Natural killer cells modulate motor neuron-immune cell cross talk in models of Amyotrophic Lateral Sclerosis

Stefano Garofalo1✉, Germana Cocozza2, Alessandra Porzia3, Maurizio Inghilleri4, Marcello Raspa5, Ferdinando Scavizzi5, Eleonora Aronica6, Giovanni Bernardini7, Ling Peng8, Richard M. Ransohoff9, Angela Santoni2,7 & Cristina Limatola2,10✉

In amyotrophic lateral sclerosis (ALS), immune cells and glia contribute to motor neuron (MN) degeneration. We report the presence of NK cells in post-mortem ALS motor cortex and spinal cord tissues, and the expression of NKG2D ligands on MNs. Using a mouse model of familial-ALS, hSOD1G93A, we demonstrate NK cell accumulation in the motor cortex and spinal cord, with an early CCL2-dependent peak. NK cell depletion reduces the pace of MN degeneration, delays motor impairment and increases survival. This is confirmed in another ALS mouse model, TDP43A315T. NK cells are neurotoxic to hSOD1G93A MNs which express NKG2D ligands, while IFNγ produced by NK cells instructs microglia toward an inflammatory phenotype, and impairs FOXP3+/Treg cell infiltration in the spinal cord of hSOD1G93A mice. Together, these data suggest a role of NK cells in determining the onset and progression of MN degeneration in ALS, and in modulating Treg recruitment and microglia phenotype.
Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease characterized by the progressive degeneration of motor neurons (MN)\(^1\). In a subgroup of familial ALS (fALS), mutant superoxide dismutase 1 (SOD1) is expressed. In this form of ALS and in the corresponding mouse models, microglia acquire an inflammatory phenotype, affecting MN death\(^1\)–\(^3\), and myeloid cells expressing mutated SOD1 promote neurotoxicity\(^4,5\). As in other CNS disorders, microglia exert protective or detrimental functions in different disease phases, depending on cell-autonomous features and local microenvironmental cues\(^6\)–\(^8\). As one example, the expression level of mutant SOD1 in microglia correlates with the late phase of disease\(^3\), while microglial phenotypes in hSOD1\(^{G93A}\) mice are modulated by infiltrating Treg cells, through cytokine production\(^1\)–\(^2\). Moreover, Treg number and FOXP3 expression are reduced in rapidly progressing ALS patients, and it has been shown that the passive transfer of Tregs prolongs survival, suppressing neuroinflammation\(^10\).

NK cells regulate both the adaptive and innate immune responses, and CNS infiltrating NK cells modulate neuroinflammation in neurodegenerative diseases\(^11\)–\(^14\), and affect microglial phenotype in cerebral tumors\(^3\)–\(^5\),\(^16\). In addition, it was recently described that in pathological conditions, damaged neurons express NKG2D ligands and selectively succumb to NK cell-mediated degeneration\(^17\). Previous longitudinal cohort studies of peripheral blood from ALS patients revealed an increased number of NK cells in patients\(^18\); additionally, end-stage hSOD1\(^{G93A}\) mice showed high NK cell frequency in the spinal cord\(^19\).

Here, we investigate the functional role of NK cells in disease onset and progression in the hSOD1\(^{G93A}\) mice. We found that NK cells directly kill spinal cord motor neurons in a NKG2D-dependent manner. In addition, we characterized NK cell cross talk with both resident microglia and infiltrating Treg cells in the hSOD1\(^{G93A}\) mice. We propose that these communication mechanisms orchestrate a vicious cycle of inflammatory signals contributing to onset of motor impairment but not disease progression. Given NK cell presence in postmortem sALS motor cortex and spinal cord, and the expression of NKG2D ligands on postmortem sALS motor neurons, these data suggest a key role of NK cells in ALS onset, highlighting the importance of immune cells in motor neuron loss.

Results

NK cell analyses in ALS patients. To determine whether NK cells were altered in ALS patients (Tables 1, 2), we analysed the CNS tissues and blood of sporadic ALS patients (sALS) for NK cell presence and for frequency of NK cell subtypes. NKP46\(^+\) cells were detected in sALS spinal cord and cerebral motor cortex postmortem tissues while they were not observed in the spinal cord or motor cortex of controls (patients listed in Table 1, Fig. 1a). In addition, peripheral blood from sALS patients showed a reduction of circulating CD56\(^+\)/CD3\(^–\) cells, both in the CD56\(^{high}\) and CD56\(^{low}\) cell populations, in comparison with controls (patients listed in Table 2, Fig. 1b).

| Patients | Clinical diagnosis | Gender | Site of onset | Age | Disease duration (months) | C9orf72 repeat expansion FUS and SOD1 mutation, TDP43 pathology |
|----------|--------------------|--------|--------------|-----|---------------------------|---------------------------------------------------------------|
| 1        | sALS               | M      | Spinal       | 53  | 33                        | C9orf72, TDP43                                               |
| 2        | sALS               | M      | Bulbar       | 58  | 11                        | TDP43                                                        |
| 3        | sALS               | M      | Spinal       | 66  | 22                        | TDP43                                                        |
| 4        | sALS               | F      | Spinal       | 64  | 34                        | C9orf72, TDP43                                               |
| 5        | sALS               | M      | Spinal       | 53  | 33                        | C9orf72, TDP43                                               |
| 6        | sALS               | M      | Bulbar       | 74  | 31                        | C9orf72, TDP43                                               |
| 7        | sALS               | F      | Bulbar       | 65  | 24                        | TDP43                                                        |
| 8        | sALS               | M      | Spinal       | 58  | 12                        | TDP43                                                        |
| 9        | sALS               | M      | Spinal       | 69  | 24                        | TDP43                                                        |
| 10       | sALS               | F      | Bulbar       | 40  | 35                        | TDP43                                                        |
| 11       | sALS               | M      | Bulbar       | 71  | 33                        | TDP43                                                        |
| 12       | Control            | M      | n/a          | 54  | n/a                       |                                                               |
| 13       | Control            | M      | n/a          | 56  | n/a                       |                                                               |
| 14       | Control            | M      | n/a          | 66  | n/a                       |                                                               |
| 15       | Control            | F      | n/a          | 79  | n/a                       |                                                               |
| 16       | Control            | F      | n/a          | 35  | n/a                       |                                                               |
| 17       | Control            | M      | n/a          | 49  | n/a                       |                                                               |
| 18       | Control            | M      | n/a          | 75  | n/a                       |                                                               |
| 19       | Control            | M      | n/a          | 74  | n/a                       |                                                               |

Site of onset (region in which first symptoms occurred): bulbar onset, spinal onset. Age: years. Disease duration: time from diagnosis until death in months. Fused in sarcoma/translocated in liposarcoma (FUS), chromosome 9 open reading frame 72 (C9orf72). The postmortem delay for ALS patients and controls was of 8 ± 2 h. ALS Amyotrophic lateral sclerosis, sALS sporadic ALS, M male, F female, n/a not applicable.
Table 2 List of patients for peripheral blood NK cell analyses.

| Patients | Clinical diagnosis | Gender | Site of onset | Disease duration | Score |
|----------|-------------------|--------|---------------|-----------------|-------|
| 1        | sALS              | F      | Spinal        | 18              | C     |
| 2        | sALS              | M      | Spinal        | 9               | A     |
| 3        | sALS              | F      | Spinal        | 16              | A     |
| 4        | sALS              | M      | Bulbar        | 7               | C     |
| 5        | sALS              | F      | Spinal        | 98              | B     |
| 6        | sALS              | M      | Bulbar        | 8               | A     |
| 7        | sALS              | F      | Spinal        | 39              | C     |
| 8        | sALS              | M      | Bulbar        | 44              | C     |
| 9        | sALS              | M      | Bulbar        | 22              | C     |
| 10       | sALS              | F      | Spinal        | 24              | A     |
| 11       | sALS              | M      | Spinal        | 25              | B     |
| 12       | sALS              | M      | Spinal        | 50              | C     |
| 13       | sALS              | M      | Bulbar        | 16              | B     |
| 14       | sALS              | F      | Bulbar        | 12              | C     |

Site of onset (region in which first symptoms occurred): bulbar onset, spinal onset.

