CD95-Ligand on Peripheral Myeloid Cells Activates Syk Kinase to Trigger Their Recruitment to the Inflammatory Site

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SUMMARY

Injury to the central nervous system initiates an uncontrolled inflammatory response that results in both tissue repair and destruction. Here, we showed that, in rodents and humans, injury to the spinal cord triggered surface expression of CD95 ligand (CD95L, FasL) on peripheral blood myeloid cells. CD95L stimulation of CD95 on these cells activated phosphoinositide 3-kinase (PI3K) and metalloproteinase-9 (MMP-9) via recruitment and activation of Syk kinase, ultimately leading to increased migration. Exclusive CD95L deletion in myeloid cells greatly decreased the number of neutrophils and macrophages infiltrating the injured spinal cord or the inflamed peritoneum after thioglycollate injection. Importantly, deletion of myeloid CD95L, but not of CD95 on neural cells, led to functional recovery of spinal injured animals. Our results indicate that CD95L acts on peripheral myeloid cells to induce tissue damage. Thus, neutralization of CD95L should be considered as a means to create a controlled beneficial inflammatory response.

INTRODUCTION

Accumulating evidence indicates that the once-called death receptor, CD95 (Fas, APO-1), fulfils a wide range of physiological nonapoptotic functions in vivo. In the central nervous system (CNS), CD95 increases branching of developing neurons, axonal growth of dorsal root ganglion (DRG) cells, migration of malignant glioma cells, and differentiation of neural stem cells (NSCs) (Corsini et al., 2009; Desbarats et al., 2003; Kleber et al., 2008; Zuliani et al., 2006). Whereas in DRGs, the CD95L-CD95 system is thought to mediate axonal growth via ERK kinase activation, in NSCs and malignant glioma cells, CD95 activates the Src-PI3K pathway (Corsini et al., 2009; Desbarats et al., 2003; Kleber et al., 2008). In the immune system, activation-induced cell death (AICD) has been thoroughly described in activated cycling T cells as a CD95-dependent process (Dhein et al., 1995). However, further studies have indicated that the CD95L-CD95 system is also involved in T cell proliferation (Kennedy et al., 1999). Solid evidence that the CD95 ligand (CD95L, FasL) can also act as a proinflammatory mediator came from studies where tissue engineered to overexpress CD95L was colonized by neutrophils (Kang et al., 1997; Seino et al., 1997). However, the molecular mechanism by which CD95 induces inflammation has remained elusive.

Injury to the spinal cord elicits an inflammatory response within the first hours after injury that lasts for several weeks. This response includes endothelial damage, release of proinflammatory mediators, changes in vascular permeability, infiltration of peripheral inflammatory cells, and activation of astrocytes and microglia. Infiltrating inflammatory cells can, on one hand, promote wound healing events but, on the other hand, release toxic factors that amplify tissue damage (Jones and Tuszynski, 2002; Rolls et al., 2009). Yet, the precise signals leading to leukocyte infiltration are still unknown.

Neutralization of CD95L markedly reduces death of neurons and oligodendrocytes and improves functional recovery of spinal injured animals (Demjen et al., 2004). These results have been further confirmed in CD95-deficient mutant mice (lpr) (Casha et al., 2005; Yoshino et al., 2004) and in rats treated with a CD95-Fc reagent (Ackery et al., 2006). However, the actual source of CD95L and the mechanism by which the CD95L-CD95 system induces damage following injury has not been addressed yet.

Here, we report that the source of CD95L following spinal cord injury (SCI) were infiltrating myeloid cells. Stimulation of CD95 on peripheral myeloid cells increased their migration via activation of the Syk-PI3K-MMP pathway. In vivo, CD95-induced migration of myeloid cells was observed in an animal model of SCI and of thioglycollate-induced peritonitis. Exclusive deletion of CD95L on myeloid cells reduced death of neurons and oligodendrocytes
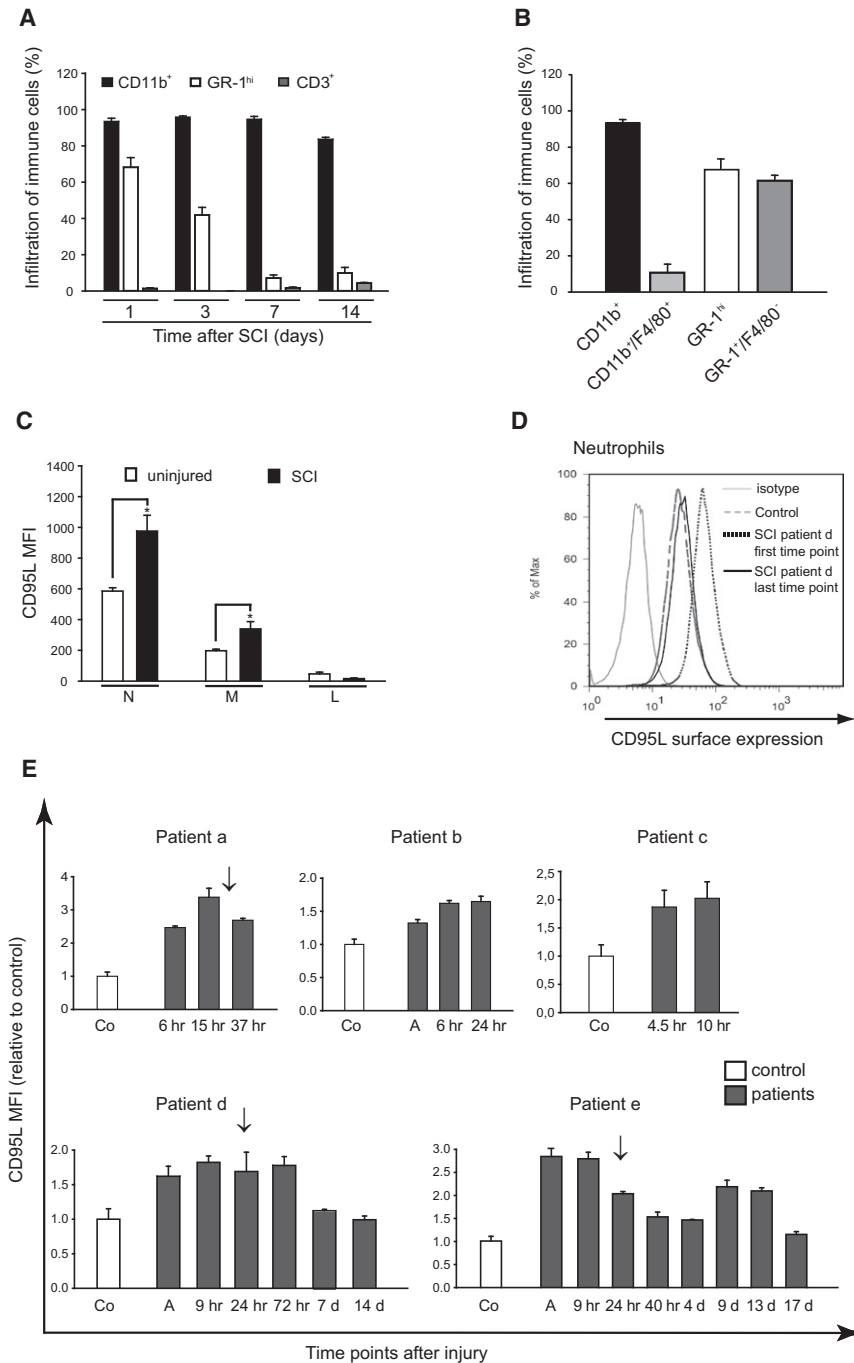
Figure 1. Increased Cell Surface Expression of CD95L on Mouse and Human Myeloid Cells after SCI

(A) Time kinetics of infiltrating immune cells into the injured spinal cord 1 to 14 days after SCI in bone marrow chimeras from eGFP donor mice and lethally irradiated WT recipient mice (BMT-eGFP).

(B) Immune cell types present at the lesion site 24 hr after SCI.

(C) Constitutive and SCI-induced expression of CD95L on peripheral blood neutrophils and monocytes. N, neutrophils; M, monocytes; L, lymphocytes; n = 4/group; *p < 0.05; **p < 0.01. Data are representative of at least three independent experiments.

(D) Representative histogram of CD95L surface expression on neutrophils from a spinal cord (SC)-injured patient (patient d: first and last time point after injury) or a healthy control.

(E) Quantification of CD95L expression on neutrophils from five SC-injured patients relative to rates in healthy controls. A, admission to the hospital (2–5 hr postinjury); d, days after injury; Arrow, Surgery. Data are presented as mean ± SEM; CD95L expression in patient's blood is representative of at least three independent stainings.

and improved functional recovery following SCI. Taken together, our data demonstrate that CD95L-detrimental function following SCI is due to activation of the innate inflammatory response rather than to direct apoptosis of CD95-bearing CNS cells.

