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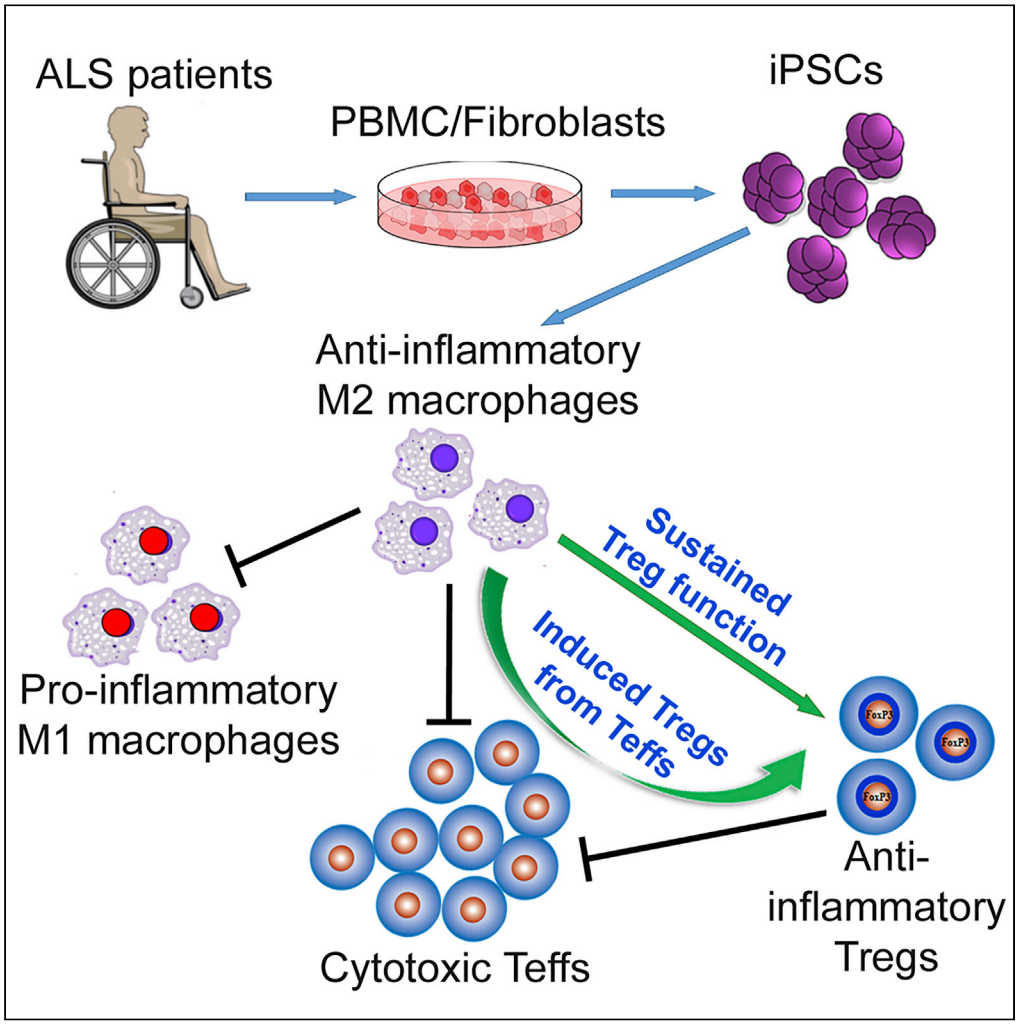


Weihua Zhao, David R. Beers, Jason R. Thonhoff, ..., Helen S. Goodridge, Clive N. Svendsen, Stanley H. Appel

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## HIGHLIGHTS

ALS iPSC-derived M2 inhibit M1 activation and proliferation of ALS effector T cells

ALS iPSC-derived M2 induce functional Tregs from ALS effector T cells

ALS iPSC-derived M2 sustain ALS Tregs' function and phenotypic expression

Functions of sporadic ALS, C9 mutant ALS, and control iPSC-derived M2 are comparable

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# Immunosuppressive Functions of M2 Macrophages Derived from iPSCs of Patients with ALS and Healthy Controls