ALS Amyotrophic lateral sclerosis, sALS sporadic ALS, M male, F female.

Disease duration: months from diagnosis to blood sample. Score: ALS patient deficit was scored according to the following scale: A—moderate; B—medium-severe; C—severe; D—complete.

The age of ALS patients was 58.37 ± 3.2 years. Healthy donors were recruited between 50.26 ± 10.2 years, of different gender (n = 14).

hSOD1G93A mice (Fig. 2b, c); PRF was expressed in all the NK cells. To better characterize the position of NK cells in the CNS, we calculated the mean distance of NKp46 + NK cells to vessels, we found that the involvement of adaptive immune response does not affect the proximity of vessels (i.e. perivascular) but entered the brain in hSOD1G93A mice showed a delay in onset of motor impairment and an increase in mean survival time in comparison with vehicle-treated mice (Fig. 2g), a delay in the onset of the paralysis (in hSOD1G93A mice, Fig. 2h), with no effect on disease progression. The timing of NK cell depletion is crucial, because when NK1.1 treatment of hSOD1G93A mice started at 13 weeks of age, no effects on survival time was observed (hSOD1G93A vehicle: 139.8 ± 1.9 days, n = 5; Ab-NK1.1: 136.8 ± 1.6 days, n = 4), in accordance with another study. Behavioural tests to evaluate the changes in muscle strength and motor coordination show that NK cell-depleted mice preserved motor functions for a longer time, with no gender-related differences (Supplementary Fig. 2a, b). These results demonstrate the involvement of NK cells in the onset of motor signs and survival in two different mouse models of ALS, without effect on disease progression.

NK cell depletion did not alter the accumulation of CD4+ and CD8+ T cells either in motor cortex or spinal cord of hSOD1G93A mice (Supplementary Fig. 2c), so we can exclude that the observed effects are due to altered infiltration of these T cells.

Table 2 List of patients for peripheral blood NK cell analyses.

NK cell depletion increases survival in mouse models of ALS hSOD1G93A and TDP43A315T mice. To assess the possible role of NK cells in disease onset and progression, we also used another animal model, the TDP43A315T mice, which express a mutated TAR-DNA binding protein, accumulate pathologic aggregates of ubiquitinated proteins in specific neurons, and activate local astrocytes and microglia, with the loss of both upper and lower motor neurons. We treated 8-week-old hSOD1G93A and TDP43A315T mice with an antibody against NK1.1, to deplete the NK cell population (see scheme in Fig. 2f). The efficacy of this treatment in depleting the NK cell (CD3+/NK1.1−) population was already shown, and preliminarily confirmed in these new experiments. hSOD1G93A and TDP43A315T mice depleted from NK cells showed a delay in onset of motor impairment and an increase in mean survival time in comparison with vehicle-treated mice (Fig. 2g), a delay in the onset of the paralysis (in hSOD1G93A mice, Fig. 2h), with no effect on disease progression. The timing of NK cell depletion is crucial, because when NK1.1 treatment of hSOD1G93A mice started at 13 weeks of age, no effects on survival time was observed (hSOD1G93A vehicle: 139.8 ± 1.9 days, n = 5; Ab-NK1.1: 136.8 ± 1.6 days, n = 4), in accordance with another study. Behavioural tests to evaluate the changes in muscle strength and motor coordination show that NK cell-depleted mice preserved motor functions for a longer time, with no gender-related differences (Supplementary Fig. 2a, b). These results demonstrate the involvement of NK cells in the onset of motor signs and survival in two different mouse models of ALS, without effect on disease progression.

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hSOD1G93A mice (Fig. 2b, c); PRF was expressed in all the NK cells. To better characterize the position of NK cells in the CNS, relative to vessels, we calculated the mean distance of NKp46+ cells from CD31+ (endothelial) cells, that was 152 ± 29 μm (Fig. 2d), indicating that many NK cells observed were not in proximity of vessels (i.e. perivascular) but entered the brain parenchyma. Since the involvement of adaptive immune response in ALS is debated, we also investigated the infiltrating lymphocyte populations. Spinal cord and motor cortex of hSOD1G93A mice showed increased accumulation of CD19+ B cells, CD8+ and CD4+ T cells, with a time dependent reduction of CD8+ T cells at later stages (Supplementary Fig. 1b). To identify possible chemotactic signals involved in NK cell recruitment, we focused our attention on CCL2, a chemokine whose expression increases both in ALS patients and in hSOD1G93A mice and plays a major role in NK cell recruitment.

At this aim, hSOD1G93A mice were treated (i.p.) with a CCL2 blocking antibody (Ab-CCL2) and data reported in Fig. 2e show that Ab-CCL2 treated mice had a significant reduction of NK cells in the CNS at early disease stages. In addition, Ab-CCL2 treatment decreased the number of CD11b+ myeloid cells (motor cortex: Veh 1099 ± 134; Ab-CCL2 341 ± 12; spinal cord: Veh 2169 ± 271; Ab-CCL2 460 ± 132, n = 2–6 mice per condition), and the number of CD3+ cells infiltrating the CNS in hSOD1G93A mice (motor cortex: Veh 53 ± 26.3; Ab-CCL2 2.7 ± 1.2; spinal cord: Veh 22.3 ± 7.8; Ab-CCL2 4 ± 1.3, n = 3–6 mice per condition). These low numbers hampered further subpopulation analysis. These results demonstrate that NK cells populating the CNS affected regions of hSOD1G93A mice are recruited to the brain by CCL2, directly or through other cell recruitment.

NK cells exert cytotoxic function against MNs in ALS. To investigate the mechanisms of NK cell contribution to motor sign progression, we assayed their cytotoxic function in hSOD1G93A mice. We first analysed NKG2D ligands (mult-1 and rae-1) and DNAM-1 (DNAX Accessory Molecule-1) ligands (nectin-2 and pvr) expression in the spinal cord and motor cortex of hSOD1G93A mice: data in Fig. 3a, b show that Smi32+ (a neuronal marker of neurofilament H) cells in the spinal cord co-stain with a murine ligand for NKG2D, Mult-1, only in hSOD1G93A and not in wt mice. In accordance, qRT-PCR analysis revealed that neurons (NeuN+ cells), sorted from the spinal cord of hSOD1G93A mice, overexpressed mult-1 and nectin-2 but not rae-1 and pvr, in comparison with wt neurons (Fig. 3c). This selective overexpression may reflect the functional redundancy of NKG2D and DNAM-1 ligands. Since NKG2D ligands are involved in triggering the cytotoxicity of NKG2D receptor-expressing NK cells, we performed a cytotoxic assay with primary neuronal cultures obtained from the lumbar region of the spinal cord of 13-week-old (early phase) hSOD1G93A and wt mice, using as effector cells, NK cells isolated from the spleen of wt mice (Fig. 3d). We first demonstrated that, also in primary cultures, hSOD1G93A Smi32+ cells exhibited an increased expression of Mult-1 protein, which was absent in primary wt cultures (Supplementary Fig. 3a). Co-incubation of wt NK cells with primary spinal hSOD1G93A neurons resulted in an increased percentage of CD107a+ (a lysosomal marker of active degranulation) NK cells, in comparison with wt neurons, across a variety of ratios (Fig. 3d and Supplementary Fig. 3b). Consistently, exposure to NK cells only affected the viability of hSOD1G93A neurons, not of wt neurons (Fig. 3d and Supplementary Fig. 3b).

To investigate whether the expression of mutated hSOD affected NK cell functionality, NK cells isolated from the spleen of hSOD1G93A mice were tested for cytotoxicity against neurons. The data shown in Supplementary Fig. 3c indicated that hSOD1G93A NK cells exhibited similar cytotoxic activity against wt or hSOD1G93A neurons, as shown for wt NK cells. To determine the involvement of NKG2D in the neurotoxic activity of NK cells, NK cell-neuron co-culture experiments were performed in the presence of anti-NKG2D blocking antibody; under these conditions the NK cell-mediated cytotoxic activity on...
neurons was abolished (Fig. 3d). We then investigated the molecular mechanisms involved in the neurotoxic activity of NK cells, interfering with specific stages of lysosome exocytosis. At this aim we performed in vitro neurotoxicity assay in the presence of dynasore (dynamin inhibitor) or wiskostatin (N-WASP inhibitor). Both dynamin and N-WASP inhibition abolished NK cells-mediated hSOD1G93A neuron death (Fig. 3d). To evaluate the role of perforin in the lytic activity, NK cells isolated from prf1 ko mice were incubated, in a cytotoxicity assay, with wt or hSOD1G93A neurons. Results shown in Fig. 3d demonstrated that the absence of perforin abolished NK cells-mediated hSOD1G93A neuron death.