RESULTS

Injury to the CNS Increases CD95L Surface Expression on Peripheral Blood Cells in Rodents and Humans

We have previously shown that systemic neutralization of CD95L improves functional recovery of spinal-injured mice by reducing the number of neurons and oligodendrocytes undergoing apoptosis (Demjen et al., 2004). Yet, the actual source of CD95L had remained elusive. CD95L is poorly expressed in the naive adult spinal cord, and it can be synthesized by resident spinal cord cells and/or infiltrating leukocytes. To characterize the different populations of immune cells recruited to the injured spinal cord, we generated eGFP-bone marrow (BM) chimeras by transferring bone marrow cells from eGFP donor mice into WT lethally irradiated recipient mice. In these mice, every immune cell is eGFP$^+$ (Table S1 available online). Using flow cytometry, we analyzed the infiltration of immune cells gating on the GFP$^+$ cells and using defined cell markers for different immune cell types. Nearly every immune cell infiltrating the lesion site at 24 hours after SCI were CD11b$^+$, a marker for myeloid cells (Figures 1A–1B). From these CD11b$^+$ cells, neutrophils accounted for more than 65% (GR-1$^+$F4/80$^-$ or GR-1^{hi}) and macrophages (CD11b$^+$F4/80$^+$) for 15% (Figure 1B). The proportion of neutrophils rapidly diminished within the first week, when macrophage numbers increased (Figure 1A). Infiltration of T cells (CD3$^+$) started after 7 days (Figure 1A). In summary, among leukocytes, neutrophils and macrophages, cells involved in the innate inflammatory response are the first to infiltrate the injured spinal cord. During the period of myeloid infiltration, amounts of CD95L mRNA and active caspase-3 reached maximum amounts (Figure S1), suggesting that these cells might

represent a major source of CD95L. Indeed, CD95L expression at the surface of peripheral blood neutrophils and monocytes significantly increased 24 hours after SCI (Figure 1C). Most importantly, increased surface levels of CD95L on peripheral blood neutrophils were also observed in spinal injured patients at early time points following injury. CD95L expression returned to control amounts at 1 week following injury (Figures 1D and 1E and Table S2). In patient e, who was followed for 17 days after injury, a second wave of CD95L expression on the surface of neutrophils was observed in the second week postinjury (Figure 1E). By contrast, CD95 surface expression on peripheral blood cells (PBCs) was not changed upon injury to the spinal cord (Figure S1).

CD95L Induces Activation of PI3K and Metalloproteinases via Syk Kinase in Myeloid Cells

We next addressed whether CD95L is acting on CD95 expressed by myeloid cells. The response to exogenously given CD95L was studied in bone-marrow-derived neutrophils and mature macrophages. Induction of apoptosis via CD95 occurs through the recruitment of the adapter protein *F*as-*A*ssociated *D*eath *D*omain (FADD) to the *D*eath *D*omain (DD) of CD95. Thus, we first examined FADD association with CD95 on primary macrophages treated with recombinant CD95L. Yet, whereas CD95L treatment induced efficient recruitment of FADD to CD95 in the CD95 apoptosis sensitive thymoma E20 cells, recruitment of FADD to CD95 could not be detected in stimulated primary macrophages (Figure S2). Consistently, macrophages are resistant to CD95-induced cell death (Altemeier et al., 2007; Park et al., 2003; Shimizu et al., 2005). There is increasing evidence that CD95L is involved in processes other than apoptosis (Sancho-Martinez and Martin-Villalba, 2009). In malignant glioma cells, we have recently reported increased migration upon CD95L stimulation (Kleber et al., 2008). In these cells, the Src family kinase Yes and the p85 subunit of PI3K are recruited to CD95 and activated upon CD95L binding. To address whether PI3K is also involved in our system, AKT phosphorylation upon CD95L treatment was assessed. Phosphorylation and, thus, activation of AKT was induced upon CD95L treatment in both neutrophils and macrophages (Figures 2A and 2B). Moreover, phosphorylation of Src family kinases (SFKs) also increased upon CD95 stimulation in primary macrophages (Figure 2B). With regard to the neutrophil cell population, further biochemical studies were performed in DMSO-differentiated HL-60 cells (dHL-60), a human neutrophil-like cell line. As in the case of primary macrophages, stimulation of CD95 led to increased phosphorylation of SFKs (Figure S2). Antibodies used to measure Y416 phosphorylation of SFKs are crossreactive with the different family members of this kinase. To address the identity of the involved SFK, we performed depletion experiments by pulling down the different SFKs with specific antibodies. Comparison of representative nondepleted samples with the depleted lysates allows identification of the SFKs that get phosphorylated upon CD95 stimulation. Accordingly, depletion experiments with Lyn antibodies and not with antibodies to other SFKs significantly reduced the double band visualized with the phospho-SFK antibody. This double band appears in the range of 55-53 kDa, which corresponds to the estimated molecular weight of Lyn (Figure S2 and data not shown).

We next addressed the molecular determinants of PI3K and SFKs activation upon stimulation of CD95 in immune cells. As the YXXL motif in CD95 was first described to get phosphorylated in primary neutrophils upon CD95 stimulation (Daigle et al., 2002), we decided to investigate potential CD95 interactors with this motif by using an SH2 array (Figure 2C, upper panel). As shown, CD95, or a CD95-containing multiprotein complex, could interact with the SH2 domain of the nonreceptor tyrosine kinase Zap70-Syk (Figure 2C, lower panel). To validate the results obtained from the protein array, we performed peptide-binding experiments, in which the corresponding sequence of CD95 containing the YXXL motif was incubated with CD95L-stimulated or nonstimulated lysates. In dHL-60 cells, incubation of the phosphorylated CD95 peptide resulted in increased binding of Syk compared to the nonphosphorylated CD95 peptide, as well as a scrambled phosphorylated peptide, which was used as negative control for sequence specificity (Figure 2D). Treatment with CD95L further enhanced binding of Syk to the phosphorylated CD95 peptide (Figure 2D). These results suggest the presence of adapter proteins and/or the requirement of posttranslational modifications, which cannot be mimicked by the peptide itself. Binding of Syk to the phosphorylated CD95 peptide was also observed in primary macrophages (Figure 2E). However, contrary to the results obtained in dHL-60, we did not observe differences in the binding upon treatment with CD95L (Figure 2E). These results were confirmed in CD95L-stimulated primary macrophages in which association of CD95 with Syk kinase was detected by coimmunoprecipitation studies (Figure 2F). Further, stimulation of CD95 led to increased phosphorylation of Syk in both dHL-60 and primary macrophages (Figure 2G and Figure S2). In B-cells, SFKs get activated by stimulation of the B-cell receptor (BCR), leading to activation of Syk, which can further activate SFKs by phosphorylation of the activation loop, thus creating a positive feedback loop between both molecules (Tamir and Cambier, 1998). To analyze possible similarities between CD95 and the BCR, we first studied the effect of SFKs on Syk phosphorylation. Inhibition of SFKs with the specific inhibitor PP2 blocked CD95L-induced phosphorylation of Syk in dHL-60 and primary macrophages (Figure S2). Knockdown of Syk in primary macrophages also abolished CD95-induced phosphorylation of SFKs and AKT (Figures 2H–2J). Taken together, these results demonstrate that Syk kinase couples CD95 to PI3K activation in myeloid cells.

