapeutic implications for neuronal injury/diseases	Current Signal Transduction Therapy		in press
Kadoyama K., Kadoyama K., Funakoshi H., Nakamura T., and Sakaeda T.	Therapeutic Potential of Hepatocyte Growth Factor for Treating Neurological Diseases	Current Drug Therapy		in press

研究成果の刊行に関する一覧

著者	論文タイトル	掲載誌名	巻 頁	出版年
青木正志、割田　仁、糸山泰人	ALSに対するヒト型組み換えHGF蛋白を用いた第1相試験（治験）	JALSA	82 10-12	2011
肝細胞増殖因子（HGF）	広範囲血液・尿検査、免疫学的検査（４） ーその数値をどう読むかー	日本臨床	68巻 増刊号7 121-130	2010

Experimental Neurology 224 (2010) 403–414

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Anti-IL-6-receptor antibody promotes repair of spinal cord injury by inducing microglia-dominant inflammation

Masahiko Mukaino [a,b], Masaya Nakamura [c,*], Osamu Yamada [b], Seiji Okada [d], Satoru Morikawa [e], Francois Renault-Mihara [b], Akio Iwanami [c,f], Takeshi Ikegami [c,f], Yoshiyuki Ohsugi [g], Osahiko Tsuji [c], Hiroyuki Katoh [f], Yumi Matsuzaki [b], Yoshiaki Toyama [c], Meigen Liu [a], Hideyuki Okano [b,*]

[a] Department of Rehabilitation Medicine, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan
[b] Department of Physiology, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan
[c] Department of Orthopedic Surgery, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan
[d] Department of Research Superstar Program Stem Cell Unit, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan
[e] Department of Dentistry and Oral Surgery, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan
[f] National Hospital Organization, Murayama Medical Center, Musashimurayama, Tokyo 208-0011, Japan
[g] Chugai Pharmaceutical Co. Ltd., Chuo, Tokyo 103-8324, Japan

ARTICLE INFO

Article history:
Received 16 October 2009
Revised 29 April 2010
Accepted 30 April 2010
Available online 16 May 2010

Keywords:
Spinal cord injury
Interleukin-6
Antibody
Inflammation
Microglia
Hematogenous macrophages

ABSTRACT

We previously reported the beneficial effect of administering an anti-mouse IL-6 receptor antibody (MR16-1) immediately after spinal cord injury (SCI). The purpose of our present study was to clarify the mechanism underlying how MR16-1 improves motor function after SCI. Quantitative analyses of inflammatory cells using flow cytometry, and immunohistochemistry with bone marrow-chimeric mice generated by transplanting genetically marked purified hematopoietic stem cells, revealed that MR16-1 dramatically switched the central player in the post-traumatic inflammation, from hematogenous macrophages to resident microglia. This change was accompanied by alterations in the expression of relevant cytokines within the injured spinal cord; the expression of recruiting chemokines including CCL2, CCL5, and CXCL10 was decreased, while that of Granulocyte/Macrophage-Colony Stimulating Factor (GM-CSF), a known mitogen for microglia, was increased. We also showed that the resident microglia expressed higher levels of phagocytic markers than the hematogenous macrophages. Consistent with these findings, we observed significantly decreased tissue damage and reduced levels of myelin debris and Nogo-A, the axonal growth inhibitor, by MR16-1 treatment. Moreover, we observed increased axonal regeneration and/or sprouting in the MR16-1-treated mice. Our findings indicate that the functional improvement elicited by MR16-1 involves microglial functions, and provide new insights into the role of IL-6 signaling in the pathology of SCI.

Introduction

In the pathology of spinal cord injury (SCI), the primary mechanical injury is followed by post-traumatic inflammation, in which inflammatory cells such as neutrophils, hematogenous macrophages (blood-borne macrophages), and resident microglia accumulate at the lesion site. These inflammatory cells release reactive oxygen, nitrogen radicals, and proteases, which exacerbate tissue damage (Hausmann, 2003). Because the inflammation is regulated by pro-inflammatory cytokines, such as TNFα, IL-1β, and IL-6, these cytokines have been targets for potential pharmaceutical interventions for SCI (Nesic et al., 2001; Okada et al., 2004; Sharma et al., 2003; Tuna et al., 2001). Among these cytokines, IL-6 is known to promote the activation and infiltration of macrophages/microglia (Hurst et al., 2001; Van Wagoner and Benveniste, 1999), which are the major inflammatory cells in the injured spinal cord, and the overexpression of IL-6 extends the inflammation to worsen the tissue injury (Klusman and Schwab, 1997; Lacroix et al., 2002). Hypothesizing that a blockade of IL-6 signaling might reduce the extension of injury by post-SCI inflammation, we previously administered the anti-mouse IL6-receptor antibody (MR16-1) after SCI and demonstrated reduced inflammation, decreased astrogliosis, and enhanced tissue sparing, leading to improved functional recovery (Okada et al., 2004). As a humanized antibody for the human IL-6 receptor (MRA; tocilizumab) is already in clinical use for the treatment of Castleman's disease and rheumatoid arthritis (Choy et al., 2002; Nishimoto et al., 2000; Sato et al., 1993), this drug might represent a new option for the treatment of SCI.

* Corresponding authors. H. Okano is to be contacted at the Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan. M. Nakamura, Department of Orthopedic Surgery, Keio University School of Medicine, Shinjuku, Tokyo 160-8582, Japan. Fax: +81 3 3357 5445.
E-mail addresses: masa@sc.itc.keio.ac.jp (M. Nakamura), hidokano@sc.itc.keio.ac.jp (H. Okano).

0014-4886/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2010.04.020

However, recent studies using gene-knockout animals revealed that the continuous inhibition of IL-6 signaling is detrimental to functional recovery, by inhibiting axonal regeneration or causing failed gliosis, implying that IL-6 may also have a beneficial function in spinal cord repair (Cafferty et al., 2004; Okada et al., 2006). Furthermore, numerous studies suggest that inflammation is beneficial or even essential for spinal cord repair, because it clears tissue debris and involves the secretion of various neurotrophic factors (Donnelly and Popovich, 2008; Hashimoto et al., 2005; McTigue et al., 2000). This discrepancy prompted us to investigate how the administration of an anti-IL-6 receptor antibody immediately after SCI promotes the repair process.

One of the important determinants of the extent of secondary damage by inflammation is the nature of the recruited inflammatory cells. For example, the transplantation of macrophages that have been co-incubated with peripheral nerves or skin improves spinal cord repair (Bomstein et al., 2003; Schwartz et al., 1999). On the other hand, zymosan-activated macrophages have neurotoxic properties, although they can also have proregenerative effects (Gensel et al., 2009; Popovich et al., 2002; Steinmetz et al., 2005). Previous reports have shown that these differences in the characteristics of inflammatory cells depend not only on their state of activation, but also on their origin. A subpopulation of hematogenous macrophages is more cytotoxic than microglia, and their excessive infiltration into a lesion is detrimental to spinal cord repair (Gris et al., 2004; Popovich et al., 1999). Since IL-6 is known to promote macrophage infiltration after central nervous system (CNS) trauma (Klusman and Schwab, 1997; Lacroix et al., 2002), here we focused on the effect of the temporary inhibition of IL-6 signaling by MR16-1 on macrophages and microglia after SCI. The administration of MR16-1 reduced the infiltration of macrophages into the injured spinal cord, but increased the number of microglia residing there, thus switching the major inflammatory cell type at the lesion from hematogenous macrophages to resident microglia. A comparison of the expression of phagocytic markers by hematogenous macrophages and microglia revealed that the microglia had greater phagocytic ability against myelin debris after SCI. Consequently, this switch in major inflammatory cell type resulted in improved tissue sparing and debris clearance, which promoted neural repair after SCI.

Materials and methods

Animals

74 adult female C57BL/6 J mice (8–10 weeks old) were used. C57BL/6 background CAG-EGFP transgenic mice that ubiquitously express EGFP under the control of the CAG promoter (Kawamoto et al., 2000) were kindly provided by Professor Jun-ichi Miyazaki (Osaka University, Osaka, Japan) and were bred in our animal facility. The ethics committee of our institution approved all the surgical and animal care procedures, in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals (National Institutes of Health), and the Guidelines and Policies for Animal Surgery provided by the Animal Study Committees of the Central Institute for Experimental Animals and of Keio University.

Rat anti-mouse IL-6 receptor monoclonal antibody (MR16-1)

The rat anti-mouse IL-6 receptor monoclonal antibody MR16-1 was prepared as described previously (Tamura et al., 1993). MR16-1 binds to the mouse IL-6 receptor and suppresses IL-6-induced cellular responses in a dose-dependent manner (Okazaki et al., 2002). Other basic characterizations of this antibody were reported previously (Okazaki et al., 2002; Tamura et al., 1993).

Purification and transplantation of genetically marked hematopoietic stem cells (HSCs)

In the present study, we produced bone marrow-chimeric mice using highly purified, genetically marked hematopoietic stem cells by the method reported by Koide et al. (2007). This method enabled us to limit our observation specifically to the hematopoietic cell lineage. The femurs and tibias were dissected from donor CAG-EGFP transgenic mice and crushed with a pestle. The marrow cells were collected in HBSS+ (Hanks-balanced salt solution supplemented with 2% FCS, 10 mM HEPES, and 1% penicillin/streptomycin), filtered through a cell strainer (Falcon 2350) to remove debris, and suspended in 50 ml of ice-cold HBSS+. The cells were collected by centrifugation at 280 g for 6 min at 4 °C, resuspended in HBSS+ at 1×10^6 cells/ml, and incubated with 5 μg/ml Hoechst 33342 (Sigma Chemical Co.) for 60 min at 37 °C. A parallel aliquot was stained with Hoechst dye in the presence of 50 μM reserpine (Sigma Chemical Co.). The cells were spun down, resuspended in 5 ml of ice-cold HBSS+, and layered on top of 5 ml of Ficoll-Paque™ Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden). After centrifugation at 630 g at 4 °C for 20 min, the mononuclear cells were collected from the intermediate layer and immediately washed with 10 ml of ice-cold HBSS+. The Hoechst-stained cells were resuspended in ice-cold HBSS+ at $1–5\times10^7$ cells/ml and stained for 30 min on ice with one of the following monoclonal antibodies: biotinylated CD34 (RAM34), PE-conjugated Sca-1 (Ly6A/E), or APC-conjugated c-Kit (2B8). Biotinylated antibodies were visualized with Cy7-APC-conjugated streptavidin. All of these reagents were purchased from eBioscience (San Diego, CA). An aliquot of cells was also stained with a mouse isotype control conjugated with FITC, PE, or APC. After antibody staining, the cells were washed in an excess volume of HBSS+ and resuspended at 1×10^7 cells/ml in HBSS+ containing 2 μg/ml propidium iodide (PI; Sigma Chemical Co.). Genetically marked, highly purified HSCs ($CD34^-$ $Sca\text{-}1^+$ $c\text{-}kit^+$ SP cells (Matsuzaki et al., 2004)) derived from donor CAG-EGFP transgenic animals were sorted by flow cytometry. A 100-μl aliquot of unfractionated marrow cell suspension (2×10^5 cells) from donors not carrying the CAG-EGFP transgene was added to provide competitor cells, which is the minimum dose to keep the animals alive during the period required for bone marrow reconstitution. A suspension of 100 $CD34^-$ $Csa\text{-}1^+$ $c\text{-}kit^+$ SP cells and 2×10^5 unfractionated marrow cells was then intravenously injected into the retro-orbital plexus of an etherized recipient mouse that had been lethally irradiated at 10.5 Gy. The successful induction of chimerism was confirmed by a Dual-laser FACS Calibur (Becton and Dickinson, CA) analysis of the peripheral blood.