To further investigate in vivo the possible cytotoxic activity of NK cells against MNs, we verified the presence of potential physical contacts between NK cells and MNs in the spinal cord of hSOD1G93A mice, as shown in Fig. 3e. We then analysed the viability of MNs in the ventral horns of the lumbar spinal cord of hSOD1G93A mice and observed a reduction of their number in comparison with wt mice, already at 13 weeks (Fig. 3f). This effect was attenuated in NK cell-depleted hSOD1G93A mice, where a higher number of MNs (Smi32 and ChAT cells) was observed, in comparison with vehicle-treated hSOD1G93A mice (Fig. 3f and Supplementary Fig. 3d). At later stages (16 weeks), the spinal cord of NK cell-depleted hSOD1G93A mice showed the same number of MNs, as compared with vehicle-treated hSOD1G93A mice, indicating that the effect of NK cell depletion has a limited time window of efficacy (Supplementary Fig. 3e). Similar results were observed in male hSOD1G93A mice soon after disease onset (Supplementary Fig. 3f). The role of NKG2D in NK cell-mediated MN death was further demonstrated knocking down NKG2D, treating mice with dendrimers-loaded with NKG2D-specific siRNA. The efficacy of siRNA silencing was verified in vitro on human and murine NK cells: Supplementary Fig. 4a shows siRNA uptake by NK cells after 48 h and the effects on klrk1 gene and NKG2D protein expression is shown in Supplementary Fig. 4b, c. Functionally, dendrimer-siRNA NKG2D-transfected NK cells have a reduced cytotoxic activity against the murine tumour cells GL261 (Supplementary Fig. 4d). In vivo, in hSOD1G93A mice, siRNA treatment reduced the circulating NKG2D+ NK cells by 26% ± 5.5%, and silenced NKG2D expression (MFI) by 29% ± 5.5% in cells that remained NKG2D-positive (Supplementary Fig. 4e). Motor neuron count, in the spinal cord of siRNA-loaded mice was normal compared to vehicle-treated hSOD1G93A mice, indicating that the effect of siRNA treatment has a limited time window of efficacy (Supplementary Fig. 3f).
dendrimer-treated mice, revealed increased numbers in comparison with empty or scrambled siRNA-dendrimer-treated mice (Fig. 3f).

Taken together, these data demonstrate the cytotoxic activity of NK cells against motor neurons expressing NKG2D ligands in the hSOD1G93A mouse model.

NK cell depletion induces a protective microglia phenotype. We previously demonstrated that NK cells modulate microglia phenotype in the context of glioma16. To investigate the possible cross talk between NK cells and microglia in hSOD1G93A mice, we analysed the microglia phenotype upon NK cell depletion. To this end, we analysed Iba

\[ Iba^+ \] (a microglia/macrophage marker26)
cells in spinal cord tissue by two-photon microscopy, measuring cellular branching and the total area covered by the single cells. Our data show that hSOD1G93A microglia, in NK cell-depleted mice, have reduced soma area, increased length of branches and, in general, cover a wider parenchymal region, compared to hSOD1G93A microglia from vehicle-treated mice (Fig. 4a). Moreover, NK cell-depleted hSOD1G93A mice had less Iba1+ cells, in the ventral horns of the spinal cord (Fig. 4b). To further study NK cell-microglial communication, we isolated microglial cells from the spinal cord of wt and hSOD1G93A mice, treated with Ab-NK1.1 or vehicle at 13 or 16 weeks and analysed the expression of IFNγ as compared with untreated hSOD1G93A mice (Fig. 4c, d), with the simultaneous increase of expression of the anti-inflammatory markers Arg1 and TGFβ, of the ROS scavenger msod (Fig. 4e, f) and the modulation of other genes associated with a homeostatic pro- and anti-inflammatory microenvironment, including il-6, il-1β, ifn-α and no2 (Fig. 4c) and ROS production (nox2, p47phox) (Fig. 4d). In NK cell-depleted hSOD1G93A mice, the expression of these genes was significantly reduced (Fig. 4c, d), with the simultaneous increase of expression of the anti-inflammatory markers chil3, arg-1, and tgfβ, of the ROS scavenger msod (Fig. 4e, f) and the modulation of other genes associated with a homeostatic pro- and anti-inflammatory microenvironment, including il-6, il-1β, ifn-α and no2 (Fig. 4c) and ROS production (nox2, p47phox) (Fig. 4d).

IFNγ mediates NK cell activities in ALS. Investigating the potential mediators of NK cell effects in ALS, we focused our attention on IFNγ produced by NK cells. We compared the expression level of IFNγ in the spinal cord of hSOD1G93A and wt mice, and detected higher levels of IFNγ in hSOD1G93A, in comparison with wt mice (Fig. 5a); NK cell depletion significantly reduced the cytokine levels (Fig. 5a). In accordance, in hSOD1G93A mice, the frequency of NK cells infiltrating in the spinal cord and expressing IFNγ was 14.25% ± 2.68% (Fig. 5a) higher than what reported in brain tumour16. To investigate the possible relevance of IFNγ produced by NK cells, we treated mice with the IFNγ-blocking antibody XMG1.2 (see scheme in Fig. 5c). The efficacy of XMG1.2 treatment was previously shown in mouse brain16, and was verified in the spinal cord by MHCIi expression analysis in microglia and antibody staining (Supplementary Fig. 6a, b).

We observed that IFNγ immunodepletion increased the mean survival time of hSOD1G93A mice (Fig. 5d) and delayed paralysis onset (Fig. 5e) without affecting disease progression. Mice were longitudinally evaluated for muscle strength, locomotor activity and motor coordination: IFNγ-depleted mice showed better score in the hindlimb extension reflex, in the inverted grid and in the Rotarod tests (Supplementary Fig. 6c). Blocking IFNγ had consequences similar to NK cell depletion on microglial phenotype, modulating inflammatory gene expression (Fig. 5f, g).

Since IFNγ stimulates the neuronal release of CCL2 which in our mouse model contributes to NK cell accumulation in the CNS (shown in Fig. 2e), we investigated the neuronal expression of ccl2 in Neun+ cells isolated from the spinal cord of hSOD1G93A mice, and observed an increased expression in comparison with wt (Fig. 5h). The expression of ccl2 in Neun+ cells was abolished in neurons isolated from the spinal cord of NK cell- and IFNγ-depleted hSOD1G93A mice (Fig. 5h). At difference, microglial ccl2 expression increased in NK cell-depleted hSOD1G93A mice compared to vehicle hSOD1G93A and wt mice (Fig. 5i).

NK cells and IFNγ affect Treg number in hSOD1G93A mice. IFNγ has a crucial role in driving regulatory T cell (Tregs) differentiation and proliferation28 and Treg become reduced and dysfunctional in ALS patients10. We performed an immunofluorescence analysis of FOXP3+ cells (Tregs) in the spinal cord of NK cell-depleted hSOD1G93A mice and observed an increased number of FOXP3+ cells in comparison with control mice (Fig. 6a). This was confirmed by mRNA analysis, where foxp3 expression, in the spinal cord of hSOD1G93A mice, was significantly increased upon NK cell depletion (Fig. 6b). In line with these results, blocking IFNγ also increased foxp3 expression demonstrating the role of this cytokine in suppressing Treg cells in hSOD1G93A mice (Fig. 6b).