CD95L Mediates Migration of Myeloid Cells via Syk-MMP Activation

We next studied CD95L-CD95-induced migration of neutrophils and macrophages via an in vitro transmigration assay through matrigel-coated transwells. Bone-marrow-derived murine neutrophils and macrophages, as well as dHL-60 cells, were plated on the coated transwells and stimulated with CD95L. After 3 hours (neutrophils), 4 hours (dHL-60), and 24 hours (macrophages), transmigrated cells were determined. Migration of neutrophils, dHL-60 cells, and macrophages significantly increased upon treatment with CD95L (Figure 3A). In glioblastoma cells, we have recently reported that CD95 induces migration via activation of MMPs (Kleber et al., 2008). In myeloid cells, pharmacological inhibition of MMP-9 and -2 also abolished CD95L-induced migration (Figure 3B). Importantly, basal migration of
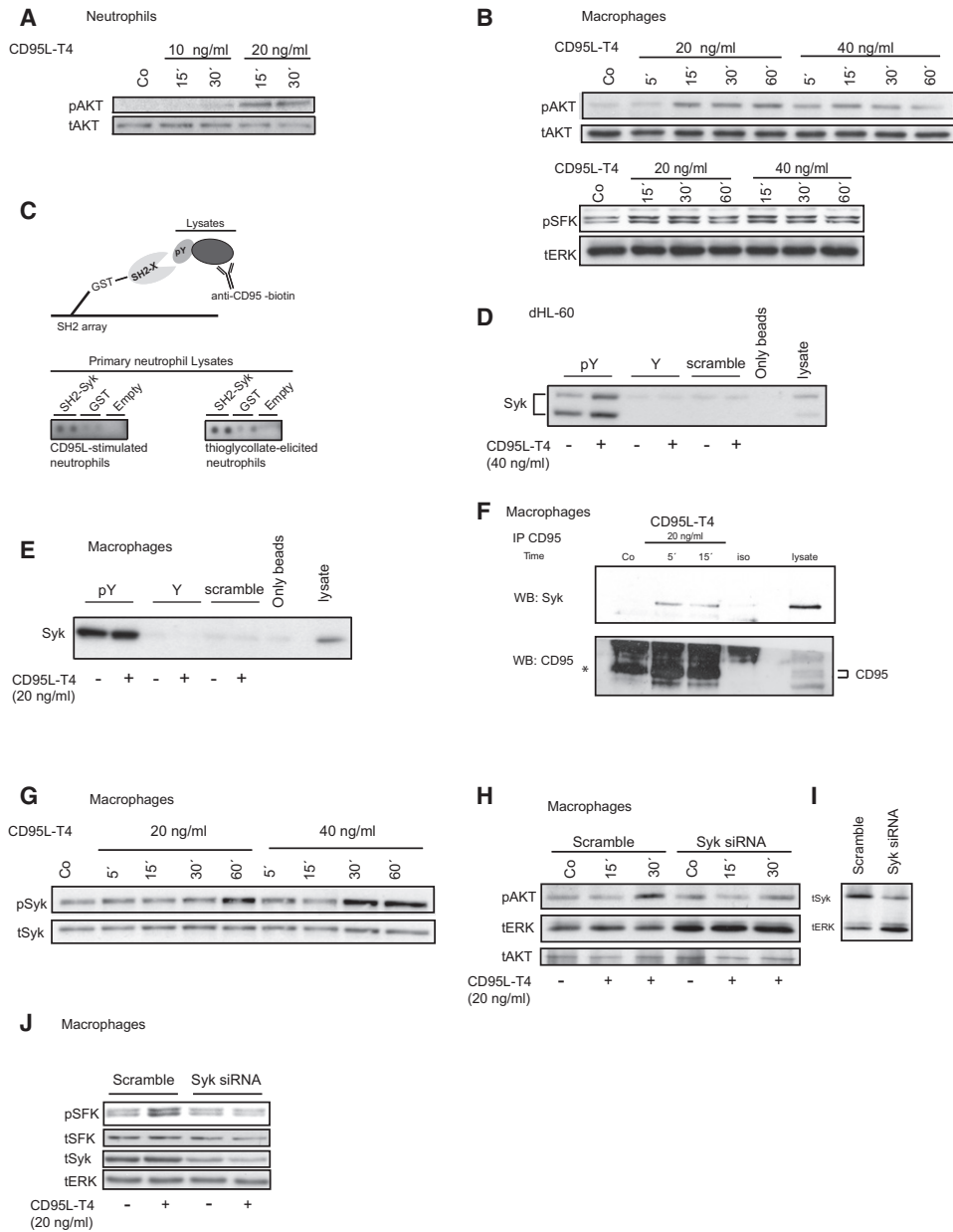
Figure 2. Stimulation of CD95 on Myeloid Cells Activates the Src/Syk/PI3K Pathway

(A–C) CD95L-T4 induces phosphorylation of AKT in (A) neutrophils and (B) macrophages and phosphorylation of Src in primary macrophages. (C) Experimental layout for SH2 arrays: detection of direct or indirect CD95 binding to an SH2-containing protein in CD95L-stimulated bone marrow-derived neutrophils or in vivo thioglycollate-activated peritoneal neutrophils. Note binding of CD95 to the SH2-domain of Syk (lower panel).

(D and E) Peptide-receptor competition experiments: peptides with the sequence of CD95 containing the phosphorylated or unphosphorylated YXXL motif were incubated with lysates from CD95L-treated or untreated (D) dHL-60 or (E) primary macrophages. Note exclusive binding of Syk kinase to the phosphorylated peptide.

(F) Primary macrophages were stimulated with CD95L-T4 for the indicated time and concentration. Immunoprecipitated CD95 was immunoblotted with antibodies against Syk and CD95.

(G) Phosphorylation of Syk kinase in primary macrophages upon CD95 stimulation.

(H–J) Knockdown of Syk kinase (I) in primary macrophages abolishes CD95L-induced phosphorylation of (H) AKT and (J) Src family kinases (SFKs). All data are representative of at least two independent experiments. t, total; p, phosphorylated; Iso, Isotype control; *specific band.

primary macrophages was reduced after neutralization of CD95L and in macrophages deficient in CD95 (*lpr*) or CD95L (*Fasl*$^{-/-}$) (Figure 3C). Thus, exogenous and endogenous CD95L increased macrophage migration via CD95 in vitro. To address the role of

Syk in CD95L-induced migration, we knocked down Syk in dHL-60 cells and primary macrophages. Reduced expression of Syk decreased CD95L-induced migration in dHL-60 and macrophages (Figure 3D and Figure S2). Accordingly, CD95L-induced
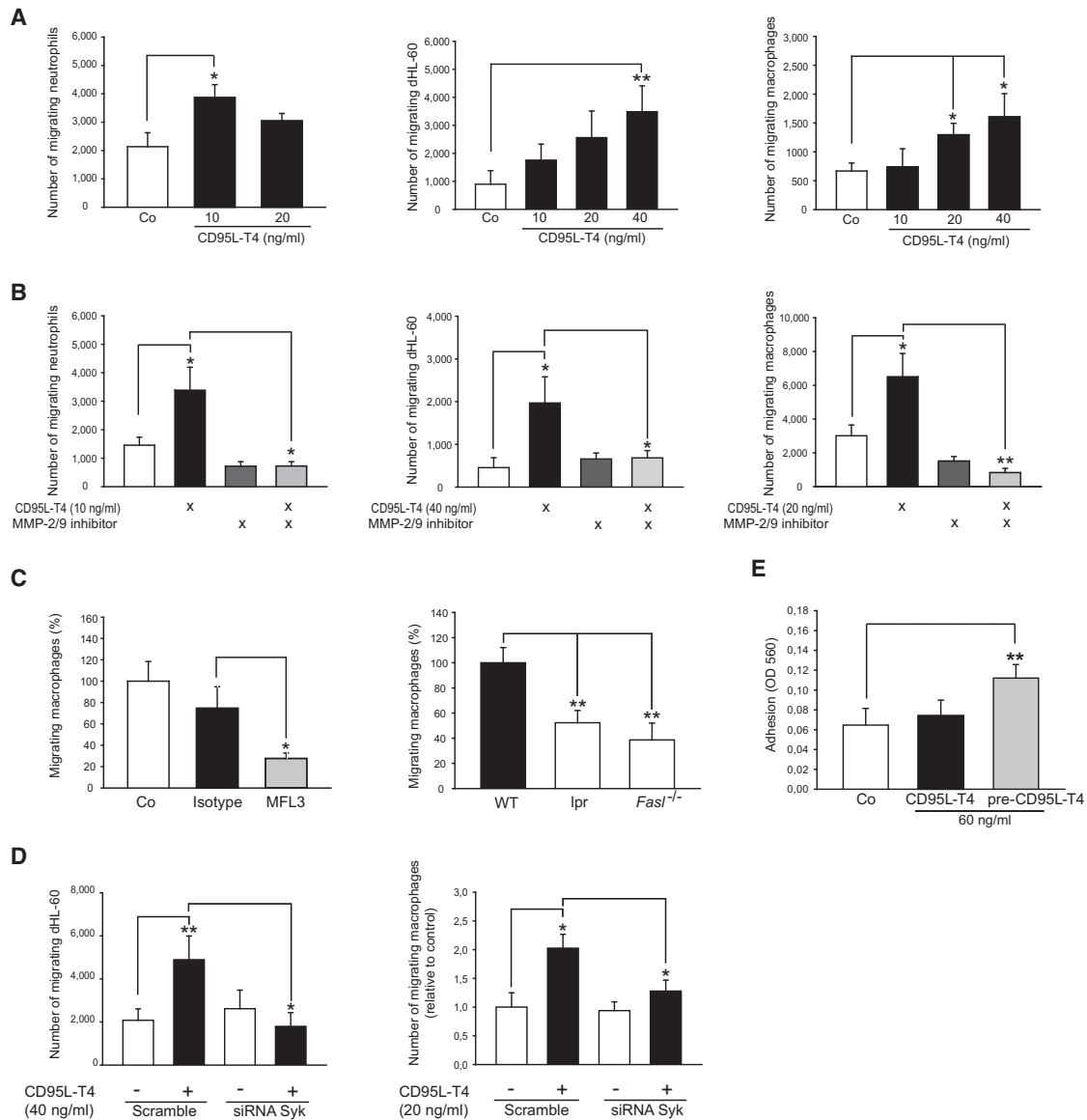
Figure 3. The CD95L-CD95 System Signals Migration of Myeloid Cells via Syk-MMP Activation
(A) CD95L-T4 induced migration of primary neutrophils, dHL-60, and primary macrophages.
(B) MMP-2 and 9 inhibitor blocked CD95L-T4 induced migration of neutrophils, dHL-60, and macrophages.
(C) Basal migration of macrophages was reduced by neutralizing antibodies to CD95L (MFL3) or in macrophages derived from *lpr* or *Fasl*$^{-/-}$ mice.
(D) Syk knockdown reduced CD95L-induced migration of dHL-60 and macrophages.
(E) CD95L pretreatment of macrophages significantly increases adhesion to ICAM-1. All data are representative of at least two independent experiments with at least three wells per condition. Data are presented as mean ± SEM; *p < 0.05; **p < 0.01.