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## SUMMARY

**Amyotrophic lateral sclerosis (ALS) is a disorder with immune alterations that augment disease severity. M2 macrophages benefit diabetic and nephrotic mice by suppressing the pro-inflammatory state. However, neither have M2 cells been investigated in ALS nor have human induced pluripotent stem cell (iPSC)-derived M2 cells been thoroughly studied for immunosuppressive potentials. Here, iPSCs of C9orf72 mutated or sporadic ALS patients were differentiated into M2 macrophages, which suppressed activation of pro-inflammatory M1 macrophages as well as proliferation of ALS $CD4^+CD25^-$ Tc (Teffs). M2 cells converted ALS Teffs into $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) and rescued Tregs of ALS patients from losing CD25 and Foxp3. Furthermore, Tregs induced or rescued by iPSC-derived M2 had strong suppressive functions. ALS iPSC-derived M2 cells including those with C9orf72 mutation had similar immunomodulatory activity as control iPSC-derived M2 cells. This study demonstrates that M2 cells differentiated from iPSCs of ALS patients are immunosuppressive, boost ALS Tregs, and may serve as a candidate for immune-cell-based therapy to mitigate inflammation in ALS.**

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig disease, is a common, devastating, and invariably fatal adult neurodegenerative disease. In addition to the loss of upper and lower motor neurons, ALS is now regarded as a disorder with immune dysregulation, which is characterized by alterations/activation of inflammatory cells that augment disease burdens and rates of disease progression. Earlier studies documented the presence of activated pro-inflammatory microglia/macrophages and lymphocytes in the central nervous system (CNS) of patients with ALS (Appel et al., 2010, 2011; Beers and Appel, 2019; Butovsky et al., 2012; Keizman et al., 2009; Zhao et al., 2017). Furthermore, a meta-analysis reported that cytokine levels of interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α were increased in the blood of patients, implicating a peripheral systemic pro-inflammatory immune response (Hu et al., 2017). Similar CNS and peripheral inflammatory responses have been reported in transgenic animal models of ALS (Beers et al., 2008; Chiu et al., 2008). Unfortunately, no treatments are presently available to arrest or substantially delay these inexorable inflammatory responses in patients with ALS.

Monocytes are myeloid cells circulating in the blood. When monocytes enter tissues, they become macrophages and the tissue milieu can induce changes in these cells. Therefore, monocytes and macrophages are closely related. Microglia are the innate immune myeloid cells within the CNS. Although microglia originate from the yolk sac and enter the CNS during embryonic development, microglia and macrophages have similar functions, such as patrolling for pathogens, responding to damage signals, performing phagocytosis of microbes and cellular debris, and acting as professional antigen-presenting cells to stimulate T cell responses. During chronic neuroinflammation, CNS-infiltrating macrophages acquire microglial markers (Grassivaro et al., 2020) and also retain significant differences (Ajami et al., 2018). Based on different activation stimuli, both microglia and macrophages can be polarized to M1 or M2 cells that produce pro-inflammatory or anti-inflammatory cytokines.

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Polarization of CNS microglia and macrophages is a spectrum categorized by the subjective nomenclature M1 and M2 cells that represent the two extreme pro- and anti-inflammatory phenotypes, respectively (Ransohoff, 2016). Classical activation of M1 cells can be generated in response to interferon (IFN)-γ and lipopolysaccharide (LPS). They possess anti-microbial and cytotoxic properties due to secretion of reactive oxygen species and pro-inflammation cytokines (Nathan and Shiloh, 2000). The persistence or escalation of the inflammatory processes mediated by M1 macrophages can result in cytotoxicity (Laskin et al., 2011). In animal models of ALS, activated microglia are pro-inflammatory and neurotoxic during the rapidly progressing phase of disease (Beers et al., 2006; Boillee et al., 2006; Liao et al., 2012; Meissner et al., 2010; Zhao et al., 2010). In the periphery, monocytes of patients with ALS are also skewed toward a pro-inflammatory M1 state and potentially contribute to disease progression (Zhao et al., 2017). In contrast, M2 cells can be established after exposure to IL-4/IL-10/IL-13, TGF-β, or glucocorticoids. M2 cells secrete high levels of anti-inflammatory cytokines and neurotrophic factors, and have high endocytic clearance capacities (Tiemessen et al., 2007). M2 cells have been reported to promote blockade of inflammatory responses and enhance tissue repair (Bai et al., 2017; Cherry et al., 2014).