NK cell-motor neuron interactions in ALS patients. To investigate the possible interaction among MNs and NK cells also in humans, we determined the expression of NKG2D ligands in the spinal cord and the cortical motor cortex MNs of sALS patients and found immunoreactivity for UL16 binding protein 3 (ULBP3), which colocalized with Sml2+ cells and was completely absent in controls (Fig. 7a, b). Furthermore, Fig. 7c shows the occurrence of physical contacts between NK cells and MNs in the spinal cord of ALS patients. In these same tissues, the mean distance of NKP46+ cells from vessels (CD31+ cells) is 188 ± 43 μm, indicating cells in brain parenchyma. Similarly to what observed in hSOD1G93A mice, higher IFNγ levels were detected in the spinal cord of sALS patients in comparison with controls (Fig. 7e), and more IFNγ+ NK cells were present in the peripheral blood (Fig. 7f). On the other hand, no differences were detected in the cytotoxic activity of NK cells isolated from the peripheral...
blood of sALS patients and controls, seen as CD107a expression upon interaction with NK-susceptible tumor (K562) target cells (Fig. 7g), and also the expression of GZMb and PRF did not differ (Fig. 7h, i).

**Discussion**

We identified a new potential mechanism to explain the non-cell-autonomous motor neuron degeneration which occurs in ALS. We demonstrated that, in sALS patients and in the mouse...
model hSOD1\(^{G93A}\), NK cells infiltrate the CNS affected regions. We observed that NeuN\(^+\) cells from the spinal cord of hSOD1\(^{G93A}\) mice expressed increased levels of the chemokine CCL2, and that CCL2 neutralization reduced CNS infiltration by NK cells. In addition, NK cell depletion increased survival in hSOD1\(^{G93A}\) and TDP43\(^{A315T}\) mice and modulated the activation of microglia and the infiltration of Treg cells in hSOD1\(^{G93A}\) mice. Finally, we reported that both motor neurons of ALS patients and those of the hSOD1\(^{G93A}\) mice express higher level of NK2GD ligands, important for NK cell cytotoxic activity. These observations support the knowledge of ALS as a non-cell-autonomous disease, where motor neuron degeneration results from communication defects with immune and glial cells.

Recent data have described that NK cells are present in the spinal cord of end-stage mSOD1 mice\(^{29,28}\), and increased NK cell number is reported in the blood of ALS patients\(^{16}\). At difference, we considered the relative amount of NK cells with respect to other populations of PBMC and demonstrated that the frequency of NK cells in peripheral blood of ALS patients is reduced. Even if a direct relation among NK cells number and their frequency is not obvious, further experiments should be performed to establish possible correlations with disease stage at the moment of blood collection, also considering ongoing pharmacological therapies. However, the function of NK cells in disease progression had never been addressed. We demonstrated that NK cell recruitment in the CNS of hSOD1\(^{G93A}\) mice is CCL2-dependent; however, we cannot discriminate among a direct effect of CCL2 on NK cells and an indirect effect, coordinating the recruitment of other cells\(^{30}\) (such as monocytes) and the production of additional chemoattractants (such as CXCL1)\(^{12,13}\). The neuronal production of CCL2 has been associated with neuronal injury in different models of trauma, pain or viral infection\(^{31-33}\), and CCL2 level increases in the spinal cord tissue of SOD1\(^{G93A}\) mice\(^{29,39}\). These data identify CCL2 as an early damage signal of neural tissue.

Motor neuron degeneration in ALS has been correlated with the presence of an uncontrolled inflammatory environment\(^{34,35}\) or with an inappropriate microglia activity, which impairs axonal regeneration\(^{36,37}\). Our results point toward the importance of re-educating microglia toward a homeostatic phenotype to reduce motor neuron loss and identify NK cells as possible therapeutic targets. While Treg and microglia modulation correlates with disease progression in mutant SOD1 mice and ALS patients\(^{32,31,30,38}\), we show that NK cell-mediated modulation of these cell types only affects survival and onset time, possibly due to specific or partial alteration of microglia or Treg phenotype in the different experimental settings.

A new paradigm that emerged from our data is that motor neuron degeneration results from direct cell-to-cell contacts with NK cells, with mechanisms involving granule exocytosis and perforin expression. A recent report on a model of peripheral nerve injury, describes that the NKG2D ligand Rae1 is upregulated in the lumbar DRG of adult mice upon cutting the corresponding spinal nerve, and that neurite fragmentation is boosted by IL-2 stimulated NK cells\(^{17}\). Our data that both in CNS tissue of ALS patient and in hSOD1\(^{G93A}\) mice, motor neurons express different NKG2D ligands, identify NKG2D on NK cells as possible molecular targets to reduce motor neuron loss. Furthermore, the observation that hSOD1\(^{G93A}\) NK cells do not exert cytotoxic activity toward wt motor neurons demonstrate that the kill-me signal comes from mutant motor neurons.

Recent data identify Treg cells as suppressor of microglia toxicity in mSOD\(^{39,38}\), mice; and ALS patient infusion with expanded autologous Treg slows disease progression\(^{18}\). In addition to a direct neurotoxic activity of NK cells, we demonstrate an IFN\(\gamma\) dependent recruitment of Foxp3\(^+\) Treg cells in the spinal cord of hSOD1\(^{G93A}\) mice, which could explain the positive effects of NK cell depletion on mice survival\(^{39,39}\) and microglia phenotype\(^{9}\). The mechanisms of Treg and NK cells cross talk in hSOD1\(^{G93A}\) mice are not known, but could be mediated by IFN\(\gamma\)\(^{39,39}\), since IFN\(\gamma\) blockade also increased ifopx3 expression in the spinal cord, or by TGF\(\beta\)\(^{39}\). In contrast, NK cell depletion did not alter CD4\(^+\) or CD8\(^+\) T cell infiltrate, although we cannot exclude a possible cytotoxic role of CD8\(^+\) T cells in ALS\(^{31,42}\), also as source of IFN-\(\gamma\).

Altogether, our data support the critical role of innate immunity in ALS-associated neurodegeneration and demonstrate the neurotoxic properties of NK cells in the hSOD1\(^{G93A}\) mouse model. Targeting NK cells could revert the vicious cycle among NK cells (producing IFN\(\gamma\)) and neurons (producing CCL2), re-boosting the homeostatic functions of microglia and sustaining Treg cell recruitment in the spinal cord (see graphic summary in Fig. 8). Together with the data obtained in ALS patients, our results point to innate immunity as a key co-factor in this neurodegenerative disease. It remains to be investigated whether a direct neurotoxic role of NK cells could be relevant to other neurodegenerative diseases.

**Methods**

**Materials.** All cell culture media, foetal bovine serum (FBS), goat serum, penicillin G, streptomycin, Na pyruvate, glutamine, the Thermo Script RT-PCR System, secondary Abs, and Hoechst (catalogue #33342, RRID:AB1261756) were from Gibco Invitrogen (Carlsbad, CA, USA). Glucose, Percoll, Papain (#P3125), secondary Abs, and Hoechst (catalogue #33342, RRID:AB1261756) were from GIBCO Invitrogen (Carlsbad, CA, USA). Glucose, Percoll, Papain (#P3125), secondary Abs, and Hoechst (catalogue #33342, RRID:AB1261756) were from Life technologies Invitrogen (Carlsbad, CA, USA). Microbeads CD11b\(^+\) were from Miltenyi Biotec (Bologna, Italy). IFN\(\gamma\), streptomycin, Na pyruvate, glutamine, the Thermo Script RT-PCR System, secondary Abs, and Hoechst (catalogue #33342, RRID:AB1261756) were from GIBCO Invitrogen (Carlsbad, CA, USA). Glucose, Percoll, Papain (#P3125), secondary Abs, and Hoechst (catalogue #33342, RRID:AB1261756) were from Life technologies Invitrogen (Carlsbad, CA, USA). Microbeads CD11b\(^+\) were from Miltenyi Biotec (Bologna, Italy). Non-immune sera were obtained from adult mice and used as source of IFN-\(\gamma\) (producing IFN\(\gamma\)). The mechanisms of Treg and NK cells cross talk in hSOD1\(^{G93A}\) mice are not known, but could be mediated by IFN\(\gamma\)\(^{39,39}\), since IFN\(\gamma\) blockade also increased ifopx3 expression in the spinal cord, or by TGF\(\beta\)\(^{39}\). In contrast, NK cell depletion did not alter CD4\(^+\) or CD8\(^+\) T cell infiltrate, although we cannot exclude a possible cytotoxic role of CD8\(^+\) T cells in ALS\(^{31,42}\), also as source of IFN-\(\gamma\).