transmigration was comparable to transmigration induced by a known Syk-dependent promigratory signal, CX3CL-1 (Fractalkine; Figure S2). These data demonstrate that CD95L acts on CD95 of neutrophils and macrophages in order to increase their migration via SFK-Syk-PI3K-AKT-MMPs.

It is important to note that leukocyte migratory capability is only the last step in the overall process of cell trafficking, related to microenvironmental repositioning. Other crucial events are involved, such as rolling, integrin triggering, arrest, and transendothelial migration (TEM). CD95L-triggered signaling pathway could potentially influence all these events. To test whether

CD95L may behave as a direct integrin agonist or prime to a subsequent proadhesive signal, we performed a static adhesion assay on purified ICAM-1. To this end, adhesion of macrophages, either untreated or treated with CD95L at the time of plating or 30 min before, was assessed at 3 min after plating. At this time, pretreatment with CD95L significantly increased the number of macrophages adhering to ICAM-1 (Figure 3E). This data indicates that prior to triggering transmigration, CD95L primes macrophages to adhesion signals. Unraveling the signaling events involved in priming will be subject of future studies.
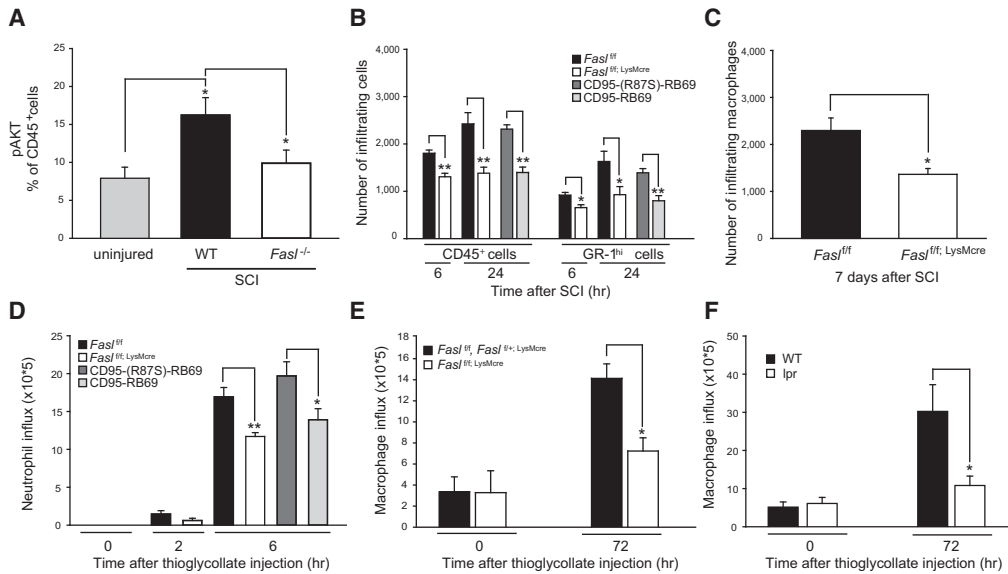
Figure 4. CD95L on Peripheral Myeloid Cells Is Involved in Their Recruitment to the Site of Injury In Vivo

(A) Phosphorylation of AKT in peripheral blood cells was assessed by flow cytometry. Injury to the spinal cord increased the percentage of pAKT-positive cells in WT, but not in $Fasl^{-/-}$ mice.

(B) Infiltration of immune cells, especially of neutrophils (CD45:GR-1^{high}), was reduced as compared to their respective controls in the injured spinal cord of $Fasl^{f/f;LysMcre}$ mice and in mice acutely treated with CD95L-neutralizing CD95 trimer (CD95-RB69) after SCI.

(C) Reduced infiltration of macrophages (CD45: CD11b$^+$, F4/80$^+$) in the injured spinal cord of $Fasl^{f/f;LysMcre}$ mice 7 days following SCI.

(D) Infiltration of neutrophils into the inflamed peritoneum was reduced in $Fasl^{f/f;LysMcre}$ mice and WT mice acutely treated with CD95-RB69 after thioglycollate injection.

(E and F) Infiltration of macrophages into the inflamed peritoneum was reduced in (E) $Fasl^{f/f;LysMcre}$ mice and (F) lpr mice. Data are presented as mean ± SEM. *p < 0.05; **p < 0.01.

CD95L on Myeloid Cells Is Involved in Their Recruitment to the Site of Injury In Vivo

To address if CD95L is also involved in AKT activation in peripheral myeloid cells in vivo, we first analyzed the activation status of AKT after SCI in wild-type (WT) and $Fasl^{-/-}$ mice. Injury to the spinal cord induced AKT phosphorylation in WT, but not CD95L-deficient PBCs (Figure 4A). To further analyze the role of CD95L in myeloid cells in vivo, we specifically deleted CD95L in neutrophils and macrophages ($Fasl^{f/f;LysMcre}$). Verified deletion of CD95L in myeloid cells with regard to mRNA and protein amounts did not influence percentages or absolute numbers of blood leukocytes in naive or injured animals, the phagocytic function of neutrophils and monocytes, or CD95 surface expression on peripheral blood cells (PBCs) (Figure S3 and data not shown).

In this mouse line, we analyzed the number of immune cells (CD45$^+$) present in the spinal cord following transection injury of the spinal cord. Six and 24 hours, as well as 7 days, after SCI, the number of infiltrating immune cells present in the injured spinal cord was assessed by flow cytometry as previously described (Stirling and Yong, 2008). In $Fasl^{f/f;LysMcre}$ mice, a significant reduction in infiltrating CD45$^+$ cells, largely identified as neutrophils (CD45: GR-1^{hi}), was observed (Figure 4B). Reduced infiltration of neutrophils could already be observed 6 hours after injury, long before the onset of apoptosis (Figure 4B). Infiltrating macrophages (CD45: CD11b$^+$, F4/80$^+$) were also markedly reduced 7 days after injury in $Fasl^{f/f;LysMcre}$ mice (Figure 4C). These data indicate that following SCI, CD95L acts

in a paracrine and/or autocrine fashion on neutrophils and macrophages in order to allow their recruitment to the injured spinal cord. To exclude any possible developmental role of CD95L in neutrophil maturation that could explain their lower infiltration rate into the site of injury, we acutely inhibited CD95L. In previous studies we used neutralizing antibodies to CD95L (Demjen et al., 2004). However, these antibodies greatly varied in their ability to neutralize CD95L. Thus, we generated a stable CD95L-neutralizing CD95 trimer, CD95-RB69. As a control protein, we also generated a mutated form of the neutralizing trimer, CD95-(R87S)-RB69, which is unable to bind CD95L (Figure S4 and Supplemental Information). Treatment with the CD95L-neutralizing trimer did not influence the phagocytic function of thioglycollate-elicited neutrophils or monocytes (Figure S3). Systemic administration of CD95-RB69, but not of the mutated form, decreased the infiltration of neutrophils into the lesion site 24 hours after injury (Figure 4B). Thus, CD95L on myeloid cells triggers their self-recruitment to the lesion site in vivo.