Spinal cord injury model

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The dorsal surface of the dura mater at the T10 level was exposed by laminectomy, and a moderate (impact force = 60 kdyn) contusion injury was induced using an IH impactor, as reported previously (Cafferty et al., 2004; Glass et al., 2001). The muscles and skin were closed in layers, and the animals were placed in a temperature-controlled chamber until thermoregulation was reestablished. Manual voiding of the bladder was performed twice per day until reflex bladder emptying was reestablished.

Injection of MR16-1 and BrdU

Immediately after SCI, mice were given a single intraperitoneal injection of MR 16-1 (100 μg/g body weight; MR16-1-treated group) or the same volume and concentration of purified rat IgG (ICN/Cappel Ohio; control group). To label the cells that divided after the injury, a sterile solution of bromodeoxyuridine (BrdU; 50 μg/g body weight; Sigma) was injected intraperitoneally immediately after the injury, and then every 24 h for 4 days after SCI (a total of 5 times).

Immunohistochemistry

At 4, 7, 14, and 42 days after SCI, animals in the MR16-1-treated and control groups were deeply anesthetized by inhalation of diethyl ether and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). The spinal cord tissue was removed and post-fixed in 4% paraformaldehyde in PBS for a few hours at room temperature. The tissue samples were immersed in 10% sucrose in PBS at 4 °C for 24 h, then placed in 30% sucrose in PBS for 48 h, and embedded in OTC compound. The embedded tissue was immediately frozen in liquid nitrogen and stored at −80 °C until use. Frozen spinal cord tissues were sectioned on a cryostat at 20 μm, either in the axial or sagittal plane. Luxol fast blue and Oil red O staining were performed to evaluate the spared myelin and myelin debris.

For immunohistochemistry with fluorescent antibodies, spinal cord sections were permeabilized with 0.03% Triton X-100 and 10% normal goat serum in 0.01 M PBS, pH 7.4, for 30 min. The following primary antibodies were applied overnight at 4 °C: rat anti-CD11b, 1:200 (Serotec, Oxford, United Kingdom); rabbit anti-Iba-1, 1:200 (Wako Pure Chemical Industries, Osaka, Japan); rat anti-LAMP2, 1:200 (Abcam, Cambridge, UK); rat anti-Mac2, 1:200 (Cedarlane, Hornby, Ontario); rabbit anti-GFP, 1:500 (MBL, Nagoya, Japan); goat anti-GFP, 1:500 (Rockland Immunochemicals, Gilbertsville, PA); or chick anti-GFP, 1:500 (Aves Lab, Tigard, OR). The sections were then incubated for 1 h at room temperature with secondary antibodies conjugated with Texas red or fluorescein isothiocyanate (FITC; all from Jackson Immunoresearch, West Grove, PA). The sections were then washed, wet-mounted, and examined by epi-fluorescence. Multiple staining with Oil red O was performed by the method reported by Koopman et al. (2001).

For diaminobenzidine (DAB; Sigma) staining, mouse anti-Neurofilament 200kD antibody, 1:200 (Chemicon, Temecula, CA) or goat anti-5HT, 1:200 (Immunostar, Hudson, WI) was used as the primary antibody, followed by horseradish peroxidase (HRP)-labeled goat anti-mouse IgG or donkey anti-goat IgG as the secondary antibody. The staining was visualized with DAB, and the sections were washed, dehydrated, cleared in xylene, and mounted.

Quantitative analyses

For quantitative analyses, three representative midsagittal or axial sections through the injured portion of the spinal cord of each mouse were selected randomly and captured at 50× magnification. The areas of tissue immunopositive for CD11b, Iba-1, LAMP2, Mac2, Neurofilament 200kD, and Nogo-A, and those stained with Oil red O (ORO) and Luxol Fast Blue (LFB) were quantified using the ImageJ software and MCID system (Imaging Research, Inc., St. Catharines, Ontario, Canada). To count the number of macrophages/microglia, three midsagittal sections through the injured portion of the spinal cord of each mouse were selected randomly, and the number of CD11b- or Iba-1- and/or EGFP-immunopositive cells contained within a cephalocaudal stretch of 500 μm at the indicated levels was counted. To confirm the CD11b-Iba-1 double-staining as a marker of macrophages/microglia the ratio of CD11b-Iba-1 double-stained cells to total CD11b-positive cells was also quantified. To count the LAMP2- andMac2-positive cells, three midsagittal sections through the injured portion of the spinal cord of each mouse were selected randomly, and a threshold was determined from the basal fluorescence of a portion of intact tissue. From the epicenter area (0–1-mm caudal and rostral to the epicenter), 15 non-overlapping high-power fields were chosen at random (630× magnification; total area 0.39 mm^2). An immunopositive cell was defined as a cell with staining over 10% of its soma, as determined by the MCID system, and the number of stained and unstained cells was counted manually. For the quantification of $5HT^+$ fibers, five regions (the dorsal horn and ventral horn of both sides, and the site around the central canal) from each axial section of the cord, 2-mm caudal to the epicenter, were captured at ×200 magnification, and the area of the $5HT^+$ tissue in each field was quantified using the MCID system. The light intensity and threshold values were maintained at constant levels for all analyses.

Flow cytometry

Mice were transcardially perfused with 0.1 M phosphate-buffered saline, and the spinal cords were harvested. The injured portion of each spinal cord (6 mm) was surgically dissected, digested with collagenase, mechanically homogenized, and passed through a wire mesh screen (Sigma-Aldrich Canada Ltd., Ontario, Canada) to obtain a single-cell suspension. The cells were washed in PBS containing 3% FBS, and incubated for 5 min on ice with Fc Block and 30 min on ice with fluorescent antibodies. Flow cytometric analysis was performed using a FACS Calibur (Becton Dickinson) and MoFlo (Dakocytomation), and the data were analyzed using Cell Quest software. The cells were stained with antibodies against CD11b-PE, CD45-FITC, and CD45-APC (eBioscience, San Diego, CA), and were classified according to their expression level of CD45 (common leukocyte antigen) and CD11b (complement 3 receptor), with $CD11b^+$ $CD45^{high}$ indicating hematogenous macrophages and $CD11b^+$ $CD45^{low}$ indicating resident microglia, as reported previously (Sedgwick et al., 1991). At least 1.0×10^6 cells were analyzed for each spinal cord sample.

Western blot analysis

Twenty-four hours after the injury, the spinal cord tissue at the lesion epicenter (6 mm in length) was dissected from the mice (four animals per group and four sham-operated animals), homogenized in MAPK lysis buffer containing protease inhibitors, sonicated, and spun at 15,000 rpm. The proteins in the supernatants were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane by electrophoresis. The membranes were blocked for 1 h at room temperature in TBST buffer containing 4% non-fat milk, NaCl (150 mM), and 0.05% Tween 20. The blots were then incubated with primary polyclonal rabbit anti-CCL2 antibody (1:2000; Abcam, Cambridge, MA), goat anti-CCL5 antibody, 1:500 (eBioscience, San Diego, CA); rabbit anti-CXCL-10 antibody, 1:500 (Cedarlane, Hornby, Ontario); rabbit anti-GM-CSF antibody, 1:2000 (Abcam, Cambridge, MA); or mouse anti-α-tubulin antibody, 1:500 followed by a secondary HRP-conjugated anti-rabbit, goat, or mouse IgG antibody. The blots were visualized with the ECL Blotting Analysis System (Amersham, Arlington Heights, IL).

Real time RT-PCR

A 4-mm-long spinal cord segment at T10 was collected at the indicated times, the total RNA was isolated using an RNeasy Kit (Qiagen Science, Maryland, USA), and cDNA was obtained by reverse transcription. The cDNA synthesis was performed at 42 °C for 50 min in a final volume of 20 μl, following the manufacturer's instructions for Superscript II RNase H Reverse Transcriptase (Invitrogen). The template cDNA was normalized to the β-actin mRNA. Real time RT-PCR was performed using an Mx3000P thermal cycler (Stratagene) with SYBR green (TaKaRa RR041A). For every set of RT-PCR analyses, at least three independent experiments were performed. The amplification was performed using the following primers: CCL2, sense 5′-GCATCCACGTGTTGGCTCA-3′, antisense 5′-CTCCAGCCTACTCATTGGGATCA-3′; CCL5, sense 5′-AGATCTCTGCAGCTGCCCTCA-3′, antisense 5′-GGAGCACTTGCTGCTGGTGTAG-3′; CXCL10, sense 5′-TGAATCCGGA ATCTAAGACCATCAA-3′, antisense 5′-AGGACTAGCCATCCACTGG GTAAAG-3′ (purchased from Takara, Kyoto, Japan) and GM-CSF, sense 5′-AAGGTCCTGAGGAGGA TGTG-3′, antisense 5′-GAGGTTCAGGGCTTCTTTGA-3′ (purchased from Hokkaido System Science, Sapporo, Japan).

Statistical analysis

Values are reported as the mean ± SEM. Statistical significance was analyzed using the unpaired Student's *t*-test, and significance was accepted at $P<0.05$.

Results

Anti-IL-6 receptor antibody treatment reduced inflammatory cell accumulation

To examine the effect of MR16-1 on the infiltration of inflammatory cells after SCI, immunostaining for CD11b and Iba-1 was performed. Although CD11b is known to be expressed by granulocytes and some T cells, 93.6 ± 3.3% of the infiltrated cells were CD11b and Iba-1 double-positive at 4, 7, and 14 days post-injury (dpi), indicating that the immunocompetent cells present at the injured site at those times after SCI were mostly hematogenous macrophages and resident microglia (Figs. 1A–E). While the CD11b-positive area increased in both the MR16-1 and control groups, with a peak at 7 dpi, the MR16-1-treated animals showed a significantly smaller $CD11b^+$ area compared to the control animals: a non-significant difference at 4 and 7 dpi that developed to a significant difference at 14 dpi (Fig. 1F). These findings suggest that MR16-1 administration reduced the accumulation of $CD11b^+$ cells at the late stage of inflammation, even though MR16-1 was only administered once, at the acute stage.

To determine whether the administration of MR16-1 alters the subtype of $CD11b^+$ cells at the acute stage, the profiles of the recruited $CD11b^+$ cells were analyzed using flow cytometry. Homogenates of the injured spinal cord were analyzed to determine the ratio of hematogenous macrophages to total $CD11b^+$ cells. We first confirmed that within the $CD11b^+$ population, hematogenous macrophages could be distinguished from the resident microglia by their expression level of CD45, as reported previously (Sedgwick et al., 1991). At 4 dpi, 37.6 ± 3.9% of the $CD11b^+$ cells were $CD45^{high}$ hematogenous macrophages in the control group, whereas only 22.3 ± 2.9% of these cells were $CD45^{high}$ in the MR16-1-treated group. Although the proportion of hematogenous macrophages had increased in both groups at 7 dpi, the difference in their proportions between the MR16-1-treated and control mice was significant (57.8 ± 1.9% in the control group, 41.0 ± 2.2% in the MR16-1-treated group) (Figs. 1G–I). MR16-1 treatment thus reduced the relative abundance of hematogenous macrophages in the injured spinal cord.