During the early stage of motor neuron injury in ALS models, the surveying microglia exhibit an M2 phenotype and react to the signals (possibly CD200 and fractalkine) with release of cytokines and trophic factors to promote repair and regeneration (Appel et al., 2010; Beers et al., 2011a; Liao et al., 2012). However, as disease progresses, the injured motor neurons release "danger signals," possibly misfolded oxidized proteins (Zhao et al., 2010), or ATP (D'Ambrosi et al., 2009), which induce microglia to release reactive oxygen species and pro-inflammatory cytokines and display an M1 phenotype. Activated M1 microglia promote further motor neuron injury, enhancing the release of pro-inflammatory signals, which activate microglia to an even greater extent, and induce a self-propagating cycle of a pro-inflammatory injurious dialog between motor neurons and microglia (Zhao et al., 2013).

The responses of the adaptive immune system also play a pivotal role regulating the rate of disease progression and survival in patients with ALS (Appel et al., 2010, 2011; Beers and Appel, 2019). It has been reported that regulatory T cells (Tregs), an adaptive immune response, had a protective role in patients with ALS; reduced Treg numbers and FOXP3 expression were associated with a more rapid disease progression and increased mortality (Henkel et al., 2013). In addition, Tregs from patients with ALS were less suppressive and the function of their Tregs correlated with disease progression rate and severity (Beers et al., 2017). Thus, the current working hypothesis is that as disease progresses, the initial supportive anti-inflammatory M2/Treg immune response is transformed to an injurious pro-inflammatory M1/Th1 response.

M2 cells derived from human blood have been shown to decrease the Th1 response to bacteria (Verreck et al., 2004). These blood-derived M2 cells also have the ability to convert $CD4^+$ T cells to Tregs with a strong suppressive function (Savage et al., 2008; Schmidt et al., 2016). However, peripheral monocytes have limited proliferation potential and do not yield sufficient numbers of M2 cells to study. Human iPSCs (induced pluripotent stem cells) can provide a source of unlimited M2 cells. Furthermore, the functions of iPSC-derived M2 have not been fully studied. In the present study, we generated M2 cells from iPSCs of patients with ALS and healthy controls and compared their immune-suppressive functions. The purpose of this study is to determine whether ALS iPSCs could be used to generate unlimited immunosuppressive M2 cells *in vitro* as an autologous cell-based therapy for patients with ALS.

## RESULTS

### M1 and M2 Macrophages Are Differentiated from iPSCs of Patients with ALS and Healthy Controls

Human fibroblasts or blood cells from six healthy controls (CTR) and seven patients with ALS (three patients with C9orf72 (C9) mutation, four patients with sporadic disease with either fast or slow disease progression) were reprogrammed to iPSCs and subsequently differentiated into monocytes. Flow cytometry revealed that more than 90% $CD14^+$ cells co-expressed CD115 and HLA-DR (Figures 1A and 1B), indicating that these $CD14^+$ cells were monocytes.

iPSC-derived $CD14^+$ monocytes were further differentiated to M1 macrophages using granulocyte-macrophage colony-stimulating factor (GM-CSF) plus LPS + IFN-γ, and in separate cultures, differentiated into M2 macrophages using macrophage-CSF (M-CSF) plus IL-4 + IL-10 + TGF-β. iPSC-derived M1 cells upregulated the pro-inflammatory cytokines IL-6, TNF-α, and IL-8 (Figures 2A–2C). iPSC-derived M2 cells

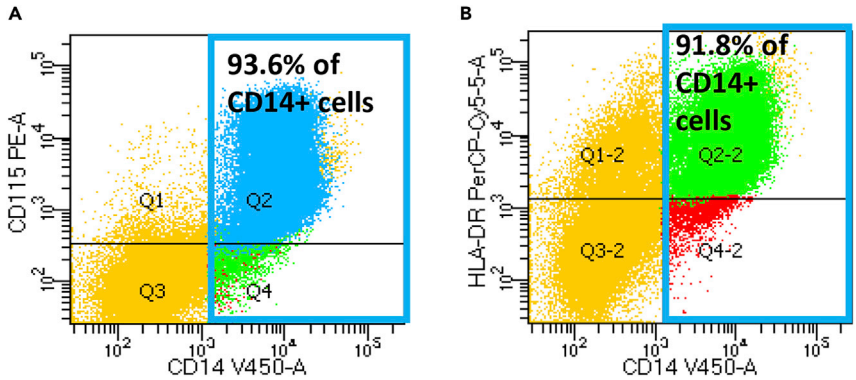


**Figure 1. Cells Differentiated from Human iPSCs Express Monocyte Markers**

After differentiation of human iPSCs, floating cells were collected and subjected to flow cytometry. More than 90% of $CD14^+$ cells (shown in blue boxes) express CD115 (A) and HLA-DR (B).