Is the proinflammatory effect of CD95L restricted to the inflammatory response elicited by the injured CNS? To address this issue, we examined the infiltration of immune cells in an animal model of peritonitis induced by intraperitoneal injection of thioglycollate, an in vivo model of leukocyte migration. At different time points after thioglycollate injection, infiltrated immune cells were removed from the peritoneum, and the amount of the different immune cell populations was assessed. A reduced infiltration of neutrophils into the peritoneum of $Fasl^{f/f;LysMcre}$

mice could already be observed 2 hours after thioglycollate injection (Figure 4D). Infiltration of neutrophils was significantly reduced 6 hours after thioglycollate injection in *Fasl*f/f;LysMcre and CD95-RB69-treated WT animals, compared to their respective controls (Figure 4D). We further assessed infiltration of macrophages in the peritoneum 72 hours after thioglycollate injection. At this time point, *Fasl*f/f;LysMcre mice showed a lower amount of infiltrating macrophages compared to control littermates, although the number of resident macrophages was not changed (Figure 4E). mRNA amounts of various proinflammatory cytokines were comparable in thioglycollate-elicited cells of control littermates and *Fasl*f/f;LysMcre mice (Figure S3), indicating that the migratory effect of CD95L is independent of cytokine production. In addition, after thioglycollate activation or SCI, the number of neutrophils undergoing apoptosis was similar in mice lacking CD95L activity and their respective controls (Figure S4). Consistent with these results, spontaneous death of neutrophils from CD95-deficient *lpr* (*l*ymphoproliferation) or CD95L-deficient *gld* (*g*eneralized *l*ymphoproliferative *d*isease) mice did not differ from the rates in WT mice (Fecho and Cohen, 1998), and blocking CD95/CD95L function with specific antagonists had no effect on the spontaneous death of neutrophils (Brown and Savill, 1999). Thus, CD95L activation of the innate immune response is not due to CD95 induction of promigratory cytokines or apoptosis. Macrophage recruitment to the inflamed peritoneum after thioglycollate injection was also assessed in *lpr* mice. To this end, we used 6-week-old *lpr* animals to avoid alteration in myeloid cell extravasation present at older stages. These sequelae of immune alterations that follow the development of autoimmunity can mask differences in thioglycollate-elicited migration (Fecho and Cohen, 1998; Gresham et al., 1991; Lowrance et al., 1994). In these mice, basal numbers of resident macrophages were not changed (Figure 4F). However, as previously observed in *Fasl*f/f;LysMcre, 72 hours following thioglycollate injection, we could observe a reduced infiltration of macrophages in *lpr* mice compared to their WT counterparts (Figure 4F).

CD95L Acts on the Innate Inflammatory Response to Induce Tissue Damage after SCI

We have demonstrated that CD95L on peripheral myeloid cells is used to facilitate their recruitment to the site of injury. Yet, what are the long term consequences of exclusive neutralization of CD95L-induced inflammation? To address this issue, we examined the long term clinical outcome and pathology of spinal injured animals with or without CD95L expression in the immune cell compartment in general or in the myeloid compartment. First, we generated bone-marrow-transplanted mice (BMT mice) from CD95L-deficient (*Fasl*−/−) or as a control, from wild-type (WT) donor mice and lethally irradiated WT recipient mice (BMT-*Fasl*−/− or BMT-WT mice, respectively) (Figure S5 and Table S1). *Fasl*−/− mice could not be used as a recipient due to defects in neuronal development that preclude significant functional recovery following SCI (Demjen et al., 2004; Zuliani et al., 2006). BMT-*Fasl*−/− mice had 25% of the CD95L mRNA as compared to BMT-WT mice and exhibited a significantly reduced caspase-3 activity in spinal cord tissue at the time at which injury-induced levels are maximal (Figure S5). In BMT-*Fasl*−/− mice, NeuN and CNPase immunoreactivity at 11 weeks after injury was higher compared to BMT-WT mice, indicating

that neurons and oligodendrocytes are rescued in BMT-*Fasl*−/− mice (Figure S5). These results clearly demonstrate that immune cells are a major source of CD95L following SCI and that the absence of CD95L in the immune cell compartment protects neurons and oligodendrocytes. To assess the long term consequences of CD95L-induced inflammation, BMT-*Fasl*−/− mice and their respective controls were subjected either to the previously used dorsal 80% transection or to the clinically more relevant crush injury of the spinal cord. Mice locomotor performance was assessed once weekly over a 10 to 11 week period in the swimming test and in the open field using the Basso Mouse Scale (BMS). Following transection or crush injury of the spinal cord, BMT-*Fasl*−/− mice exhibited a higher functional recovery than BMT-WT mice (Figure S5).

Second, we performed SCI in mice with exclusive deletion of CD95L in neutrophils and macrophages (*Fasl*f/f;LysMcre) and their control littermates. Importantly, after transection injury, spinal cord CD95L mRNA was highly reduced in *Fasl*f/f;LysMcre mice 24 hours after injury, further demonstrating that among immune cells, infiltrating myeloid cells are the major source of CD95L (Figure 5A). Besides, 3 days after injury, caspase-3 activity in the spinal cord of *Fasl*f/f;LysMcre mice was lower than in control littermates, reaching significance at 7 days (Figure 5B). Consistently, 11 weeks after injury, *Fasl*f/f;LysMcre mice had an increased number of surviving neurons and oligodendrocytes compared to their respective controls (Figures 5C and 5D). Furthermore, deletion of CD95L in the myeloid compartment allowed for a higher functional recovery following either crush or transection injury to the spinal cord in the BMS, as well as in the swimming test (Figures 5E and 5F). To analyze a possible effect of T cell-derived CD95L, *Fasl*f/f;LCKcre mice (Table S1) and control littermates underwent crush injury to the spinal cord. Contrary to *Fasl*f/f;LysMcre mice, SCI-induced neurological deficits were comparable in *Fasl*f/f;LCKcre and their respective controls (Figure S5). Thus, we clearly identified neutrophils and macrophages as the major source of CD95L, inducing death of neurons and oligodendrocytes and, therefore, participating in the pathogenesis of SCI.

Characterization of the Inflammatory Environment after Neutralization of CD95L

In order to characterize the molecular events regulated upon neutralization of CD95L on myeloid cells, we examined the gene signature of *Fasl*f/f;LysMcre mice and their littermate counterparts in the spinal cord 24 hours after transection injury. Already at this early time point, regenerative processes including organogenesis, development, and neurogenesis are switched on in *Fasl*f/f;LysMcre mice (Figure S6). Similarly, expression of genes involved in apoptosis was reduced in *Fasl*f/f;LysMcre mice or CD95-RB69-treated mice as compared to their respective controls (Figure S6). Beyond this, lack of CD95L in myeloid cells or neutralization of CD95L in CD95-RB69-treated animals resulted in downregulation of genes involved in the immune response (Figure S6). The observed downregulation of proinflammatory genes was further validated by qRT-PCR (Figure 6A and data not shown). Importantly, among these downregulated proinflammatory cytokines, neutralization of IL-6, IL-1, or CXCL10 is reported to improve functional recovery after SCI (Akuzawa et al., 2008; Gonzalez et al., 2007; Okada et al., 2004). Interestingly, 24 hours after SCI, 65.2% of genes were
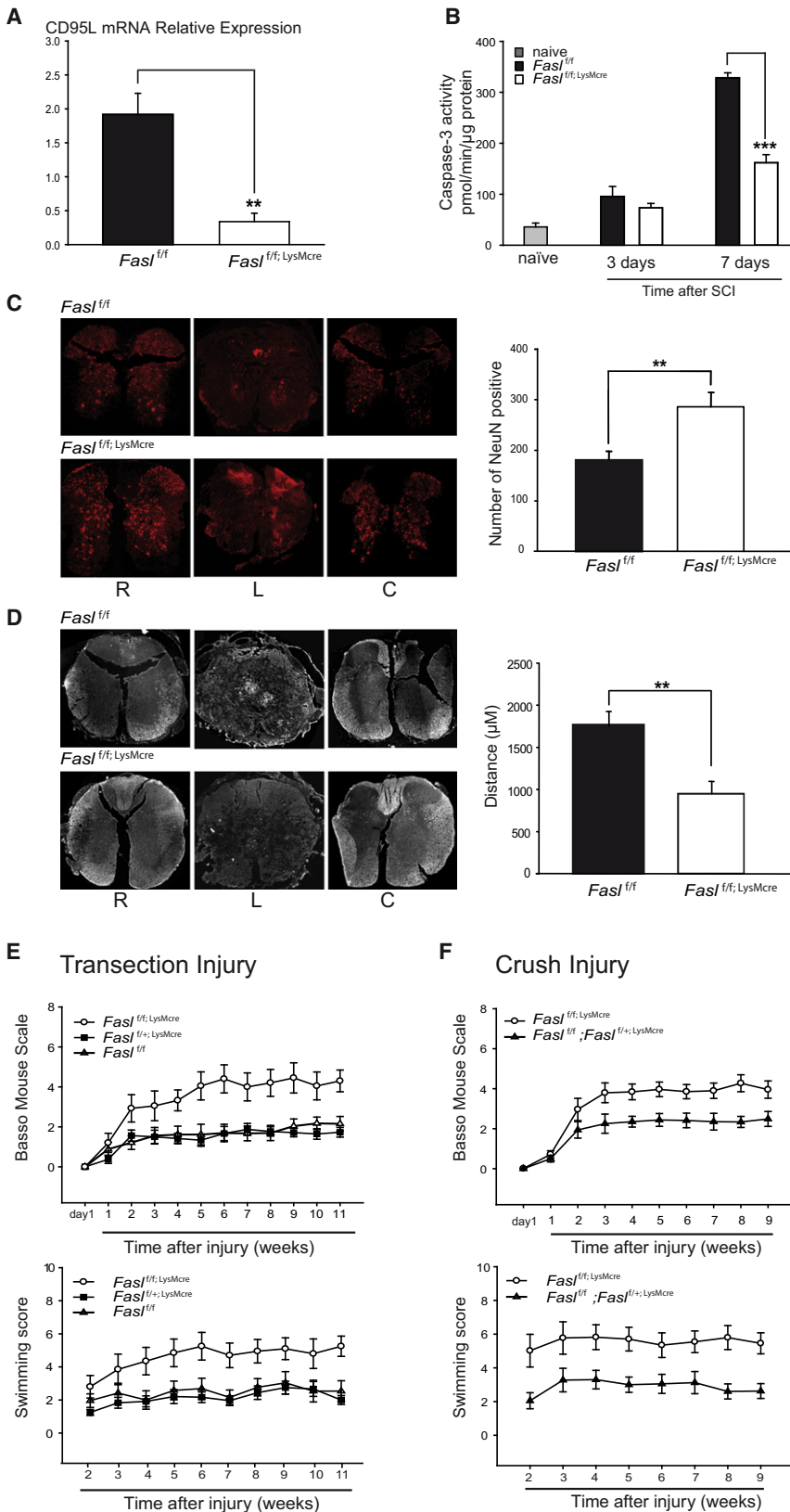
Cell PRESS