MR16-1 treatment caused the central player in the inflammation after SCI to shift from hematogenous macrophages to resident microglia

To determine whether MR16-1 specifically affects the recruitment of hematogenous macrophages into the injured spinal cord, contusive SCI was induced in chimeric mice, which were generated by irradiating

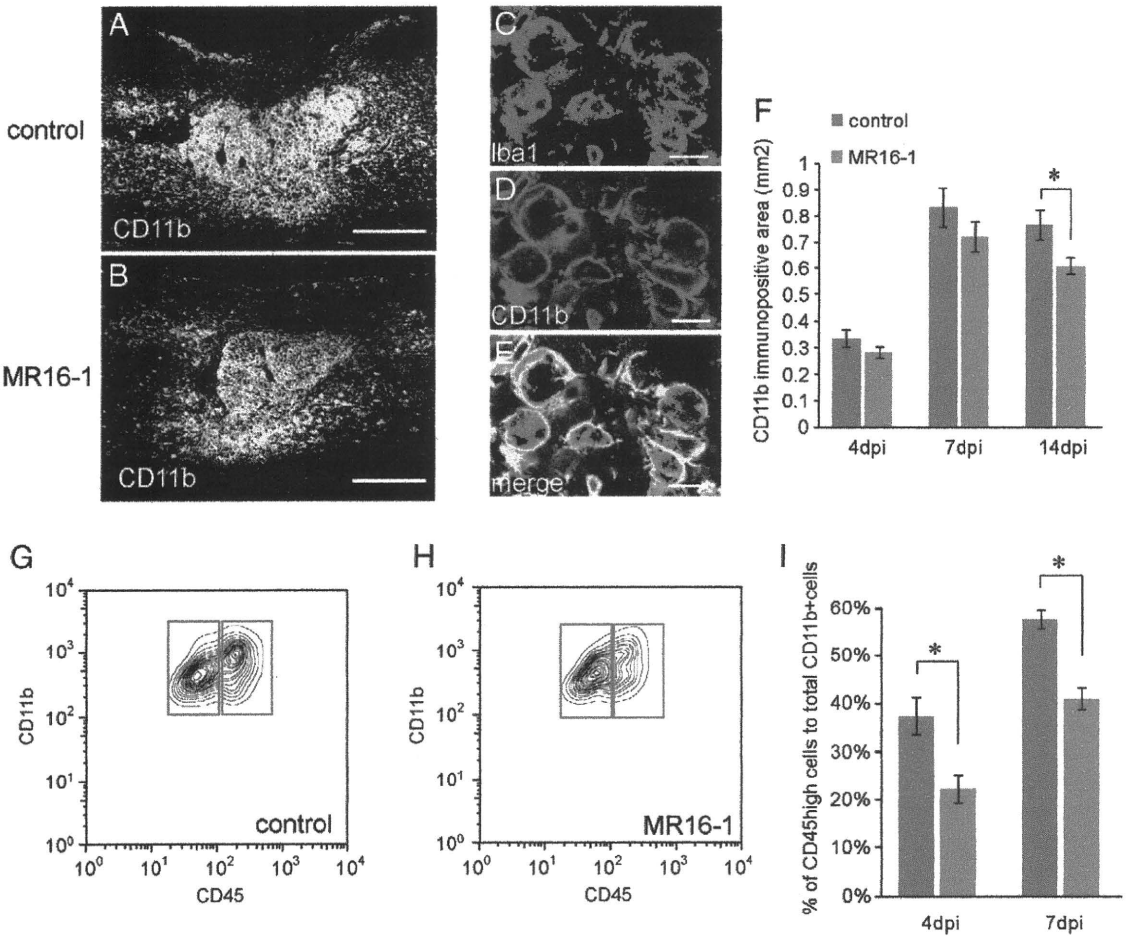

Fig. 1. MR16-1 treatment accelerates the resolution of inflammation. A, B, F: There was a significant difference in the $CD11b^+$ area between the control group (A) and the MR16-1-treated group (B) at 14 dpi, but not at 4 or 7 dpi (F). C–E: 96.9 ± 1.3% of the $CD11b^+$ cells (D) were double-labeled with Iba1 (C, E), indicating that the accumulated $CD11b^+$ cells were inflammatory macrophages/microglia. G–I: MR16-1-treatment decreased the proportion of hematogenous macrophages in the injured spinal cord at 4 and 7 dpi. Hematogenous macrophages ($CD11b^+CD45^{high}$, blue box) and microglia ($CD11b^+CD45^{low}$, red box) were identified according to their levels of CD11b and CD45 expression. I: The proportion of hematogenous macrophages within the $CD11b^+$ population was significantly lower in the MR16-1-treated animals than in the control group at 4 and 7 dpi. Values are means ± SEM. *$P<0.05$. Scale bars = 500 μm in A, B; 20 μm in C–E.

recipient mice and then transplanting purified EGFP-expressing HSCs into them (Matsuzaki et al., 2004). Flow cytometric analysis revealed that 3 months after the HSC transplantation, 88.34 ± 1.45% of the CD11b$^+$ leukocytes in the blood of the chimeric mice were EGFP-positive. In contrast, only 1.57% of the CD11b$^+$ cells in the uninjured spinal cord expressed EGFP, suggesting that the recruitment of microglia from the hematopoietic pool was a rare event. Since the irradiation dose required for this method is rather high (10.5 Gy), which could affect microglial turnover (Mildenberger et al., 1990), we quantified the accumulation of CD11b+ cells at the lesion of chimeric mice, and compared it to that of wild-type mice. There was no significant difference in the number of CD11b+ cells between the chimeric mice and wild-type mice at 4, 7, or 14 dpi ($P = 0.60$, 0.76, 0.67, respectively). This result is consistent with the previous report by Turrin et al. (2007), which showed that the chimerization with 10 Gy irradiation dose does not significantly affect the acute inflammatory response. Thus, these chimeric mice enabled us to distinguish in situ hematogenous macrophages from endogenous microglia by their EGFP immunoreactivity (Figs. 2A–C), and examine the precise spatio-temporal localization of these cell populations after SCI.

Consistent with our findings using wild-type mice, the total number of recruited CD11b$^+$ macrophages/microglia was comparable between the MR16-1-treated and control groups at 4 dpi (not shown), but the proportion and distribution of the hematogenous macrophages and microglia were completely different (Figs. 2D, E, G, and I). In the MR16-1-treated group, there were significantly fewer CD11b$^+$EGFP$^+$ hematogenous macrophages at the lesion site (Fig. 2G), and there were significantly more CD11b$^+$EGFP$^-$ resident microglia, especially in areas 1.0- to 2.0-mm away from the lesion site (Fig. 2I). Thus, at 4 dpi, the MR16-1 treatment led to a reduced accumulation of hematogenous macrophages at the lesion epicenter, and an increased number of microglia at sites rostral and caudal to the lesion epicenter.

Similarly, at 7 dpi, although the distribution of CD11b$^+$ cells was comparable in the control and MR16-1-treated groups (Fig. 2F), the composition of the CD11b$^+$ population was dramatically different. In the MR16-1-treated group, the CD11b$^+$EGFP$^+$ hematogenous macrophage accumulation at the lesion epicenter was significantly reduced (Fig. 2H), and the CD11b$^+$EGFP$^-$ resident microglia had significantly increased (Fig. 2J). These results indicate that the central player in the inflammation after SCI shifted from being hematogenous macrophages to being resident microglia, following MR16-1-treatment.

MR16-1 treatment reduced the expression of macrophage-recruiting chemokines and increased the GM-CSF level at the lesion site

Although there is no evidence that IL-6 directly stimulates the infiltration or proliferation of inflammatory cells, it does affect the expression of various cytokines; furthermore, the blockade of IL-6 signaling during inflammation causes a drastic change in the cytokine profile, including the chemokines and colony-stimulating factors (CSFs) (Matsumura et al., 1999; Romano et al., 1997). Because the infiltration of hematogenous macrophages is mediated by chemokines (Babcock et al., 2003; Romano et al., 1997) and the increased proliferation of microglia is mainly controlled by CSFs (Giulian and Ingeman, 1988; Lee et al., 1994), we examined the expression levels of CCL2 (MCP-1), CCL5 (RANTES), CXCL10 (IP-10), and GM-CSF, which are representative cytokines known to direct cell infiltration and proliferation, by quantitative real time PCR, 12 h after injury. The mRNA levels of CCL2, CCL5, and CXCL10 were significantly attenuated by MR16-1-administration compared to the control group (to 15.0%, 49.7% and 30.8% of the control levels, respectively), whereas the GM-CSF mRNA level was significantly increased (to 214% of the control level) (Figs. 3A–D). We also quantified the cytokine proteins by western blotting. The protein level of CCL2 was significantly decreased (to 80.7% of the control level), whereas the GM-CSF level was significantly increased (to 193% of the control level). Although the difference did not reach to statistical significance, we also observed a tendency for the protein levels of CCL5 and CXCL10 to decrease (Figs. 3E–H).

Microglia had higher phagocytic capacities than hematogenous macrophages

To determine whether the shift in the major inflammatory cells by MR16-1 treatment affected the inflammatory process, we further characterized the hematogenous macrophages and the microglia. The phagocytosis of tissue debris by inflammatory cells is a pivotal process for spinal cord repair after injury, as the debris includes various cytotoxic agents and axonal growth inhibitory factors. Quantitative analysis revealed that the expression of LAMP2, a marker for endosomes/lysosomes, was significantly increased at the lesion epicenter in the MR16-1-treated group compared to the control group, at 4, 7, and 14 dpi (Figs. 4A–C). Previous reports showed that the resident microglia have higher phagocytic activity than the infiltrating hematogenous macrophages (Rinner et al., 1995; Schilling et al., 2005). Consistent with these reports, we found that significantly more Iba1$^+$EGFP$^-$ resident microglia expressed LAMP2 than did the Iba1$^+$EGFP$^+$ hematogenous macrophages at 4 and 7 dpi (Figs. 4D–G).

In addition, we performed immunostaining with Mac2, which is reported to participate in the phagocytosis of myelin (Rotshenker et al., 2008). Although Mac2 was expressed on the cell membrane of most of the accumulated cells, only a portion of the macrophages/microglia expressed Mac2 in their cytoplasm. Consistent with the LAMP2 results, more of the Iba1$^+$EGFP$^-$ resident microglia than Iba1$^+$EGFP$^+$ hematogenous macrophages showed cytoplasmic expression of Mac2 (Figs. 5A–D, and I). Similarly, the average size and intracellular Mac2$^+$ area of the resident microglia were significantly greater at 7 dpi than those of hematogenous macrophages (Figs. 5J and K). We also performed triple staining for Iba-1, EGFP, and Oil red O using the method reported by Koopman et al. (2001) to observe phagocytosed lipid, which is derived from myelin. The Oil red O$^+$ area in each cell body was significantly greater in the Iba1$^+$EGFP$^-$ resident microglia than in the Iba1$^+$EGFP$^+$ hematogenous macrophages, and was increased at 7 dpi by the MR16-1 treatment (Figs. 5E–H, L).