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Italy). RNeasy Mini Kit was from Qiagen (Hilden, Germany). CD45, CD69, CD3, CD4, CD8, CD107a, CD133, NK1.1 Abs, and IL-15 were from eBioscience Inc. (San Diego, CA). Rabbit anti-Iba1 from Wako (Cat# 019-19741, RRID:AB_839504) (VA, USA). Rat anti-IFNγ monoclonal antibody (clone: XMG1.2) (Cat# BE0055, RRID:AB 1107694), anti-CCL2 (clone: 2H5) (Cat# BE0185, RRID:AB_10950302), and anti-NK1.1 (Cat# BE0036, RRID:AB_1107737) were from Bioxcell (West Lebanon, USA). ULBP-3 Ab (Cat# MAB1517, RRID:AB_2272829) was from R&D systems (Minneapolis, MN, CANADA). Foxp3 Ab (Clone: FJK-16s) (Cat# 53-5773-82, RRID:AB_763537) was from Thermo Fisher (San Diego, CA). CD31 Ab (Cat# 3568 S, RRID:AB_10694616) was from Cell Signaling (WZ Leiden, The Netherlands). SMI-32 Ab was from BioLegend (San Diego, CA). siRNA-NKG2D ALEXA 488 (CODA10433) and scrambled siRNA were from Life technologies Invitrogen (Carlsbad, CA, USA). Dendrimers were synthesized for siRNA delivery as in13.
Fig. 4 NK cells modulate microglia phenotype in hSOD1G93A mice. a Quantification of soma and scanning domain area, branch numbers and length of Iba1+ cells in slices obtained from the spinal cord of wt and hSOD1G93A mice (13 weeks) treated with vehicle or Ab-NK1.1. Right: representative images (original magnification, ×600; Scale bar: 10 μm) of the maximal intensity projections of confocal z-stack images of Iba1+ cells in the different conditions, converted to binary images and then skeletonized. (n = 3 mice per treatment, 30 cells per mice in at least six slices. **P < 0.01 power 0.921 one-way ANOVA). Error bars show mean ± S.E.M. B Mean (±s.e.m.) area of Iba1+ cells (expressed as % of spinal cord area) in wt or hSOD1G93A mice (13 weeks) treated with vehicle or Ab-NK1.1 (n = 4, **P < 0.01 power 1 one-way ANOVA). Right: representative immunofluorescence image of Iba1+ cells in spinal cord sections. Scale bar: 50 μm. c-g RT-PCR of il-6, il-1β, tnfα, nos2, rox2, p47phox, chi3, soc3a, arg-1, tygb, msodl, p2y12, trem2, knn4, bdnf, il-15 gene expression in microglial cells isolated from the lumbar spinal cord of wt or hSOD1G93A mice (13 weeks) treated with vehicle or Ab-NK1.1 (n = 5 mice. Data are the mean ± S.E.M. *P < 0.05 **P < 0.01 power >0.85 one-way ANOVA). Source data are provided as a Source Data file.

Animals. Experiments described in the present work were approved by the Italian Ministry of Health (authorization n. 78/2017-PR) in accordance with the ethical guidelines on the use of animals from the European Community Council Directive of September 22, 2010 (2010/63/EU), and from the Italian D.Leg 26/2014. All possible efforts were made to minimize animal suffering, and to reduce the number of animals used per condition by calculating the necessary sample size before performing the experiments. Male and female hSOD1G93A (B6.Cg-Tg(SOD1-G93A) iGur/J) [13] mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and Charles River (Calco, Italy). hSOD1G93A mice were also maintained as hemizygotes by breeding transgenic males with wild-type C57BL/6J females from Charles River Laboratories, both maintained on C57BL/6J genetic background. Age-matched non-transgenic C57BL/6J mice were always used as controls. Transgenic mice were identified by PCR on DNA obtained from tail biopsies. Briefly, tail tips were digested (overnight, 58 °C) in a buffer containing 100 mM Tris–HCl pH 8, 0.1% SDS, 20 mM EDTA pH 8, 200 mM NaCl and 20 mg/ml protease K (Ambion-Thermo Fisher, Germany, #2548) and the genomic DNA was amplified with SsoFast Eva Green Supermix (Bio-Rad Laboratories, California, #172-5201) using the following primers: SOD1 forward 5′-TAATCCATCTGA-3′ and reverse 5′-CCCGACTAAACAACTTGGTA-3′. Mice were housed in standard breeding cages at a constant temperature (22 ± 1 °C) and relative humidity (50%), with a 12:12 light-dark cycle (light on 07:00–19:00 h). Food and water were available ad libitum. Microbiological analyses were routinely performed (each 3–4 months) and defined endemic Norovirus and Helicobacter in our conventional animal facility. Mice were evaluated for motor deficits with a behavioural score system: 0 = Full extension of hind legs away from the lateral midline when the mouse is suspended by tail; the mouse must hold this position for 20 s and then fixate its eyes when the mouse is not supported by the experimenter. 1 = Collapse or partial collapse of leg extension towards lateral midline (weakness) or trembling of hind legs during tail suspension; 2 = Curling of the toes and dragging of at least one limb during walking; 3 = Rigid paralysis or minimal joint movement; foot not used for forward motion; 4 = Mouse cannot stand up in 20 s from either side, euthanasia.

Patients enrolment. We studied 15 patients with sporadic (mean age 61.3 ± 5.2 years, probable (clinically or laboratory supported) or definite ALS44, recruited from theRare Neuromuscular Diseases Centre of Umberto I Hospital in Rome (ref. 3314/25.09.14, protocol n. 1186/14). The study was conducted according to the declaration of Helsinki and was approved by the institutional ethics committee. Informed consent was obtained from all the subjects. Patients were evaluated using the ALS Functional Rating Scale- Revised (ALSFRS-R), the Medical Research Council (MRC) score. All patients took Riluzole 50 mg BID. Healthy Donors stratified for age and sex were also enrolled as control. Table 2 resume the characteristics of ALS patients and healthy donors enrolled in the study.

Human brain and spinal cord tissues. Postmortem material was obtained at autopsy from 12 ALS patients at the department of (Neuro)Pathology of the Amsterdam UMC, Academic Medical Center, (University of Amsterdam, the Netherlands). All patients fulfilled the diagnostic criteria for ALS (El Escorial criteria46) as reviewed independently by two neuropathologists. All patients with ALS died from respiratory failure. Control spinal cord tissue was obtained from eight patients who had died from a non-neurological disease. Both ALS and control patients included in the study displayed no signs of infection before death. Informed consent was obtained for the use of brain tissue and for access to medical records for research purposes and was obtained from the relevant local ethical committees for medical research. All autopsies were performed within 10 hours after death. ALS sporadic patients did not show mutation for SOD1 or FUS proteins. Four patients were positive for the C9ORF72 hexanucleotide repeat expansion.

Table 1 resume the characteristics of ALS patients and control enrolled in the study.

Tissue preparation: paraffin-embedded tissue was sectioned at 6 μm and mounted on pre-coated glass slides (StarFrost, Waldemar Knittel Glasbearbeitungs GmbH, Braunschweig, Germany). Representative sections of all specimens were processed for haematoxylin and eosin, Klüver-Barrera and Nissl stains.

Primary spinal cord neurons culture from adult mice. Neurons cultures were obtained from spinal cord 13w C57BL6/J or hSOD1G93A mice. Mice were euthanized by cervical dislocation and the spinal cords were removed; tissues were digested with 360 U/ml papain at 37 °C. After 20 min the reaction was stopped by adding papain inhibitor and pipetting 10–15 times with a glass Pasteur pipette, until no more cell aggregates were visible. Cells were filtered with 100μm cell-strainer and centrifuged at 300 × g for 8 min. The pellet was resuspended in complete Neurobasal medium ( Gibco, part of Thermo Fisher, Germany #10888-022) (2 mM glutamine, 1% B27, 100 U/ml penicillin and 0.1 mg/ml streptomycin, BDNF 10 ng/ml) and plated (2 × 10^6 cells/well) onto poly-L-lysine coated glass cover slips. After 14 days in culture, purity of neuronal cells (analysed as Smi32+ cells) ranges between 50 and 60%.