A

CD95L mRNA Relative Expression

B

C

Fasl f/f

Fasl f/f; LysMcre

R   L   C

D

Fasl f/f

Fasl f/f; LysMcre

R   L   C

E   Transection Injury

F   Crush Injury

Figure 5. Exclusive Deletion of CD95L in Myeloid Cells Reduces Death of Neurons and Oligodendrocytes and Improves Functional Recovery after SCI

(A and B) Twenty-four hours after transection-SCI, *Fasl* f/f;LysMcre mice exhibited lower amounts (A) of CD95L mRNA (n = 6/group) and (B) of caspase-3 activity (n = 4/group) compared to control littermates. mRNA amounts were normalized to naïve WT animals.

(C) Ten to eleven weeks after crush-SCI, *Fasl* f/f;LysMcre mice exhibited increased number of NeuN+ cells (NeuN: a marker for mature neurons) compared to *Fasl* f/f control littermates (n = 6/group).

(D) White matter sparing was determined by the distance between the rostral loss and reappearance caudal to the lesion site of the CNPase staining in the dorsal funiculus of the spinal cord. Ten to eleven weeks after crush-SCI, *Fasl* f/f;LysMcre mice exhibited a shorter distance as compared to the respective controls, indicating increased white matter sparing (n = 6/group).

(E and F) Following transection-SCI (E) or crush-SCI (F), functional improvement over time, as assessed by Koziol test of BMS and swimming score, was higher in *Fasl* f/f;LysMcre than in *Fasl* +/f LysMcre and *Fasl* f/f control littermates (n = 10–12/group; p < 0.01). R, rostral to the lesion site; L, lesion site; C, caudal to the lesion site Data are presented as mean ± SEM. *p < 0.05; **p < 0.01.
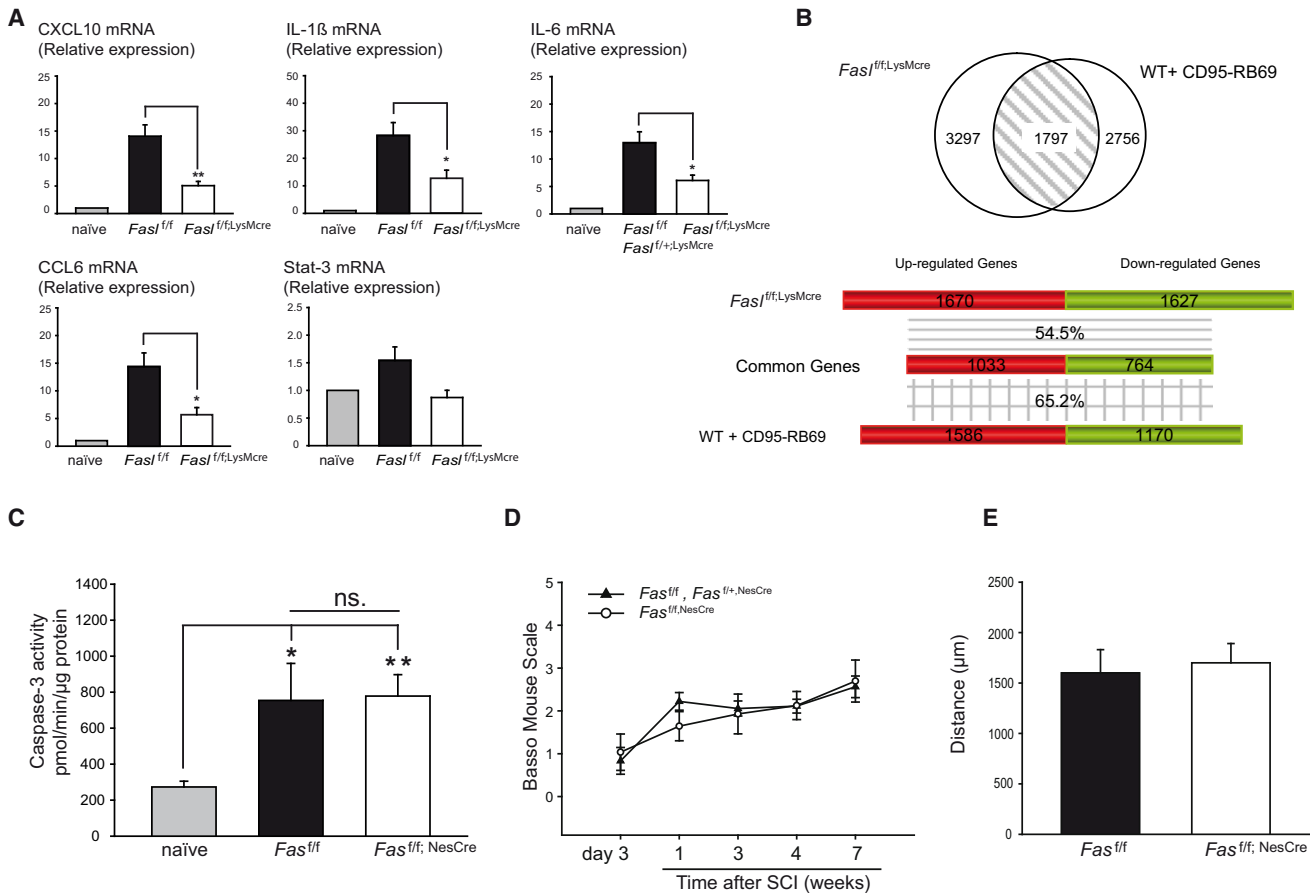
Figure 6. Deletion of CD95L in Myeloid Cells Regulates the Inflammatory Environment following SCI

(A and B) Gene-expression profiling was performed in Fas$^{f/f;LysMcre}$ mice and CD95-RB69-treated mice and their respective controls 24 hr after SCI (n = 3/group).

(A) Validation of microarray data by qRT-PCR: mRNA amounts of CXCL10, IL-1β, IL-6, CCL6, and Stat-3 24 hr after SCI. (n = 4/group; *p < 0.05; **p < 0.01).

(B) 65.2% of genes were commonly regulated on the data set of Fasl$^{f/f;LysMcre}$ compared to Fasl$^{f/f}$ littermates and CD95-RB69-treated compared to vehicle-treated mice 24 hr after SCI.

(C) Caspase-3 activity 7 days following SCI in Fas$^{f/f;Nescre}$ and their respective littermate controls, Fas$^{f/f}$ (n=4 to 5/group; *p < 0.05; **p < 0.01).

(D) Assessment of functional recovery after crush injury of the spinal cord of Fas$^{f/f;Nescre}$ and respective littermate controls.