MR16-1 treatment promotes repair of the spinal cord

To examine the effect of the altered inflammatory response on spinal cord repair, we evaluated the clearance of myelin debris by Oil red O staining and Nogo-A immunostaining, as well as the sparing of myelin sheath, which is evaluated by Luxol Fast Blue staining. At 14 and 42 dpi, the Oil red O$^+$ area was significantly decreased in the MR16-1-treated group compared to the control group (Figs. 6A–C). The deposition of Nogo-A, the major myelin-derived axonal growth inhibitor, was also decreased by MR16-1 treatment (Figs. 6D–F). Furthermore, we evaluated the spared myelin sheath using Luxol fast blue staining. At the lesion epicenter, the area of the spared myelin sheath in the MR16-1-treated group was significantly greater than in the control group at 14 and 42 dpi (Figs. 6G–I).

To determine the effect of MR16-1 on repair of neural tissue, we quantified the RT-97$^+$ (Neurofilament 200kD) fibers at the lesion epicenter and the 5-HT$^+$ (serotonergic) fibers that were caudal to the lesion site. There was no significant difference in the area of RT-97$^+$ fibers between the two groups at 14 dpi, but a significantly larger RT-97$^+$ area was observed in the MR16-1-treated group at 42 dpi (Figs. 6J–K). Similarly, there was no significant difference in the 5-HT$^+$ area between the two groups at 14 dpi. Although there was a slight increase in 5-HT$^+$ fibers at 42 dpi, even in the control group, which is characteristic of contusive SCI, a significantly larger area of 5-HT$^+$

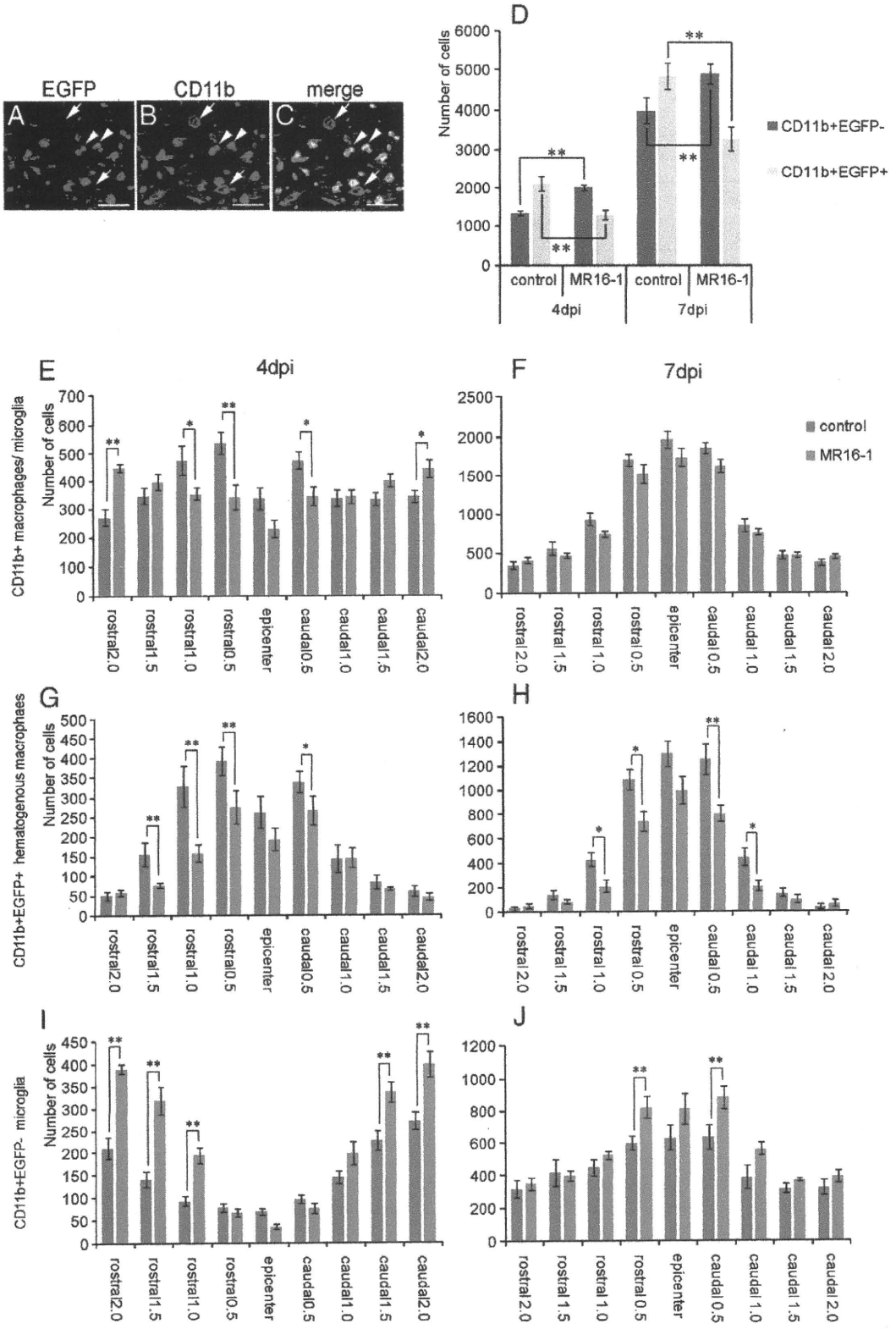

Fig. 2. MR16-1 treatment switches the major inflammatory cell type, from hematogenous macrophages to resident microglia. Analysis using chimeric mice. A–C: Immunostaining for EGFP (A, green) and CD11b (B, red), showing the distribution of $CD11b^{+}EGFP^{+}$ hematogenous macrophages (arrowheads) and $CD11b^{+}$ $EGFP^{-}$ resident microglia (arrows) in the injured spinal cord (C, merged). D: In the MR16-1-treated group, the major player in the inflammation switched from $CD11b^{+}EGFP^{+}$ hematogenous macrophages (light gray) to $CD11b^{+}EGFP^{-}$ resident microglia (dark gray) at 4 and 7 dpi. Number and distribution of macrophages/microglia in the spinal cord at 4 and 7 dpi. E, F: MR16-1 did not significantly affect the total number of inflammatory cells ($Cd11b^{+}$) at 4 (E) or 7 (F) dpi. G, H: MR16-1 reduced the recruitment of $CD11b^{+}EGFP^{+}$ hematogenous macrophages at 4 (G) and 7 (H) dpi. I, J: MR16-1 treatment increased the number of $CD11b^{+}EGFP^{-}$ resident microglia 1.0 to 2.0 mm rostral and caudal to the lesion epicenter at 4 dpi (I). At 7 dpi, both groups showed a shift in $CD11b^{+}EGFP^{-}$ microglia to the lesion epicenter, where the number of $CD11b^{+}EGFP^{-}$ microglia was significantly higher in the MR16-1-treated animals (J). Values are means ± SEM. $^{*}P<0.05$. $^{**}P<0.01$. Scale bars = 50 μm in A–C.

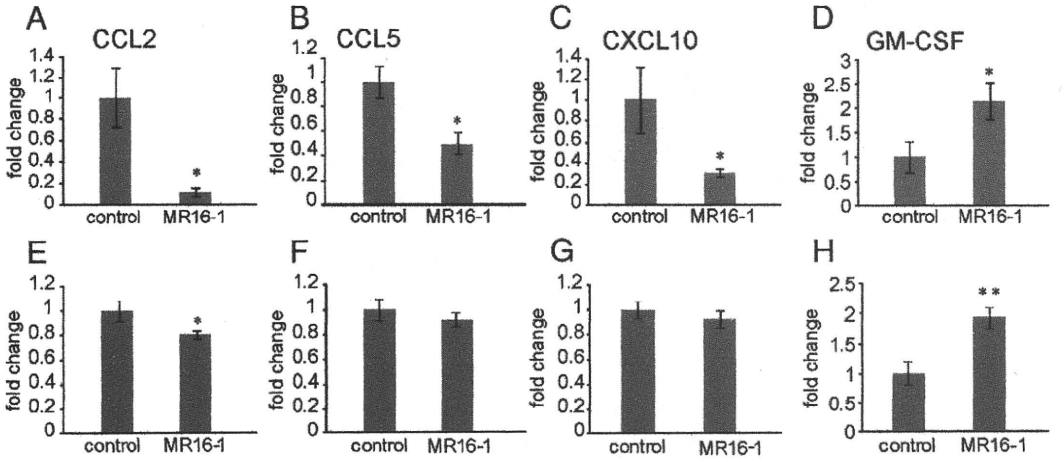

Fig. 3. MR16-1 decreases the expression level of macrophage-recruiting chemokines, while increasing that of GM-CSF. A–D: The CCL2 (A), CCL5 (B), CXCL10 (C), and GM-CSF (D) mRNA expression levels in the spinal cord tissue 12 h after injury were determined using quantitative RT-PCR. Macrophage-recruiting chemokines had significantly decreased, while the expression of GM-CSF, a known mitogen for microglia, had increased. E–H: The protein levels of the CCL2(E), CCL5(F), CXCL10(G) and GM-CSF(H) 24 h after injury were determined by western blot analysis. The protein level of CCL2 (E) was significantly decreased, whereas the GM-CSF (H) level was significantly increased. There was a tendency for the protein levels of CCL5 (F) and CXCL10 (G) to decrease, although the change was not statistically significant. Values are means ± SEM. $^{*}P<0.05$.

fibers was observed in the MR16-1-treated group than in the control group (Figs. 6M–O).

Discussion

IL-6 is a pro-inflammatory cytokine that triggers secondary injury in the pathophysiology of SCI. IL-6 binds to soluble and membrane-bound IL-6-receptor to form a complexed ligand for gp130, the common signal transducer of IL-6 and its related cytokines. MR16-1 is a neutralizing antibody for IL-6-receptor that competitively inhibits its binding to IL-6, thereby blocking IL-6-receptor-mediated cell signaling. We previously reported that the systemic administration of MR16-1 decreases the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in the injured spinal cord, demonstrating that this treatment potently affects the IL-6/JAK/STAT3 signaling pathway. Subsequently, we showed that MR16-1 administration after SCI reduces the number of inflammatory cells present 2 weeks after injury and decreases the amount of reactive astrogliosis, leading to improved functional recovery (Okada et al., 2004). Our present study extends these findings, showing that MR16-1 treatment alters the nature of the inflammatory response after SCI.

Here we found that the temporary inhibition of IL-6 signaling by MR16-1 treatment caused a significant reduction in the macrophage/microglia accumulation at 14 dpi, but not at 4 or 7 dpi. We had expected MR16-1 to have an anti-inflammatory effect, because IL-6 is a pro-inflammatory cytokine, so it was unclear why the immediate administration of MR16-1 affected only the late phase of the inflammatory response after SCI. We hypothesize that the response was delayed because the effects of MR16-1 treatment were owed to changes in the nature of the inflammatory response, rather than to the immediate effects of inhibiting the IL-6 signal.