Immunofluorescence and FACS analysis. Immune cells from wt or hSOD1G93A mice were enriched by centrifugation on percoll 40%, washed in PBS and immunostained with fluorochrome-conjugated anti-CD3, anti-CD19 and anti-CD4 antibodies to identify T cells (CD3+ NK1.1+) NK cells (NK1.1+ CD3−) and B cells (CD19+) and analyzed by flow cytometry using a FACS Canto II (BD Biosciences). Data were elaborated using FlowJo Version 9.3.2 software (TreeStar).

NK cells purification from blood patients. Peripheral blood mononuclear cells (PBMCs), freshly isolated by lymphoprep (Nycomed AS, Oslo, Norway) were frozen and stored at −80 °C for up to 2 months. The day before the experiment, cells were thawed and kept in RPMI (75 μl) for recovering. These cells were removed, and cells were stained with NK cell subset specific antibodies or were used for functional assays. To determine NK cell degranulation potential, PBMCs were co-cultured with K562 cells at 1:1 effector/target ratio for 3 h in complete RPMI 1640 medium (Hepes 10 mM + Monensin 100 μM), and degranulation was assessed by evaluation of the lysosomal marker CD107a expression on CD56+ CD3− cells by FACS analysis. To determine intracellular IFNγ production, cells were maintained in culture for 6 h in the presence of Brefeldin A (10 μg/ml), stained with anti-CD56 and -CD3 and subsequently fixed and permeabilized using Cytofix/cytoperm kit (BD Biosciences). After permeabilization, cells were stained with anti-INF-γ specific mAb and analyzed by FACS.

Mice treatment. Starting at 8 weeks of age, male and female hSOD1G93A, TDP43A315T or non-transgenic C57BL/6 mice were randomly grouped for the treatments. NK cell depletion was performed using a blocking Ab against NK1.1, which recognizes an epitope of the NKR1Pc-activating receptor (PK136). Mice were i.p. injected with 200 μg (in 100 μl) of anti-NK1.1 Ab every 2 days the first week, every 4 days the second week and then repeated once a week until the age described in the text or until sacrifice. Control mice were treated with the corresponding IgG.

Survival analysis. hSOD1G93A and TDP43A315T mice were treated with Ab anti-NK1.1, XMG1.2 or vehicle as described above and were monitored daily. The endpoint was fixed when animals were unable to stand up within 20 s after being placed on either side, or death. The probability of survival was calculated using the Kaplan–Meier method, and statistical analyses were performed using a log–rank test.

Behavioural tests. Behavioural tests started when mice were 8-week-old. All animals were handled for at least 5 min/day for 2–3 days before starting the experiments. For the hindlimb extension reflex, mice were suspended by the tail, and scored for hindlimb extension reflex deficits. The scores were recorded from 0 to 2 as follows: 2, normal extension reflex in both hind limbs; 1.5, imbalanced extension in the hind limbs; 1.0, extension reflex in only one hind limb; 0.5, the absence of any hindlimb extension; and 0, total paralysis. For the inverted grid test mice were placed in the center of a wire grid (40 × 60 cm, suspended 50 cm above a
cushioned table) and then the grid was inverted (maximum time allowed 60 s). The time spent hanging on to the grid was measured. For the Rotarod test, motor coordination, strength and balance were assessed using a rotarod apparatus (Ugo Basile, Gemonio Italy, #47650). Animals were placed onto the cylinder at a constant speed of 15 rpm. The arbitrary cut-off time was 300 s., and the longest latency was recorded. For the hanging wire test mice were allowed to grab a horizontal wire with their front paws and the time spent hanging measured (maximum time allowed 60 s). Behaviour was scored according to the following scale: 1, hanging onto the bar with both forepaws; 2, in addition to 1, attempted to climb onto the bar; 3, hanging onto the bar with two forepaws and one or both hindpaws; 4, hanging onto the bar with all four paws with tail wrapped around the bar; 5, able to walk on the bar to escape.

**Immunostaining.** In different stages of pathology, mice were overdosed with chloral hydrate (400 mg/kg, i.p.) and then intra-cardially perfused with PBS and then PFA 4%; brains and spinal cord were then isolated, fixed in 4% formaldehyde and snap frozen. Cryostat sections (20 μm) were washed in PBS, blocked (3% goat serum in 0.3% Triton X-100) for 1 h, at RT, and incubated overnight at 4 °C with specific antibodies diluted in PBS containing 1% goat serum and 0.1% Triton X-100. The sections were incubated with the following primary Abs: anti-NKp46 (1:50), anti-CD31 (1:200), anti-Smi32 (1:500), anti-ChAT (1:100), anti-Mult-1 (1:100), anti-Foxp3 (1:100) and anti-Iba1 (1:500). For immunostaining on paraffin-embedded human motor cortex section, tissues were placed at 60 °C for 15 min, incubated in xylene at RT for 25 min, and then transferred sequentially into 100% EtOH, 95% EtOH, 70% EtOH, and 50% EtOH for 4 min at RT. The sections were
rinsed in deionized water and incubated with Abc anti-Smi32 (1:500), anti-NKp46 (1:50) and anti-ULBP3 (1:100). After several washes, sections were stained with the fluorochrome-conjugated antibody and Hoechst for nuclei visualization and analysed using a fluorescence microscope. For co-immunofluorescence, the secondary antibody was subsequently used. For Iba1 and Smi32 staining, coronal sections were then blunted with a pipette to obtain single-cell suspensions, which were used for the experiments. Purity of MNs was evaluated counting only Smi32-positive cells isolated from the lumbar spinal cord of wt or hSOD1G93A mice (13 weeks) treated with control IgG, Ab-NK1.1 or XMG1.2 (n = 5). Representative FACs analysis is shown on the right. Error bars show mean ± SEM. Scale bar: 20 μm. ** P < 0.01 power 0.859 vs wt mice, one-way ANOVA). Each dot (13 weeks) treated with vehicle or Ab-NK1.1, expressed as % of spinal cord area ± s.e.m. (n = 7 vehicle; 18.6 ± 0.3 weeks; n = 6 XM1G2: 20.0 ± 0.3 weeks. Data are the mean ± s.e.m. ** P < 0.01 one-way ANOVA). Source data are provided as a Source Data file.

**Fig. 6 NK cells mediate Treg recruitment in the spinal cord of hSOD1G93A mice.** a) Analysis of Foxp3+ cells in the spinal cord of hSOD1G93A mice (13 weeks) treated with vehicle or Ab-NK1.1, expressed as % of spinal cord area ± s.e.m. (n = 5, ** P < 0.01 power 1 two-tailed Student’s t-test). Representative immunofluorescence on the right. Error bars show mean ± SEM. Scale bar: 20 μm. b) RT-PCR analysis of foxp3 expression in the lumbar spinal cord of hSOD1G93A mice (13 weeks) treated with control IgG (n = 5), Ab-NK1.1 (n = 6) or XM1G2 (n = 5), (** P < 0.01 power 921 vs control, one-way ANOVA). Source data are provided as a Source Data file.

Real-time PCR. Microligial cells and NeuN+ cells sorted from spinal cord of mice were lysed in Trizol reagent for isolation of RNA. Reverse transcription reaction was performed in a thermocycler (MJ Mini Personal Thermal Cycler; Biorad) using iScript TM Reverse Transcription Supermix (Biorad) according to the manufacturer’s protocol, under the following conditions: incubation at 25 °C for 5 min, reverse transcription at 42 °C for 30 min, inactivation at 85 °C for 5 min. Real-time PCR (RT-PCR) was carried out in a C1000 Multicolor RT-PCR Detection System (Biorad) using SoFast EvaGreen Supermix (Biorad) according to the manufacturer’s instructions. The PCR protocol consisted of 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 30 s. For quantification analysis, the comparative Threshold Cycle (Ct) method was used. The Ct values from each gene were normalized to the Ct value of GAPDH in the same RNA samples. Relative quantification was performed using the 2^(-ΔΔCt) method and expressed as fold change in arbitrary values. The primers used were listed in Supplementary Table 1.