(E) White matter sparing was determined by the distance between the rostral loss and reappearance caudal to the lesion site of the CNPase staining in the dorsal funiculus of the spinal cord. Fourteen weeks after crush-SCI, this distance did not differ between Fas$^{f/f;Nescre}$ mice and respective controls, indicating that deletion of CD95 on neural cells does not influence white matter sparing (n = 3 to 4 per group). Data are presented as mean ± SEM. ns, not significant.

commonly regulated between the group with genetic deletion of CD95L on myeloid cells (Fasl$^{f/f;LysMcre}$ versus Fasl$^{f/f}$ littermates) and the group with pharmacological inhibition of CD95L (CD95-RB69-treated versus vehicle-treated mice), indicating that at this time point, the gene signature is due to the exclusive deletion of CD95L in the immune cell compartment (Figure S6). Furthermore, we compared the data sets from the following animals and their respective control counterparts 24 hours following SCI: Fasl$^{f/f;LysMcre}$, Fasl$^{-/-}$, and CD95-RB69-treated mice (Table S1). In contrast to Fasl$^{f/f;LysMcre}$ animals, in the latter two groups, CD95L from resident spinal cells is also deleted. For statistically based meta-analysis of combined microarray of our three microarray data sets, the GeneMeta package from Bioconductor (http://bioconductor.org) was applied (Supplemental Information). This analysis provides a single estimate of the degree of differential expression for each gene while simultaneously accounting for the detection of differences between

each experiment and animal background (Supplemental Information). Comparison of these three data sets allowed the detection of 612 genes that were consistently and significantly differentially regulated in the spinal cord 24 hours after injury (Figure S6 and Table S3). The identification of a common gene signature regardless of the site of CD95L inhibition implies that the initial cause of CD95L-induced damage is the activation of the innate inflammatory response.

To finally assess the contribution to tissue damage of CD95L-induced inflammation versus direct CD95L-induced apoptosis, we examined caspase activity in mice deficient in CD95 in resident neural cells (Fas$^{f/f;NesCre}$) and their littermate controls (Fas$^{f/f}$). The extent of caspase-3 activity did not differ between the two groups (Figure 6C and Figure S6). Most importantly, the functional recovery or loss of CNPase immunoreactivity following SCI was not influenced by the lack of CD95 in resident CNS cells (Figures 6D and 6E). These data demonstrate that

CD95L detrimental function following SCI is due to its influence on the innate inflammatory response and not to direct CD95-induced apoptosis of CD95-bearing resident neural spinal cells.

DISCUSSION

Our results reveal a mechanism by which the CD95L-CD95 system on peripheral myeloid cells mediates their recruitment to the inflammatory site via the SFK-Syk-PI3K-AKT-MMP pathway. We show that an injury to the CNS increases expression of CD95L on peripheral myeloid cells in rodents and humans. This system is also involved in the recruitment of myeloid cells to the inflamed peritoneum after thioglycollate injection. Further, we show that neutralization of CD95L reduces the initial infiltration of inflammatory cells, creating an inflammatory response that facilitates recovery of locomotor function after SCI. Importantly, lack of CD95 on CNS resident neural cells did not result in improved functional recovery following SCI, indicating that apoptosis of CD95-bearing cells does not contribute to the pathophysiology of SCI.

Until the mid-90s, the dogma that apoptosis does not induce inflammation was strongly anchored in the scientific community. It was generally believed that CD95L resolves inflammation by inducing activation-induced cell death (AICD) of T cells (Griffith et al., 1995, 1996; Nagata, 1999). Along this line, constitutive expression of CD95L by cells in the eye and testis was thought to contribute to the immune-privileged status of these organs (Griffith et al., 1995, 1996). It was further suggested that constitutive CD95L expression by a variety of tumor populations would lead to immune evasion (Hahne et al., 1996; O'Connell et al., 1996; Strand et al., 1996). But unexpectedly, most cell types and tissues genetically engineered to express CD95L undergo destruction through neutrophils (Allison et al., 1997; Kang et al., 1997; Seino et al., 1997). This data would indicate a role for CD95L as a chemoattractant. Alternatively, it is known that CD95L is quickly removed from the surface of the cell by metalloproteinases, and released CD95L in the blood can bind to CD95 on peripheral myeloid cells and trigger their recruitment—in this case, the engineered tissue. In the case of spinal cord injury, a yet unknown chemotactic signal induces expression of CD95L on peripheral myeloid cells, thereby leading to activation of Syk-PI3K-AKT via CD95. This signal primes cells for undirected migration, and the chemotactic gradient arising from the injured spinal cord directs those cells to the site of injury. Accordingly, we show that CD95L primes macrophages to ICAM-1-induced adhesion. Thus, the CD95-CD95L interaction takes place in the periphery and not in the CNS.

Indirect evidence for a similar role of CD95L in autoimmune disease is given by the fact that the *lpr* mutation ameliorates disease signs in mice with experimental autoimmune encephalomyelitis and collagen-induced arthritis (Hoang et al., 2004; Ma et al., 2004; Sabelko et al., 1997). However, the autoimmune disease resulting from the *lpr* mutation hampers the study of inflammation in this strain. Accordingly, though it has been reported that WT mice exhibit significantly higher thioglycollate-elicited neutrophil extravasation than *lpr* mice at 6 hours after stimulation, the absolute numbers of thioglycollate-elicited neutrophils over a period of 48 hours did not differ between WT and *lpr* animals (Fecho and Cohen, 1998). The mice used in this study were on average old enough (*gld*, 13 weeks and *lpr*, 17.5 weeks) to be showing signs of autoimmune disease and, therefore, results being masked by the autoimmune phenotype. Factors associated with autoimmunity, such as higher amounts of TGF-β found in *lpr* mice compared to littermate controls, lead to alterations in neutrophil extravasation to the inflamed peritoneum (Gresham et al., 1991; Lowrance et al., 1994).

Here, we show that exclusive deletion of CD95L on myeloid cells allows a beneficial innate inflammatory response in an animal model of peritonitis and of spinal cord injury. Accordingly, proinflammatory cytokines and chemokines, such as IL-1β, IL-6, CXCL10, and CCL6, were downregulated in the injured spinal cord of these animals. Thus, the reduced production of inflammatory mediators by CD95L-deficient myeloid cells might contribute to changes in the inflammatory environment. Indeed CD95 is described to activate signal transduction pathways leading to the induction of proinflammatory responses (Baud and Karin, 2001). Ligation of CD95 can induce proinflammatory cytokines and chemokines in different cell types, such as endothelial cells, bronchial epithelial cells, human vascular smooth muscle cells, and astrocytes (Hohlbaum et al., 2002), which could explain a reduced proinflammatory environment in *Fasl*^f/f;LysMcre^ mice. Nevertheless, we show that thioglycollate-elicited neutrophils and macrophages from *Fasl*^f/f;LysMcre^ mice expressed the same mRNA amounts of proinflammatory cytokines. Moreover, at 6 hours after SCI or 2 hours after thioglycollate-induced peritonitis, one of the earliest time points in neutrophil infiltration, reduced numbers of neutrophils in *Fasl*^f/f;LysMcre^ mice could already be detected. Immune cells that infiltrate the lesion site produce cytokines upon interaction with other immune cells or with CNS-resident cells. This leads to amplification of the chemotactic gradient and, thereby, to further infiltration of leukocytes to the lesion site. Thus, we believe that reduced amounts of proinflammatory cytokines are due to reduced migration and not to deficient induction of cytokines via CD95. Taken together, CD95 influences myeloid cell migration and thereby influence the inflammatory environment. By contrast, a recent study showing full depletion of neutrophils using an anti-Ly6G/Gr1 antibody prior to SCI reports increased amounts of several proinflammatory cytokines including IL-6 and a worsened clinical outcome following SCI of depleted animals (Stirling et al., 2009). It is noteworthy that neutrophils and macrophages not only contribute to tissue damage but also to cleaning the injury site, limiting bacterial infection and promoting wound healing. Accordingly, neutralization of CD95L led to a reduction without complete abrogation of infiltrating neutrophils and macrophages. How this residual inflammation exerts its beneficial effects remains the subject of future studies.