Previous studies showed that functional recovery after SCI is affected by the properties of the inflammatory response, which are determined by the cell types involved and their state of activation (Gris et al., 2004; Popovich et al., 1999; Rapalino et al., 1998; Saville et al., 2004; Schwartz et al., 1999). Hematogenous macrophages and microglia are the major players in the inflammatory pathology of SCI, and their characteristics have therefore been of major interest. Of the two, hematogenous macrophages are regarded as more detrimental, because removing them or preventing their infiltration into the injured tissue reduces the degree of secondary injury and improves functional recovery (Gris et al., 2004; Popovich et al., 1999). In contrast, microglia are believed to be relatively beneficial for spinal cord repair, owing to their higher phagocytotic activity and expression of various neurotrophic factors (Lalancette-Hebert et al., 2007; Schilling et al., 2005). The state of cell activation also affects the nature of the inflammation; the implantation of bone marrow-derived macrophages previously stimulated by co-incubation with peripheral nerve or skin improves spinal cord repair (Bomstein et al., 2003; Rapalino et al., 1998).

Because IL-6 has a leading role in recruiting macrophages during inflammation, we hypothesized that MR16-1 treatment would decrease the infiltration of hematogenous macrophages. Therefore, we focused on the balance between the types of inflammatory cells, i.e., the hematogenous macrophages and microglia. Flow cytometric analysis revealed that the proportion of infiltrated $CD45^{high}$ hematogenous macrophages decreased markedly following MR16-1 treatment, with the result that resident microglia replaced hematogenous macrophages as the major inflammatory cell type at the lesion site. Furthermore, we performed quantitative analyses in chimeric mice bearing transplanted EGFP-expressing, highly purified HSCs. These analyses showed that, besides the reduced infiltration of hematogenous macrophages following MR16-1 treatment, the number of resident microglia increased, contributing to the shift in the major inflammatory cell type. The increase in the number of BrdU+ microglia by MR16-1 indicated that the higher number of microglia might have resulted from their increased proliferation (Suppl. Fig. 1).

Two mechanisms appear to mediate this phenomenon. First, the number of hematogenous macrophages is reduced because fewer are recruited from the blood pool. Various chemokines are known to mediate macrophage infiltration; in particular, CCL2, CCL5, and CXCL10 have a demonstrated role in recruiting macrophages following CNS injury (Babcock et al., 2003; Ghirnikar et al., 1998; Glass et al., 2001; Liu et al., 2001). Here we observed decreased expression levels of CCL2, CCL5, and CXCL10 following MR16-1-treatment, which could account for the reduced infiltration of hematogenous macrophages. This agrees with the observation that IL-6 is a major player in directing chemokine-mediated macrophage infiltration during inflammation (Hurst et al., 2001; Romano et al., 1997).

In contrast to hematogenous macrophage recruitment, microglial proliferation is mainly regulated by colony-stimulating factors (CSFs) (Giulian and Ingeman, 1988; Lee et al., 1994), and the local increase in GM-CSF that we observed could have stimulated their proliferation, resulting in their increased numbers at the lesion site (Lee et al., 1994). Since IL-6, like other cytokines, interacts with various other

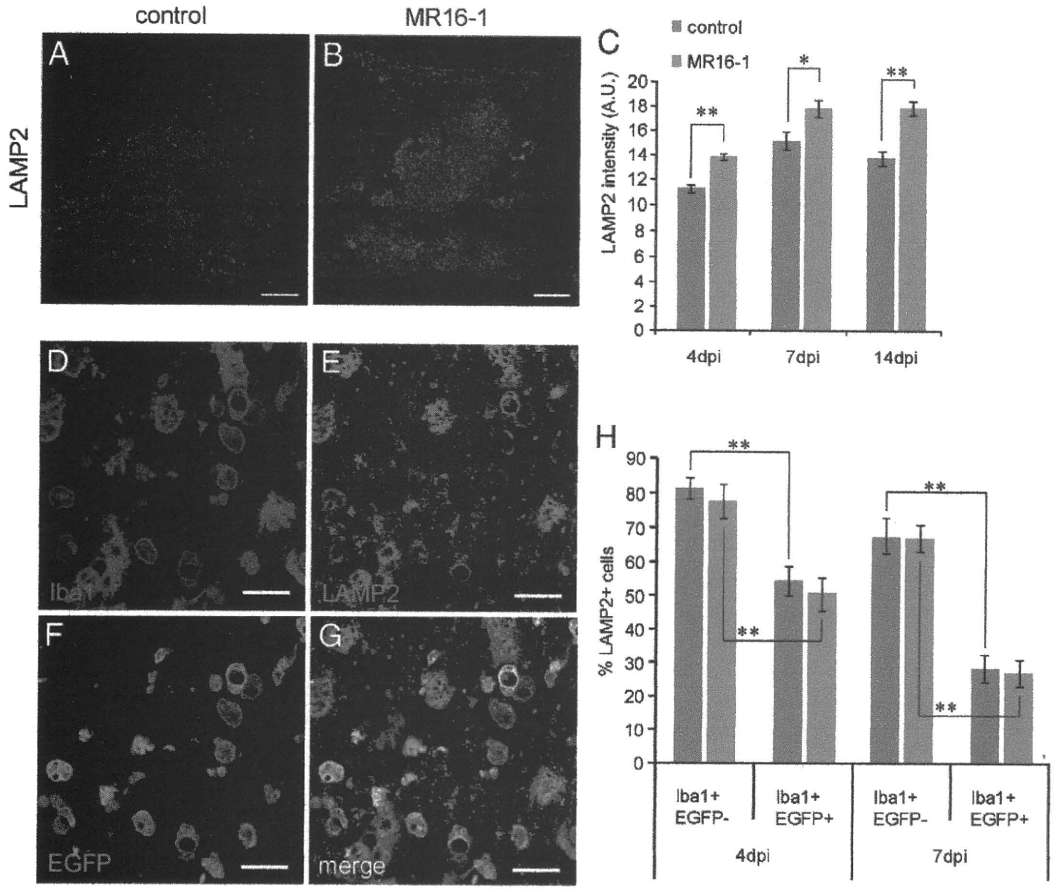

Fig. 4. MR16-1 enhances the phagocytotic activity in the injured spinal cord. A–C: Phagocytosis, indicated by LAMP2 expression, was increased by MR16-1-treatment. D–H: Quantification of the LAMP2$^+$ inflammatory subsets in injured chimeric mice indicated that the microglia had significantly higher phagocytotic activity than the hematogenous macrophages. MR16-1-treatment did not affect the phagocytotic activity of either cell subpopulation. Values are means ± SEM. *P<0.05. Scale bars = 200 μm in A, B; 20 μm in D–G.

cytokines or neurotrophic factors, and since previous reports show that the inhibition of IL-6 signaling drastically alters the expression profiles of various cytokines (Matsumura et al., 1999; Romano et al., 1997), the temporary inhibition of IL-6 signaling may, by altering the chemical milieu, underlie the shift in the dominant inflammatory cell type following MR16-1 treatment.

Given the characteristics of hematogenous macrophages vs. those of resident microglia, the MR16-1-induced switch in the central player in post-SCI inflammation should be beneficial. The evidence bears out this prediction. In the injured spinal cord, broad destruction of the blood–spinal cord barrier (BSCB) permits the infiltration of hematogenous macrophages into the lesion. If the infiltrating neutrophils and macrophages are depleted or blocked, tissue sparing improves, as does axonal regeneration/sprouting (Gris et al., 2004; Popovich et al., 1999; Saville et al., 2004). The cytotoxicity of hematogenous macrophages may be due to their increased NO production in the injured CNS (Ponomarev et al., 2007). In addition, a recent report showed that direct physical interactions between activated hematogenous macrophages and axons causes the axons to retract; microglia have a similar effect, but it is much weaker (Horn et al., 2008).

Despite the detrimental effects of hematogenous macrophages, the accumulation of inflammatory cells is considered to be critical for the repair process, because these cells clear away tissue debris and release neurotrophic factors. Previous studies demonstrated that the resident microglia play an active role in repairing the injured CNS, through their relatively high phagocytotic activity (Schilling et al., 2005) and by releasing various neuroprotective cytokines or neurotrophic factors (Lalancette-Hebert et al., 2007; Lambertsen et al., 2009). In fact, the tissue debris within the injured spinal cord contains myelin-derived axonal growth inhibitory factors (Bregman et al., 1995; Merkler et al., 2001) that hinder the repair process after SCI, so clearing away this debris is prerequisite for axonal re-growth.

In this study, MR16-1 treatment led to an increased accumulation of microglia, which expressed higher levels of the phagocytic markers LAMP2 and Mac2 than did the hematogenous macrophages. Furthermore, the Oil red O-stained intracellular area of microglial cells was greater than that of hematogenous macrophages. These data indicate that the microglia had higher phagocytotic activity than the hematogenous macrophages, and this activity was enhanced by the MR16-1 treatment.

The enhanced phagocytosis by MR16-1 treatment, along with attenuation of injury, resulted in decreased Oil red O staining (indicating reduced deposition of myelin) and decreased immunostaining for the axonal growth inhibitor Nogo-A, at the chronic phase of post-SCI inflammation. These effects could have contributed to the formation of a permissive environment for the regeneration or sprouting of neuronal fibers. In fact, the process of spinal cord repair seemed to be enhanced, given the increase in the RT-97$^+$ fibers and 5HT$^+$ serotonergic fibers between 14 and 42 dpi. The higher density of RT-97 fibers in the penumbral area in the treated group, and the lack of RT-97+ fibers at the center of the injury site in both groups, may indicate that enhanced sprouting contributed to the increase in 5HT+ fibers caudal to the lesion site (Supplemental Fig. 2).