Cytotoxicity and degranulation assays. Cell viability of spinal cord primary culture was determined by MTT assay or by staining dead cells67. Results are expressed as percentage of cell survival, taking as 100% the cells not incubated with NK cells. In order to analyse NK cell degranulation, purified NK cells were activated O/N in IL-15 (50 ng/ml) and then co-incubated with MNs or GL261 for 4 h at the Effector:Target (E:T) ratios indicated in Fig. 3d and Supplementary Figs. 3 and 4. The last two hours, FITC-conjugated anti-mouse CD107α or IgG were added directly into each well. NK cells were collected from the non-adherent fraction and analysed by FACS. Cell viability of spinal cord primary culture was determined by MTT assay or by staining dead cells. Results are expressed as percentage of cell survival, taking as 100% the cells not incubated with NK cells.

Morphological analysis of Iba1+ cells and 3D reconstruction. Brain and lumbar spinal cord slices from perfused mice were analysed by confocal microscopy and skeleton analysis to assess Iba1+ cell morphology. Twenty mm-z-stacks were acquired at 0.5 mm intervals using an FV1000 laser scanning microscope (Olympus) at ×60 objective. Maximal intensity projections of each image were generated, binarized, and skeletonized using the Skeletonize 2D/3D plugin in ImageJ, after which the Analyze Skeleton plugin (http://imagej.net/AnalyzeSkeleton) was applied. The average branch number (process end points per cell) and length per cell were recorded for each image with a voxel size exclusion limit of 150 applied. The number of single and multiple junction points was additionally

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**Fig. 5 IFNγ mediates the effects of NK cells in ALS.** a) Expression of IFNγ in the spinal cord of wt or hSOD1G93A mice (13 weeks) treated with control IgG or Ab-NK1.1 (n = 5, ** P < 0.01 power 1 one-way ANOVA). Error bars show mean ± SEM. b) Frequency of IFNγ+NK1.1+CD3+ cell population isolated from the spinal cord of hSOD1G93A mice (n = 5). Representative FACs analysis is shown on the right. c) Scheme of mice treatment with XM1G2. d) Kaplan–Meier curve of hSOD1G93A mice treated with control IgG or XM1G2 (hSOD1G93A n = 7 vehicle: 138.7 ± 2.5 days; n = 6 XM1G2: 149.7 ± 2.3 days, data are the mean ± s.e.m. ** P < 0.01 two-sided log-rank test). e) Age of paralysis, considered as the absence of hindlimb movement, upon XM1G2 treatment, in hSOD1G93A mice (13 weeks) treated with control IgG, Ab-NK1.1 or XMG1.2 (n = 5). Data are the mean ± s.e.m. ** P < 0.01 power 0.80 one-way ANOVA). RT-PCR analysis of il-6, il-1β, ifna, nos2 (f); and bdnf, chil3, socs3, arg-1 (g) gene expression in microglial cells isolated from the lumbar spinal cord of wt or hSOD1G93A mice (13 weeks) treated with control IgG or XM1G2 (n = 5). Data are the mean ± s.e.m. wt vs hSOD1G93A and hSOD1G93A vs hSOD1G93A = XM1G2. ** P < 0.015 (h) - 0.01 power >0.9 one-way ANOVA), h RT-PCR of ccl2 expression in NeuN+ cells isolated from the lumbar spinal cord of wt and hSOD1G93A mice (13 weeks) treated with control IgG, Ab-NK1.1 or XM1G2 (n = 5, ** P < 0.01 power 0.859 vs wt mice, one-way ANOVA). h) RT-PCR of ccl2 expression in microglia isolated from the lumbar spinal cord of wt and hSOD1G93A mice (13 weeks) treated with control IgG or Ab-NK1.1 (n = 5, ** P < 0.01 power 0.859 vs wt mice, one-way ANOVA). Each dot (f, h) represents the qPCR analysis of ccl2 per mice. Source data are provided as a Source Data file.

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**Isolation of lumbar microglia cells.** Adult microglia were isolated from the lumbar spinal cord tract of age-matched non-transgenic C57BL/6j wt mice and hSOD1G93A mice48. In detail, mice were deeply anaesthetized with chloral hydrate (i.p., 400 mg/Kg) before being transcardially perfused with PBS. Spinal cords were then flushed out from the spinal canal using a 20 μl syringe filled with PBS and digested with 30 units of papain (15–23 U/mg protein) for 30 min at 37°C. Tissue was then triturated with a pipette to obtain single-cell suspensions, which were applied to 70 μm/40 μm cell strainers and used for the experiments. Purity of isolated microglia cells ranges between 70 and 90%, and were selected only the samples in which the purity was 90%.

**Isolation of NeuN-positive cells and extraction of total RNA.** The isolated spinal cords were cut in small pieces and single-cell suspension was achieved by enzymatic digestion in trypsin (0.25 mg/ml) solution in HBSS. The tissue was further mechanically dissociated using a wide-tipped glass pipette and the suspension applied to a 70 μm nylon cell strainer. Cells, obtained after a three-step Percoll gradient45,46 were stained with anti-NeuN Ab (1:1000) at 4 °C for 30 min and expressed as fold change in arbitrary values. The primers used were listed in Supplementary Table 1.
calculated to give an indication of branching complexity. The areas of the soma and scanning domain were measured for each cell. 3D reconstruction of NK cells was achieved by confocal microscopy analysis with a FV1200 (Olympus) Laser Scanning System, at ×60 magnification. NK cells were identified with an Alexa fluor 594 conjugated NKp46 antibody. Acquisition files were then processed with ImageJ software for two-dimensional analysis. Three-dimensional reconstructions were generated with Imaris software (Bitplane, Zurich, Switzerland).

**Image acquisition and data analysis.** Images were digitized using a CoolSNAP camera (Photometrics) coupled to an ECLIPSE Ti-S microscope (Nikon) and processed using MetaMorph 7.6.3.0 image analysis software (Molecular Device). Slices were scanned by consecutive fields of vision (×10 objective lens) to build a single image per section. Data were expressed as area occupied by fluorescent cells versus total area by converting pixel to mm. For comparison between different treatments, at least 12 coronal sections per lumbar spinal cord or brain were analysed.
**Fig. 7** NK cell analyses in sALS patients. a Expression of ULBP-3 in the spinal cord and cerebral motor cortex of control or sALS patients, showing co-staining with Smi32+ cells (n = 8 controls, 12 sALS patients). Scale bar: 20 μm. b Colocalization of Smi32 and ULBP-3 covered area in the spinal cord and cerebral motor cortex of control or sALS patients (n = 8 controls, 12 sALS patients; *P < 0.01 power 1 two-tailed Student’s t-test). c Representative image of NK cell–MN contacts in the spinal cord of ALS patient (original magnification, x600). Scale bar: 10 μm. (n = 8 ALS patients). For boxplots, the center line, boxes and whiskers represent the median, inner quartiles, and rest of the data distribution, respectively. d Distances of Nrpn46+ cells from vessel endothelial cells (CD31+) in the spinal cord of ALS patient (n = 12 sALS patients). Scale bar: 20 μm. Representative immunofluorescence is shown. e Expression of IFNγ in the spinal cord of control and sALS patients (n = 3; *P < 0.01 power 0.871 two-tailed Student’s t-test). Error bars show mean ± SEM. f Frequency of IFNγ+ cells in the CD56+/CD3− cell populations (NK cells) isolated from the peripheral blood of control or sALS patients (n = 6 controls, 9 sALS patients; *P < 0.05 two-tailed Student’s t-test). Note that when the highest value among sALS patients was excluded, the difference among the two groups remains significant (p < 0.034 power 0.716). g NK cells isolated from the peripheral blood of control or sALS patients, were incubated with human K562 cells. Degranulation was assessed by FACs analysis of CD107+ cells (%, minus degranulation in the absence of targets) (n = 14 controls, 11 sALS patients). h, i Frequency of GZMB+ (h) and PRF1+ (i) cells in the CD56+/CD3− cell populations (NK cells) isolated from the peripheral blood of control or sALS patients (n = 8 controls, 15 sALS patients). Source data are provided as a Source Data file.