We have previously shown that CD95L triggers invasion in a glioblastoma model and differentiation of NSCs via SFK-PI3K-AKT pathway (Corsini et al., 2009; Kleber et al., 2008). But how does CD95 induce PI3K activation in myeloid cells? In 1996, Atkinson and colleagues reported a physical interaction between CD95 and a nonreceptor tyrosine kinase, the Src familiy member Fyn in T cells (Atkinson et al., 1996). They further described the presence of a highly conserved tyrosine-containing YXXL motif located in the DD of CD95 that resembles an *i*mmunoreceptor *t*yrosine *a*ctivation *m*otif (ITAM). Six years later, Daigle and

colleagues (Daigle et al., 2002) showed that stimulation of CD95 in primary neutrophils leads to phosphorylation of this motif, thus serving as docking sites for SH2-domain-containing proteins. Phosphorylation of the receptor is thought to be driven by members of the Src family of nonreceptor tyrosine kinases (SFKs: Src, Fyn, Yes, Lck, Hck, and Lyn) (Atkinson et al., 1996). Once the YXXL motif is phosphorylated, other SH2-containing protein kinases or phosphatases could potentially bind and initiate activation of downstream signaling pathways. Here, we show that CD95L stimulation of CD95 on myeloid cells activates Syk, further leading to PI3K-MMP signaling. Accordingly, blocking PI3K has been shown to inhibit migration of immune cells (Ali et al., 2004; Frommhold et al., 2007). However, the role of SFKs and Syk in leukocyte migration is still a matter of debate (Baruzzi et al., 2008). SFK- or Syk-deficient macrophages have significantly reduced migration in vitro and in vivo, yet, same deficiency in neutrophils allows migration in vitro and into the inflamed peritoneum but impairs migration into the inflamed lung, or cremaster muscle (Baruzzi et al., 2008). These apparently contradictory results reflect the presence of different ligands in the various inflammatory scenarios, with some ligands activating non-ITAM-dependent promigratory signaling pathways, such as G-coupled protein receptors (GPCR)-driven PI3K-SFK activation. The different migratory behavior of CD95L-deficient and Syk-deficient neutrophils suggests that Syk may transduce pro- and antimigratory signaling pathways (Mócsai et al., 2002). Besides, CD95L might activate other promigratory signaling pathways that have not been addressed yet.

Do neurons and oligodendrocytes die in the injured spinal cord due to direct CD95-induced death or, rather, to CD95-elicited inflammation? It has been shown that neutrophils can kill bystander cells in coculture systems through the CD95L-CD95 system (Brown and Savill, 1999; Serrao et al., 2001). Moreover, phagocytosis triggers macrophage release of CD95L and, thus, is able to induce cell death of bystander cells (Brown and Savill, 1999). In addition, a recent study has demonstrated that CD95L is directly able to induce death of oligodendrocytes through both intrinsic and extrinsic pathways of the CD95-mediated apoptotic signaling (Austin and Fehlings, 2008). However, all these data have been generated by in vitro studies. The fact that mice with exclusive genetic deletion of CD95 in neural cells do not show a decreased caspase-3 activity or improved functional recovery following SCI demonstrates that CD95 exerts its deleterious function by acting on the innate immune response. As a consequence, pharmacological interventions aimed at neutralization of CD95 activity have to act in the periphery and not, as previously believed, in the spinal cord. This would have the additional advantage of not interfering with the recently reported CD95-regenerating signal in the CNS (Corsini et al., 2009). Beyond this, neutralization of CD95L-CD95 system emerges as a candidate therapy for inflammatory diseases in general.

EXPERIMENTAL PROCEDURES

Animals

$Fasl^{-/-}$ were described previously (Karray et al., 2004). C57BL/6J mice were purchased from Charles River Laboratories. $Fasl$-floxed mice (Karray et al., 2004) were bred with LysM Cre mice (Jackson Laboratory) and LCK Cre mice (a kind gift from G. Hämmerling). Fas-floxed mice (kind gift from K. Rajewsky) were bred with Nestin-Cre mice (kind gift from G. Schütz). Mice that ubiquitously express an enhanced green fluorescent protein were a kind gift from B. Arnold. Experimental animals were age-matched and used at 12–14 weeks of age. Animals used are described in Table S1 online. All animal experiments were performed in accordance with institutional guidelines of the German Cancer Research Center and were approved by the Regierungspräsidium Karlsruhe, Germany.

Thioglycollate-Induced Peritonitis

For thioglycollate-induced peritonitis, 1 ml of 3% thioglycollate broth (Fluka) was injected i.p. in $Fasl^{f/f;LysMcre+}$ and $Fasl^{f/f}$ mice or in WT mice acutely treated with CD95-RB69 or its respective control. In this model, neutrophils are known to start infiltrating the peritoneum within the first hours, whereas macrophage infiltration peaks at 72 hr. At the indicated times, mice were sacrificed, blood samples were collected, and peritoneal cavities were lavaged with 10 ml sterile Hank's balanced salt solution (HBSS; Invitrogen) containing 0.25% bovine serum albumin (Roche). Total cell counts were performed in a Neubauer hematocytometer (Brand), and differential cell counts were carried out by flow cytometry. Results are expressed as the absolute number of neutrophils or macrophages $\times 10^5$/cavity. For every experiment performed, blood immune cell populations were analyzed by the appropriate cell markers.

Migration Assay

Migration of bone-marrow-derived neutrophils or macrophages was assessed in vitro in a two-chamber migration assay. Transwell inserts (3 μm [BD no. 353096] or 8 μm [BD no. 353097] pore size for neutrophils or macrophages, respectively) were coated with matrigel (50 μg/ml; BD no. 354234). 5×10^5 neutrophils, 1×10^6 dHL60, or 2×10^5 macrophages were plated in 500 μl medium onto the upper chamber. Cells were left untreated or treated with CD95L-T4 (engineered $Mus\ musculus$ CD95L [Kleber et al., 2008 and Supplemental Information]) by adding 10, 20, and 40 ng/ml to the upper chamber. The number of migrated cells was counted 3 hr for neutrophils, 4 hr for dHL-60, and 24 hr for macrophages after treatment by using a hemocytometer. CD95L-induced migration of macrophages was analyzed by blocking basal migration of macrophages by using neutralizing antibodies to CD95L (MFL3, 10 μg; BD no. 555290) or the appropriate isotype control (IgG, 10 μg; BD no. 554709). Data of the migration assays are representative of at least four independent experiments with six technical replicates per condition.

The role of metalloproteinases on neutrophil and macrophage recruitment was investigated by using selective inhibitors of MMP-2 and -9. Neutrophils, dHL-60, and macrophages were preincubated with MMP-2 and -9 inhibitors (50 μM; Calbiochem no. 444251) 30 minutes prior to CD95L-T4 treatment, and the number of migrated cells was counted at the times indicated previously.

Spinal Cord Injury

SCI models: transection injury of the spinal cord was performed as previously described (Demjen et al., 2004). For the crush injury model, forceps were held on the spinal cord for 15 seconds, resulting in a lateral compression of the spinal cord (Plemel et al., 2008). Immediately following injury and for an additional week, mice received antibiotics (Gentamycin, 5 ml/kg of a 0.2 mg/ml solution) to prevent infection. Postoperative care included housing of the animals at 27°C, food and water ad libitum, and manual evacuation of the bladders once daily.

Anti-CD95L Treatment

Mice were treated intravenously 5 minutes after SCI or induction of thioglycollate-induced peritonitis with 50 μg (solved in 200 μl sterile PBS) of either a stable CD95L-neutralizing CD95 trimer, CD95-RB69, or a mutated form, CD95-(R87S)-RB69, which is unable to bind CD95L and serves as a control.

Behavioral Assessment

All behavioral tests were performed by two independent observers in a double-blind manner weekly for 9–11 weeks after injury. The general locomotor performance of the animals was assessed using the Basso Mouse locomotor rating Scale (BMS) (Basso et al., 2006) and the swimming test, assessed as previously described (Demjen et al., 2004). For the BMS, animals were additionally

tested at the first day after injury. Any mouse showing a BMS score over 0.5 at day 1 was excluded from further studies. As littermate controls (*Fasl* [f/f] and *Fasl* [f/+;LysMcre]) showed no significant difference in locomotor activity, their results were pooled in the crush injury model.

Statistical Evaluation

All statistical summaries data, including the sample size and results of statistical evaluations, are listed in Table S4. For behavioral experiments, the overall improvement in mice compared to the control group was statistically analyzed by using the Koziol test (Koziol et al., 1981), a nonparametric test appropriate for longitudinal data, which allows analysis of the combined data over time. Statistical analyses of all other endpoints were performed by using the standard unpaired Student t test. No formal test for normality was applied in view of the small sample sizes when Student's t test was applied. All data were presented as mean ± standard error of the mean (SEM). Statistical significance was reported by the p value of the statistical test procedures and was assessed as significant ($*p < 0.05$) strongly significant ($**p < 0.01$), or highly significant ($***p < 0.001$). All statistical analyses were performed with the program package ADAM from the Biostatistics Unit of the German Cancer Research Center, DKFZ.

SUPPLEMENTAL INFORMATION

The Supplemental Data include Supplemental Experimental Procedures, six figures, and four tables and can be found with this article online at doi:10.1016/j.immuni.2010.01.011.

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