Taken together, it is possible that the immediate administration of MR16-1 affected only the late phase of inflammation, because the change in cytokine profile by MR16-1 enhanced the participation of microglia in the inflammatory process, resulting in a tissue-protective inflammation. Our results with LFB, Oil red O, and immunostaining for

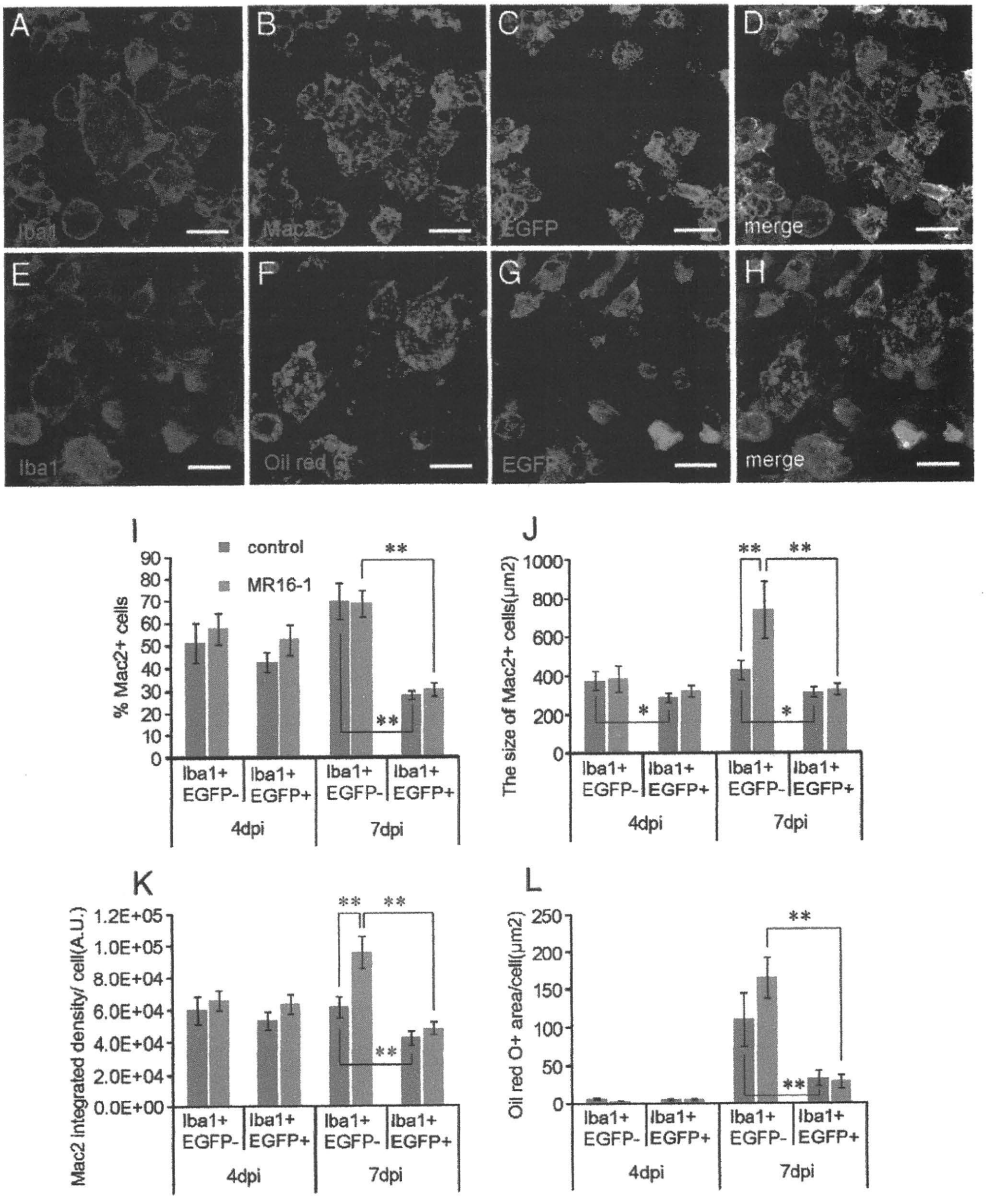

Fig. 5. Microglia have higher phagocytic activity against myelin debris than hematogenous macrophages. A–D: Mac2 expression was observed in the cells at the lesion epicenter. A portion of the macrophages/microglia expressed Mac2 in their cytoplasm. E–H: Oil red O^+ particles were observed in the cell body of macrophages/microglia at the lesion epicenter. I: The cytoplasmic expression of Mac2 was greater in $Iba1^+EGFP^-$ resident microglia than in $Iba1^+EGFP^+$ hematogenous macrophages. J, K: The average size (J) and Mac2-integrated density (K) at 7 dpi of each $Iba1^+EGFP^-$ resident microglial cell were significantly greater than those of $Iba1^+EGFP^+$ hematogenous macrophages, and were increased by MR16-1 treatment. The intracellular Oil red O-stained area was significantly greater in the $Iba1^+EGFP^-$ resident microglia than in the $Iba1^+EGFP^+$ hematogenous macrophages, and this tendency was increased at 7 dpi by MR16-1 treatment (L). Values are means ± SEM. $^*P<0.05$. $^{**}P<0.01$. Scale bars = 20 μm in A–H.

Nogo-A, which showed reduced injury and deposition of debris, support this idea. This series of immune responses takes place over a long time, which is consistent with the effects of MR16-1 treatment appearing only at the late phase.

However, despite the marked improvement in the inflammatory response obtained with MR16-1 treatment, skepticism about the therapeutic effects of IL-6 signal inhibition remains, because several studies have shown that IL-6 signaling has neuroprotective functions after CNS trauma. For example, Penkowa et al. showed that the overexpression of IL-6 results in decreased oxidative stress and apoptosis, leading to faster tissue repair after brain injury (Penkowa et al., 2003), and IL-6-deficient mice show a slower rate of recovery and healing after brain injury than wild-type mice (Swartz et al., 2001).

Although our results may seem to conflict with these reports, we believe that the apparent discrepancies are owing to the context-dependent pleiotropic actions of IL-6. Our present data revealed that the therapeutic effect of MR16-1 was achieved by inhibiting the excessive infiltration of hematogenous macrophages through the damaged BSCB, during the acute phase of SCI. This idea is corroborated by previous reports demonstrating that the overexpression of IL-6 family cytokines during the acute phase of SCI significantly increases inflammatory cell accumulation, resulting in greater damage (Kerr and Patterson, 2004; Lacroix et al., 2002). On the other hand, IL-6 can enhance spinal cord repair by modifying the migration of reactive astrocytes (Okada et al., 2006) or enhancing axonal re-growth (Miao et al., 2006), and IL-6's temporary inhibition may have little effect on these functions, because astrocyte migration and axonal re-growth are rather long-term processes. Although numerous studies have shown that the recruitment of inflammatory cells aggravates secondary injury after SCI (Ghirnikar et al., 2001; Hausmann, 2003;

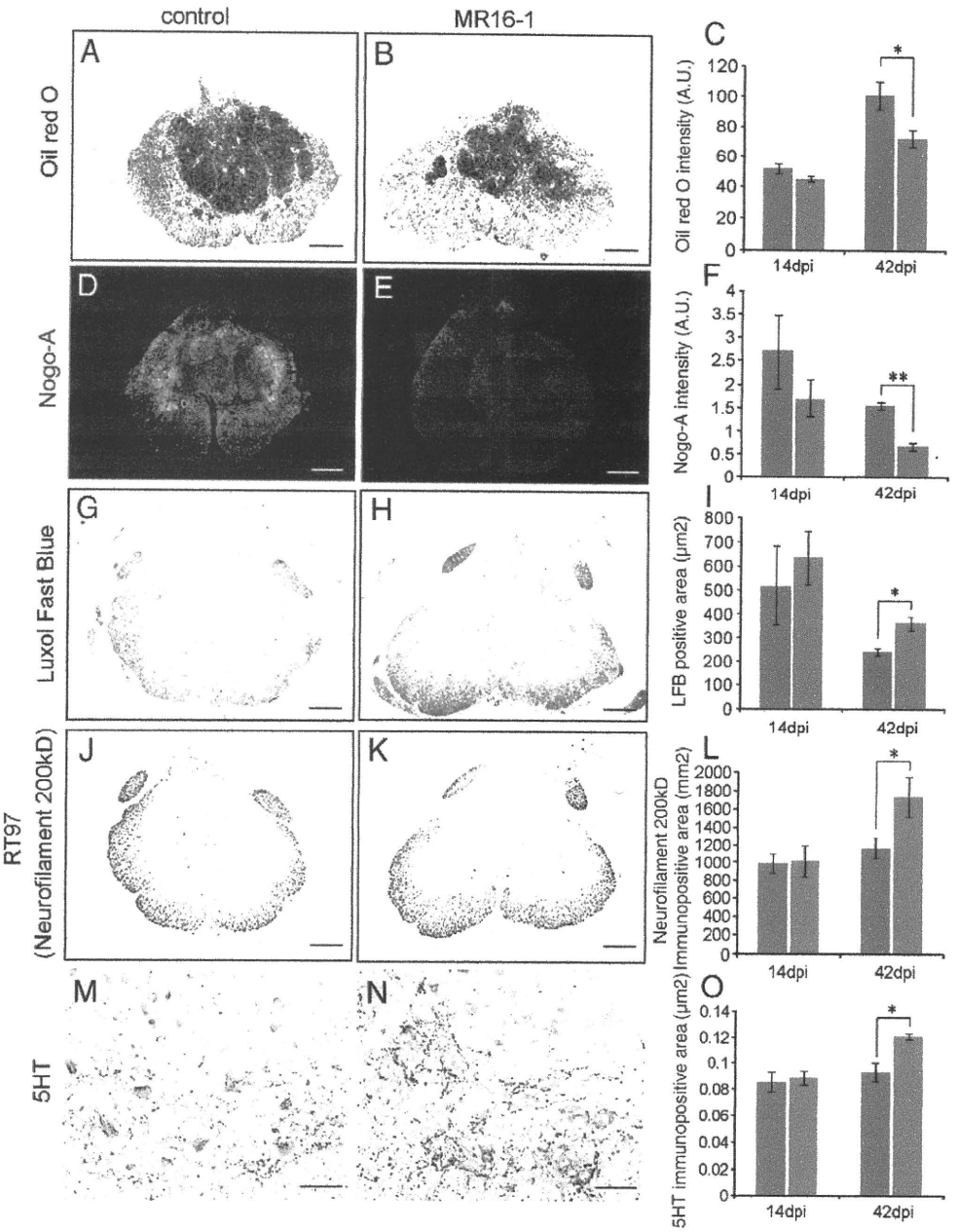

Fig. 6. MR16-1 treatment improves repair of the spinal cord. A–C: MR16-1 treatment improved the clearance of myelin debris, as seen by a significant reduction in the Oil red O-stained area. D–F: The deposition of Nogo-A was decreased in the MR16-1-treated group. G–I: The area of Luxol Fast Blue-stained tissue, which represents spared myelin sheath, was significantly increased by the MR16-1 treatment. J–L: RT-97^{+} (Neurofilament 200kD) fibers at the lesion epicenter were significantly increased in the MR16-1-treated animals at 42 dpi. M–O: 5HT-positive fibers at the ventral horn. 5HT-immunostaining of the area 2 mm caudal to the lesion revealed a significant increase in 5HT^{+} fibers at 42 dpi by the MR16-1-treatment. *$P<0.05$. Scale bars = 200 μm in A, B, D, E, G, H; 50 μm in J, K.

Saville et al., 2004), inflammation itself is important for spinal cord repair (Donnelly and Popovich, 2008), and excessive suppression of the inflammatory response can be detrimental. In the present study, we showed that anti-IL-6-receptor antibody treatment at the acute stage of injury switches the main subpopulation of inflammatory cells from hematogenous macrophages to microglia, which does not simply suppress inflammation: rather, it changes the characteristics of the post-traumatic inflammation to promote spinal cord repair.

Given that a humanized antibody for the human IL-6 receptor (MRA; tocilizumab) is already in clinical use (Choy et al., 2002; Nishimoto et al., 2000; Sato et al., 1993), the present data support the potential application of the anti-IL-6-receptor antibody for the treatment of SCI. However, in most clinical situations, it is not possible to administer an antibody immediately after SCI. Further investigation to determine the therapeutic time window will be needed before this treatment can be tried clinically. Nevertheless, these findings shed light on IL-6's role in the pathology of SCI, and suggest a new approach for SCI treatment, in which the characteristics of inflammation are adjusted to support spinal cord repair, by modifying the cytokine-mediated cellular response.