**Fig. 8** Graphic summary. (Left) NK cell’s effects in the spinal cord of hSOD1G93A mice at the early symptomatic stage. NK cells directly exert cytotoxic activity against MNs through the NKG2D-Mult-1 axis. Furthermore, IFNγ released by NK cells: (i) increases the release of CCL2 by damaged neurons; (ii) reduces the number of Treg Foxp3+ cells; (iii) shapes microglia toward a pro-inflammatory phenotype. (Right) NK cell depletion, in hSOD1G93A mice, induces neuroprotective microglia phenotype, increases the number of Treg Foxp3+ cells, protecting the MNs.

**Measurement of IFNγ by ELISA.** The spinal cord of control and sALS patients, or 13-weeks-wt and hSOD1G93A mice were disrupted with a homogenizer and analysed for IFNγ content using a sandwich ELISA, following the manufacturer’s instructions. Briefly, 96-well ELISA microplates were coated with anti-IFNγ monoclonal Ab. Samples or IFNγ standard were added at the appropriate dilution and incubated for 2 h at room temperature. After careful washing, biotinylated goat anti-human IFNγ was added to each well; horseradish-peroxidase was used as secondary Ab and optical density was read at 450 nm.

**Statistical analysis.** Data are shown as the mean ± SEM. Statistical significance was assessed by Student’s t-test, one-way ANOVA or two-way ANOVA for parametrical data, as indicated; Holm–Sidak test was used as a post-hoc test; Mann–Whitney Rank test and Kruskal–Wallis on non-parametrical data, followed by Dunn’s or Tukey’s post-hoc tests. For multiple comparisons, multiplicity-adjusted p-values are indicated in the corresponding figures (*p < 0.05, **p < 0.01). Power calculation (power) was added in all the figure legends. For the Kaplan–Meier analysis of survival, the log-rank test was used. Statistical analyses comprising calculation of degrees of freedom were done using Sigma Plot 11.0, Imaris; Origin 7, and Prism 7 software.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**
The data that support the findings of this study are available from the corresponding author upon reasonable request. The source data are provided as a Source Data file.

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**References**
1. Mitchell, J. D. & Borasio, G. D. Amyotrophic lateral sclerosis. Lancet 369, 2031–2041 (2007).
2. McGeer, P. L. & McGeer, E. G. Inflammatory processes in amyotrophic lateral sclerosis. Muscle Nerve 26, 459–470 (2002).
3. Beers, D. R. Neuroinflammation modulates distinct regional and temporal clinical responses in ALS mice. Brain Behav. Immun. 25, 1025–1035 (2011).
4. Beers, D. R. Wild-type microglia extend survival in PU.1 knockout mice with familial amyotrophic lateral sclerosis. Proc. Natl Acad. Sci. USA 103, 16021–16026 (2006).

5. Boillée, S. Onset and progression in inherited ALS determined by motor neurons and microglia. Science 312, 1389–1392 (2006).

6. Liao, B. Transformation from a neuroprotective to a neurotoxic microglial phenotype in a mouse model of ALS. Exp. Neurol. 237, 147–152 (2012).

7. Salters, K. J. Microglia emerge as central players in brain disease. Nat. Med. 23, 1018–1027 (2017).

8. Deczkowska, A. Disease-AssOCIated Microglia: a Universal Immune Sensor of Neurodegeneration. Cell 173, 1073–1081 (2018).

9. Zhao, W. Regulatory T lymphocytes from ALS mice suppress microglia and effector T lymphocytes through different cytokine-mediated mechanisms. Neurol. Dis. 48, 418–428 (2010).

10. Beers, D. R. ALS patients’ regulatory T lymphocytes are dysfunctional, and correlate with disease progression rate and severity. JCI Insight 2, e9530 (2017).

11. Infante-Duarte, C. Frequency of blood CX3CR1-positive natural killer cells correlates with disease activity in multiple sclerosis patients. FASEB J. 19, 1902–1904 (2005).

12. Huang, D. The neuronal chemokine CX3CL1/fractalkine selectively recruits NK cells that modify experimental autoimmune encephalomyelitis within the central nervous system. FASEB J. 20, 896–905 (2006).

13. Hertwig, L. CX3CR1-dependent recruitment of mature NK cells into the central nervous system contributes to control autoimmune neuroinflammation. Eur. J. Immunol. 46, 1984–1996 (2016).

14. Poli, A. NK cells in central nervous system. J. Immunol. 190, 5355–5362 (2013).

15. Poli, A. Targeting glialoblastoma with NK cells and mAb against NG2/CSPG4 prolongs animal survival. Oncotarget 4, 1527–1546 (2013).

16. Garofalo, S. Environmental stimulus shape microglial plasticity in glioma.cell 6, e33415 (2017).

17. Davies, A. J. Natural killer cells degenerate intact sensory afferents following nerve injury. Cell 176, 716–728 (2019).

18. Murdock, B. J. Correlation of peripheral immunity with rapid amyotrophic lateral sclerosis progression. JAMA Neurol. 74, 1446–1454 (2017).

19. Finkbeiner, A. Abnormal changes in NKT cells, the IGF-1 axis, and liver pathology in an animal model of ALS. PLoS ONE 6, e22374 (2011).

20. Lyon, M. S. Inflammation, immunity and amyotrophic lateral sclerosis: Etiology and Pathology. Muscle Nerve 59, 10–12 (2019).

21. Chiu, I. M. Activation of innate and humoral immunity in the peripheral nervous system of ALS transgenic mice. Proc. Natl Acad. Sci. USA 106, 20960–20965 (2009).

22. Wogorzewska, I. TDP-43 mutant mice develop features of ALS and frontotemporal lobar degeneration. Proc. Natl Acad. Sci. USA 106, 18809–18814 (2009).

23. Garofalo, S. Enriched environment reduces glioma growth through immune and non-immune mechanisms in mice. Nat. Comm. 30, 6623 (2019).

24. Kominé, O. Innate immune adenosine TRIF deficiency accelerates disease progression of ALS mice with accumulation of aberrantly activated astrocytes. Cell Death Differ. 25, 2130–2146 (2018).

25. Lanier, L. L. NK cell recognition. Annu. Rev. Immunol. 23, 225–227 (2005).

26. Chiu, I. M. A. Neurodegeneration-specific gene expression signature of acutely isolated microglia from an amyotrophic lateral sclerosis mouse model. Cell Rep. 4, 385–401 (2013).

27. Di Liberto, G. Neurons under T cell attack coordinate phagocyte-mediated synaptic stripping. Cell 175, 458–471 (2018).

28. Chang, J. H. IFN-gamma-STAT1 signal regulates the differentiation of inducible Treg potential role for ROS-mediated apoptosis. Eur. J. Immunol. 39, 1241–1251 (2009).

29. Beers, D. R. CD4+ T cells support glial neuroprotection, slow disease progression, and modify glial morphology in an animal model of inherited ALS. Proc. Natl Acad. Sci. USA 105, 15558–15563 (2008).

30. Henkel, J. S. The chemokine MCP-1 and the dendritic and myeloid cells it attracts are increased in the mSOD1 mouse model of ALS. Mol. Cell Neurosci. 31, 427–437 (2006).

31. Flügel, A. Neuronal MCP-1 expression in response to remote nerve injury. J. Cereb. Blood Flow. Metab. 21, 69–76 (2001).

32. Jung, H. Monocyte chemotactic protein-1 functions as a neuromodulator in dorsal root ganglia neurons. J. Neurochem. 104, 254–263 (2008).

33. Howe, C. L. Neuronal CCL2 expression drives inflammatory monocyte infiltration into the brain during acute virus infection. J. Neuroinfl. 14, 238 (2017).

34. Thonnhoff, J. R. Neuroinflammatory mechanisms in amyotrophic lateral sclerosis pathogenesis. Curr. Opin. Neurol. 31, 655–659 (2018).

35. Barbetto, A. G. Motor neuron-immune interactions: the vicious circle of ALS. J. Neural Transm. 117, 981–1000 (2010).