Author contributions

M.M., M.N., Y.T., M.L. and H.O. designed the research; M.M., O.Y., A. I., T.I., F.RM. and O.T. performed the in vivo experiments; S.M. and Y.M. generated the chimeric mice; Y.O. supplied the anti-IL-6 receptor antibody and analyzed the antibody results; M.M., M.N., S.O., F.RM. and H.K. analyzed the data; M.M., M.N. and H.O. wrote the paper; M.N. and H.O. supervised all the experiments.

Acknowledgments

This work was supported by grants from the Project for the Realization of Regenerative Medicine from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan to H.O.; the General Insurance Association of Japan to M.N., A.I. and Y.T.; and a Grant-in-Aid for the Global COE Program from MEXT to Keio University. This work was also supported by grants from the Grant of Orthopaedics and Traumatology Foundation, Inc. No. 0178 to A.I. We are grateful to Professor Claude Bernard at Monash University for his critical reading of the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2010.04.020.

References

Babcock, A.A., Kuziel, W.A., Rivest, S., Owens, T., 2003. Chemokine expression by glial cells directs leukocytes to sites of axonal injury in the CNS. J. Neurosci. 23, 7922–7930.
Bomstein, Y., Marder, J.B., Vitner, K., Smirnov, I., Lisaey, G., Butovsky, O., Fulga, V., Yoles, E., 2003. Features of skin-coincubated macrophages that promote recovery from spinal cord injury. J. Neuroimmunol. 142, 10–16.
Bregman, B.S., Kunkel-Bagden, E., Schnell, L., Dai, H.N., Gao, D., Schwab, M.E., 1995. Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378, 498–501.
Cafferty, W.B., Gardiner, N.J., Das, P., Qiu, J., McMahon, S.B., Thompson, S.W., 2004. Conditioning injury-induced spinal axon regeneration fails in interleukin-6 knock-out mice. J. Neurosci. 24, 4432–4443.
Choy, E.H., Isenberg, D.A., Garrood, T., Farrow, S., Ioannou, Y., Bird, H., Cheung, N., Williams, B., Hazleman, B., Price, R., Yoshizaki, K., Nishimoto, N., Kishimoto, T., Panayi, G.S., 2002. Therapeutic benefit of blocking interleukin-6 activity with an anti-interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum. 46, 3143–3150.
Donnelly, D.J., Popovich, P.G., 2008. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. Exp. Neurol. 209, 378–388.
Gensel, J.C., Nakamura, S., Guan, Z., van Rooijen, N., Ankeny, D.P., Popovich, P.G., 2009. Macrophages promote axon regeneration with concurrent neurotoxicity. J. Neurosci. 29, 3956–3968.
Ghirnikar, R.S., Lee, Y.L., Li, J.D., Eng, L.F., 1998. Chemokine inhibition in rat stab wound brain injury using antisense oligodeoxynucleotides. Neurosci. Lett. 247, 21–24.
Ghirnikar, R.S., Lee, Y.L., Eng, L.F., 2001. Chemokine antagonist infusion promotes axonal sparing after spinal cord contusion injury in rat. J. Neurosci. Res. 64, 582–589.
Giulian, D., Ingeman, J.E., 1988. Colony-stimulating factors as promoters of ameboid microglia. J. Neurosci. 8, 4707–4717.
Glass, W.G., Liu, M.T., Kuziel, W.A., Lane, T.E., 2001. Reduced macrophage infiltration and demyelination in mice lacking the chemokine receptor CCR5 following infection with a neurotropic coronavirus. Virology 288, 8–17.
Gris, D., Marsh, D.R., Oatway, M.A., Chen, Y., Hamilton, E.F., Dekaban, G.A., Weaver, L.C., 2004. Transient blockade of the CD11d/CD18 integrin reduces secondary damage after spinal cord injury, improving sensory, autonomic, and motor function. J. Neurosci. 24, 4043–4051.
Hashimoto, M., Nitta, A., Fukumitsu, H., Nomoto, H., Shen, L., Furukawa, S., 2005. Involvement of glial cell line-derived neurotrophic factor in activation processes of rodent macrophages. J. Neurosci. Res. 79, 476–487.
Hausmann, O.N., 2003. Post-traumatic inflammation following spinal cord injury. Spinal Cord 41, 369–378.
Horn, K.P., Busch, S.A., Hawthorne, A.L., van Rooijen, N., Silver, J., 2008. Another barrier to regeneration in the CNS: activated macrophages induce extensive retraction of dystrophic axons through direct physical interactions. J. Neurosci. 28, 9330–9341.
Hurst, S.M., Wilkinson, T.S., McLoughlin, R.M., Jones, S., Horiuchi, S., Yamamoto, N., Rose-John, S., Fuller, G.M., Topley, N., Jones, S.A., 2001. Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. Immunity 14, 705–714.
Kawamoto, S., Niwa, H., Tashiro, F., Sano, S., Kondoh, G., Takeda, J., Tabayashi, K., Miyazaki, J., 2000. A novel reporter mouse strain that expresses enhanced green fluorescent protein upon Cre-mediated recombination. FEBS Lett. 470, 263–268.
Kerr, B.J., Patterson, P.H., 2004. Potent pro-inflammatory actions of leukemia inhibitory factor in the spinal cord of the adult mouse. Exp. Neurol. 188, 391–407.
Klusman, I., Schwab, M.E., 1997. Effects of pro-inflammatory cytokines in experimental spinal cord injury. Brain Res. 762, 173–184.
Koide, Y., Morikawa, S., Mabuchi, Y., Muguruma, Y., Hiratsu, E., Hasegawa, K., Kobayashi, M., Ando, K., Kinjo, K., Okano, H., Matsuzaki, Y., 2007. Two distinct stem cell lineages in murine bone marrow. Stem Cells 25, 1213–1221.
Koopman, R., Schaart, G., Hesselink, M.K., 2001. Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. Histochem. Cell Biol. 116, 63–68.
Lacroix, S., Chang, L., Rose-John, S., Tuszynski, M.H., 2002. Delivery of hyper-interleukin-6 to the injured spinal cord increases neutrophil and macrophage infiltration and inhibits axonal growth. J. Comp. Neurol. 454, 213–228.
Lalancette-Hebert, M., Gowing, G., Simard, A., Weng, Y.C., Kriz, J., 2007. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. J. Neurosci. 27, 2596–2605.
Lambertsen, K.L., Clausen, B.H., Babcock, A.A., Gregersen, R., Fenger, C., Nielsen, H.H., Haugaard, L.S., Wirenfeldt, M., Nielsen, M., Dagnaes-Hansen, F., Bluethmann, H., Faergeman, N.J., Meldgaard, M., Deierborg, T., Finsen, B., 2009. Microglia protect neurons against ischemia by synthesis of tumor necrosis factor. J. Neurosci. 29, 1319–1330.
Lee, S.C., Liu, W., Brosnan, C.F., Dickson, D.W., 1994. GM-CSF promotes proliferation of human fetal and adult microglia in primary cultures. Glia 12, 309–318.
Liu, M.T., Keirstead, H.S., Lane, T.E., 2001. Neutralization of the chemokine CXCL10 reduces inflammatory cell invasion and demyelination and improves neurological function in a viral model of multiple sclerosis. J. Immunol. 167, 4091–4097.
Matsumura, M., Banba, N., Motohashi, S., Hattori, Y., 1999. Interleukin-6 and transforming growth factor-beta regulate the expression of monocyte chemoattractant protein-1 and colony-stimulating factors in human thyroid follicular cells. Life Sci. 65, PL129–PL135.
Matsuzaki, Y., Kinjo, K., Mulligan, R.C., Okano, H., 2004. Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. Immunity 20, 87–93.
McTigue, D.M., Popovich, P.G., Morgan, T.E., Stokes, B.T., 2000. Localization of transforming growth factor-beta1 and receptor mRNA after experimental spinal cord injury. Exp. Neurol. 163, 220–230.
Merkler, D., Metz, G.A., Raineteau, O., Dietz, V., Schwab, M.E., Fouad, K., 2001. Locomotor recovery in spinal cord-injured rats treated with an antibody neutralizing the myelin-associated neurite growth inhibitor Nogo-A. J. Neurosci. 21, 3665–3673.
Miao, T., Wu, D., Zhang, Y., Bo, X., Subang, M.C., Wang, P., Richardson, P.M., 2006. Suppressor of cytokine signaling-3 suppresses the ability of activated signal transducer and activator of transcription-3 to stimulate neurite growth in rat primary sensory neurons. J. Neurosci. 26, 9512–9519.
Mildenberger, M., Beach, T.G., McGeer, E.G., Ludgate, C.M., 1990. An animal model of prophylactic cranial irradiation: histologic effects at acute, early and delayed stages. Int. J. Radiat. Oncol. Biol. Phys. 18, 1051–1060.
Nesic, O., Xu, G.Y., McAdoo, D., High, K.W., Hulsebosch, C., Perez-Pol, R., 2001. IL-1 receptor antagonist prevents apoptosis and caspase-3 activation after spinal cord injury. J. Neurotrauma 18, 947–956.
Nishimoto, N., Sasai, M., Shima, Y., Nakagawa, M., Matsumoto, T., Shirai, T., Kishimoto, T., Yoshizaki, K., 2000. Improvement in Castleman's disease by humanized anti-interleukin-6 receptor antibody therapy. Blood 95, 56–61.
Okada, S., Nakamura, M., Mikami, Y., Shimazaki, T., Mihara, M., Ohsugi, Y., Iwamoto, Y., Yoshizaki, K., Kishimoto, T., Toyama, Y., Okano, H., 2004. Blockade of interleukin-6 receptor suppresses reactive astrogliosis and ameliorates functional recovery in experimental spinal cord injury. J. Neurosci. Res. 76, 265–276.
Okada, S., Nakamura, M., Katoh, H., Miyao, T., Shimazaki, T., Ishii, K., Yamane, J., Yoshimura, A., Iwamoto, Y., Toyama, Y., Okano, H., 2006. Conditional ablation of Stat3 or Socs3 discloses a dual role for reactive astrocytes after spinal cord injury. Nat. Med. 12, 829–834.
Okazaki, M., Yamada, Y., Nishimoto, N., Yoshizaki, K., Mihara, M., 2002. Characterization of anti-mouse interleukin-6 receptor antibody. Immunol. Lett. 84, 231–240.
Penkowa, M., Giralt, M., Lago, N., Camats, J., Carrasco, J., Hernandez, J., Molinero, A., Campbell, I.L., Hidalgo, J., 2003. Astrocyte-targeted expression of IL-6 protects the CNS against a focal brain injury. Exp. Neurol. 181, 130–148.
Ponomarev, E.D., Maresz, K., Tan, Y., Dittel, B.N., 2007. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells. J. Neurosci. 27, 10714–10721.
Popovich, P.G., Guan, Z., Wei, P., Huitinga, I., van Rooijen, N., Stokes, B.T., 1999. Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury. Exp. Neurol. 158, 351–365.
Popovich, P.G., Guan, Z., McGaughy, V., Fisher, L., Hickey, W.F., Basso, D.M., 2002. The neuropathological and behavioral consequences of intraspinal microglial/macrophage activation. J. Neuropathol. Exp. Neurol. 61, 623–633.
Rapalino, O., Lazarov-Spiegler, O., Agranov, E., Velan, G.J., Yoles, E., Fraidakis, M., Solomon, A., Gepstein, R., Katz, A., Belkin, M., Hadani, M., Schwartz, M., 1998. Implantation of stimulated homologous macrophages results in partial recovery of paraplegic rats. Nat. Med. 4, 814–821.
Rinner, W.A., Bauer, J., Schmidts, M., Lassmann, H., Hickey, W.F., 1995. Resident microglia and hematogenous macrophages as phagocytes in adoptively transferred experimental autoimmune encephalomyelitis: an investigation using rat radiation bone marrow chimeras. Glia 14, 257–266.
Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., van Hinsbergh, V., Sozzani, S., Bussolino, F., Poli, V., Ciliberto, G., Mantovani, A., 1997. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity 6, 315–325.
Rotshenker, S., Reichert, F., Gitik, M., Haklai, R., Elad-Sfadia, G., Kloog, Y., 2008. Galectin-3/MAC-2, Ras and PI3K activate complement receptor-3 and scavenger receptor-AI/II mediated myelin phagocytosis in microglia. Glia 56, 1607–1613.
Sato, K., Tsuchiya, M., Saldanha, J., Koishihara, Y., Ohsugi, Y., Kishimoto, T., Bendig, M.M., 1993. Reshaping a human antibody to inhibit the interleukin 6-dependent tumor cell growth. Cancer Res. 53, 851–856.
Saville, L.R., Pospisil, C.H., Mawhinney, L.A., Bao, F., Simedrea, F.C., Peters, A.A., O'Connell, P.J., Weaver, L.C., Dekaban, G.A., 2004. A monoclonal antibody to CD11d reduces the inflammatory infiltrate into the injured spinal cord: a potential neuroprotective treatment. J. Neuroimmunol. 156, 42–57.

Schilling, M., Besselmann, M., Muller, M., Strecker, J.K., Ringelstein, E.B., Kiefer, R., 2005. Predominant phagocytic activity of resident microglia over hematogenous macrophages following transient focal cerebral ischemia: an investigation using green fluorescent protein transgenic bone marrow chimeric mice. Exp. Neurol. 196, 290–297.

Schwartz, M., Lazarov-Spiegler, O., Rapalino, O., Agranov, I., Velan, G., Hadani, M., 1999. Potential repair of rat spinal cord injuries using stimulated homologous macrophages. Neurosurgery 44, 1041–1045 discussion 1045–1046.

Sedgwick, J.D., Schwender, S., Imrich, H., Dorries, R., Butcher, G.W., ter Meulen, V., 1991. Isolation and direct characterization of resident microglial cells from the normal and inflamed central nervous system. Proc. Natl. Acad. Sci. U. S. A. 88, 7438–7442.

Sharma, H.S., Winkler, T., Stalberg, E., Gordh, T., Alm, P., Westman, J., 2003. Topical application of TNF-alpha antiserum attenuates spinal cord trauma induced edema formation, microvascular permeability disturbances and cell injury in the rat. Acta Neurochir. Suppl. 86, 407–413.

Steinmetz, M.P., Horn, K.P., Tom, V.J., Miller, J.H., Busch, S.A., Nair, D., Silver, D.J., Silver, J., 2005. Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. J. Neurosci. 25, 8066–8076.

Swartz, K.R., Liu, F., Sewell, D., Schochet, T., Campbell, I., Sandor, M., Fabry, Z., 2001. Interleukin-6 promotes post-traumatic healing in the central nervous system. Brain Res. 896, 86–95.

Tamura, T., Udagawa, N., Takahashi, N., Miyaura, C., Tanaka, S., Yamada, Y., Koishihara, Y., Ohsugi, Y., Kumaki, K., Taga, T., et al., 1993. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc. Natl. Acad. Sci. U. S. A. 90, 11924–11928.

Tuna, M., Polat, S., Erman, T., Ildan, F., Gocer, A.I., Tuna, N., Tamer, L., Kaya, M., Cetinalp, E., 2001. Effect of anti-rat interleukin-6 antibody after spinal cord injury in the rat: inducible nitric oxide synthase expression, sodium- and potassium-activated, magnesium-dependent adenosine-5′-triphosphatase and superoxide dismutase activation, and ultrastructural changes. J. Neurosurg. 95, 64–73.

Turrin, N.P., Plante, M.M., Lessard, M., Rivest, S., 2007. Irradiation does not compromise or exacerbate the innate immune response in the brains of mice that were transplanted with bone marrow stem cells. Stem Cells 25, 3165–3172.

Van Wagoner, N.J., Benveniste, E.N., 1999. Interleukin-6 expression and regulation in astrocytes. J. Neuroimmunol. 100, 124–139.

Therapeutic potential of appropriately evaluated safe-induced pluripotent stem cells for spinal cord injury

Osahiko Tsuji[a,b,1], Kyoko Miura[a,c,1], Yohei Okada[a,d], Kanehiro Fujiyoshi[a,b], Masahiko Mukaino[a,e], Narihito Nagoshi[a,b,f], Kazuya Kitamura[a,b], Gentaro Kumagai[a,g], Makoto Nishino[a], Shuta Tomisato[a], Hisanobu Higashi[a], Toshihiro Nagai[h], Hiroyuki Katoh[a,b,f], Kazuhisa Kohda[a], Yumi Matsuzaki[a], Michisuke Yuzaki[a], Eiji Ikeda[i,j], Yoshiaki Toyama[b], Masaya Nakamura[b,2], Shinya Yamanaka[c], and Hideyuki Okano[a,2]

Departments of [a]Physiology and [b]Orthopedic Surgery, School of Medicine, Keio University, Shinjuku, Tokyo 160-8582, Japan; [c]Center for Induced Pluripotent Stem Cell Research and Application, Kyoto University, Kyoto 606-8507, Japan; [d]Kanrinmaru-Project and Departments of [e]Rehabilitation Medicine, [h]Electron Microscope Laboratory, and [i]Pathology, School of Medicine, Keio University, Tokyo 160-8582, Japan; [f]Department of Orthopedic Surgery, National Hospital Organization, Murayama Medical Center, Tokyo 208-0011, Japan; [g]Department of Orthopedic Surgery, Graduate School of Medicine, Hirosaki University, Aomori 036-8560, Japan; and [j]Department of Pathology, Graduate School of Medicine, Yamaguchi University, Yamaguchi 755-8505, Japan

Edited by Fred Gage, Salk Institute, San Diego, CA, and approved June 3, 2010 (received for review September 3, 2009)

Various types of induced pluripotent stem (iPS) cells have been established by different methods, and each type exhibits different biological properties. Before iPS cell-based clinical applications can be initiated, detailed evaluations of the cells, including their differentiation potentials and tumorigenic activities in different contexts, should be investigated to establish their safety and effectiveness for cell transplantation therapies. Here we show the directed neural differentiation of murine iPS cells and examine their therapeutic potential in a mouse spinal cord injury (SCI) model. "Safe" iPS-derived neurospheres, which had been pre-evaluated as nontumorigenic by their transplantation into nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse brain, produced electrophysiologically functional neurons, astrocytes, and oligodendrocytes in vitro. Furthermore, when the safe iPS-derived neurospheres were transplanted into the spinal cord 9 d after contusive injury, they differentiated into all three neural lineages without forming teratomas or other tumors. They also participated in remyelination and induced the axonal regrowth of host 5HT$^+$ serotonergic fibers, promoting locomotor function recovery. However, the transplantation of iPS-derived neurospheres pre-evaluated as "unsafe" showed robust teratoma formation and sudden locomotor functional loss after functional recovery in the SCI model. These findings suggest that pre-evaluated safe iPS clone-derived neural stem/progenitor cells may be a promising cell source for transplantation therapy for SCI.

neural stem/progenitor cell | cell transplantation | regenerative medicine | remyelination | axonal regrowth

Given their ability to generate all types of neural cells, neural stem/progenitor cells (NS/PCs) are a promising source for cell replacement therapy for various intractable CNS disorders (reviewed in refs. 1–6). Notably, ES cells have great developmental plasticity and can be induced to become NS/PCs with specific differentiation potentials (7–11), making them a major candidate for cell replacement therapies for CNS disorders (12–16). The clinical use of ES cells is complicated, however, by ethical and immunological concerns, both of which might be overcome by using pluripotent stem cells derived directly from a patient's own somatic cells (17).

We recently reported the establishment of induced pluripotent stem (iPS) cells from mouse fibroblasts by the retroviral introduction of four factors (*Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*) with selection for *Fbxo15* expression (18) and *Nanog* expression (19, 20). Compared with *Fbxo15*-selected iPS cells, *Nanog*-selected iPS cells more closely resembled ES cells' gene-expression pattern and could contribute to germline-competent adult chimeras (19–21). More recently, we and others (22, 23) generated iPS cells without using *c-Myc* retroviruses, albeit with lower efficiency. The successful establishment of these iPS cell lines, along with initial reports showing efficacy in the therapeutic use of iPS cells in rodent models of sickle cell anemia (24) and Parkinson disease (25), led us to examine the use of iPS cells as a treatment for spinal cord injury (SCI).

A number of important issues need to be addressed before a clinical trial using iPS cells as a cell-therapy source for SCI is initiated. First, a detailed evaluation of iPS cells' potential to generate neural cells compared with ES cells is required. Second, iPS cells are likely to carry a higher risk of tumorigenicity than ES cells, due to the inappropriate reprogramming of these somatic cells, the activation of exogenous transcription factors, or other reasons (25–27). Thus, it is essential to confirm the safety of grafted iPS-derived NS/PCs. Finally, the effectiveness of iPS-derived NS/PC transplantation as a treatment for SCI must be evaluated.

In the previous study, we pre-evaluated iPS clones for safety by transplanting iPS-derived neurospheres into the NOD/SCID mouse brain (27). Here, we show that the transplantation of neurospheres derived from safe iPS cell clones into the injured spinal cord promoted functional recovery without any tumor formation. In contrast, the transplantation of neurospheres derived from unsafe iPS cells, showing robust teratoma formation in the NOD/SCID mouse brain, also resulted in initial functional recovery, but was later followed by teratoma formation and deterioration of locomotor function. These data suggest that the evaluation of in vitro differentiation and in vivo tumorigenicity are important for identifying safe iPS clones for cell therapy, and that the NS/PCs derived from iPS clones deemed safe by such pre-evaluation are a promising source for cell therapy for SCI.

Results

Pre-Evaluated Safe MEF-iPS Cells Exhibit ES-Like Neural Differentiation Potentials in Vitro. We previously reported the neural differentiation of 36 independent murine iPS cell clones (27). The results of this study led us to classify several iPS clones as safe or unsafe

Author contributions: O.T., K.M., M. Nakamura, S.Y., and H.O. designed research; O.T., K.M., Y.O., K.F., M.M., N.N., K. Kitamura, G.K., M. Nishino, S.T., H.H., T.N., H.K., E.I., and H.O. performed research; O.T. and K.M. contributed new reagents/analytic tools; O.T., K.M., Y.O., K.F., M.M., N.N., K. Kitamura, G.K., H.K., K. Kohda, Y.M., M.Y., E.I., Y.T., M. Nakamura, S.Y., and H.O. analyzed data; and O.T., K.M., Y.O., K.F., H.K., E.I., M. Nakamura, and H.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]O.T. and K.M. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: hidokano@sc.itc.keio.ac.jp or masa@sc.itc.keio.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0910106107/-/DCSupplemental.