expressed the macrophage lineage marker, CD68 (Figure 3A), and typical M2 markers, CD163 and CD206 (Figures 3B, 3C, and S9). Furthermore, the production of anti-inflammatory cytokines was measured in the supernatants of M2 cultures. As the TGF-β ELISA kit also recognized the latent TGF-β complex present in culture media, enhanced TGF-β secreted by M2 macrophages was shown as subtracted levels from M0 cells cultured at the same time (M2-M0) (Figure 3E). M2 cells from CTR iPSCs, ALS C9 iPSCs, or sporadic ALS iPSCs produced more IL-10 and TGF-β than their corresponding resting M0 macrophages; the levels of IL-10 and TGF-β were not different among M2 cells derived from CTR iPSCs, ALS C9 iPSCs, and sporadic ALS iPSCs (Figures 3D and 3E).

## ALS iPSC-Derived M2 Macrophages Inhibit M1 Activation

M2 cells were cultured on coverslips so that they could be removed after co-cultured with M1 cells at different time points , and M1 and M2 cells could be investigated separately. After co-culturing with M2 cells derived from an ALS iPSC line with C9 mutation, M1 cells expressed less IL-6 and TNF-α mRNA than M1 cultures alone; M2 in the co-cultures only express minimal IL-6 and TNF-α mRNA (Figures 4A and 4C). IL-6 and TNF-α protein levels were also suppressed in M1+M2 co-cultures compared with M1 alone (Figures 4B and 4D). M2 cells suppressed M1 IL-8 mRNA at early time points (3–7 h). However, M2 cells upregulated IL-8 mRNA at 24-h time point (Figure 4E). IL-8 protein levels in M1+M2 co-cultures were not lower than those in M1 cells alone (Figure 4F).

M1 and M2 co-cultures were then performed using three CTR iPSCs and five additional ALS iPSCs from three patients with sporadic ALS and two ALS patients with C9 mutation. M2 from each of these iPSCs suppressed IL-6 and TNF-α expression, but did not change IL-8 protein of M1 in a similar pattern (Figures S1–S8). To determine whether the function of M2 cells is specific, resting M0 cells derived from same ALS iPSCs were used as controls. M2 differentiated from CTR iPSCs were also used as controls to study the influence of C9 mutation and ALS disease insults. The suppression of ALS M2 cells on IL-6, TNF-α, and IL-8 mRNA of M1 cells was significantly higher than that of resting ALS M0 cells, but similar to the suppressive function of CTR M2 cells (Figure 4G). IL-6 protein levels were reduced in ALS M1+M2 co-cultures compared with M1 alone or ALS M1+M0 cultures; M0 cells did not change IL-6 protein after co-culturing with M1 cells (Figure 4H). TNF-α protein levels increased in ALS M1+M0 co-cultures compared with M1 alone cultures, whereas TNF-α protein levels were lower in ALS M1+M2 co-cultures than ALS M1 alone or ALS M1+M0 cultures (Figure 4H). No differences were observed on IL-6 and TNF-α production between CTR M1+M2 and ALS M1+M2 co-cultures (Figure 4H), indicating that the suppressive abilities of ALS M2 were not reduced due to C9 mutation or ALS-related changes, and at same levels as control M2 on M1. However, IL-8 protein was increased in M1+M2 co-cultures compared with M1 cultures (Figure 4H).

## ALS iPSC-Derived M2 Macrophages Suppress Proliferation of ALS Teffs

After being co-cultured with increased number of M2 cells derived from three ALS iPSC lines, less proliferation of ALS Teffs was observed in a dose-dependent manner (Figure 5B). Representative flow cytometry graphs were shown in Figure 5A. ALS iPSC-derived M2 cells behaved differently from M0 cells, which

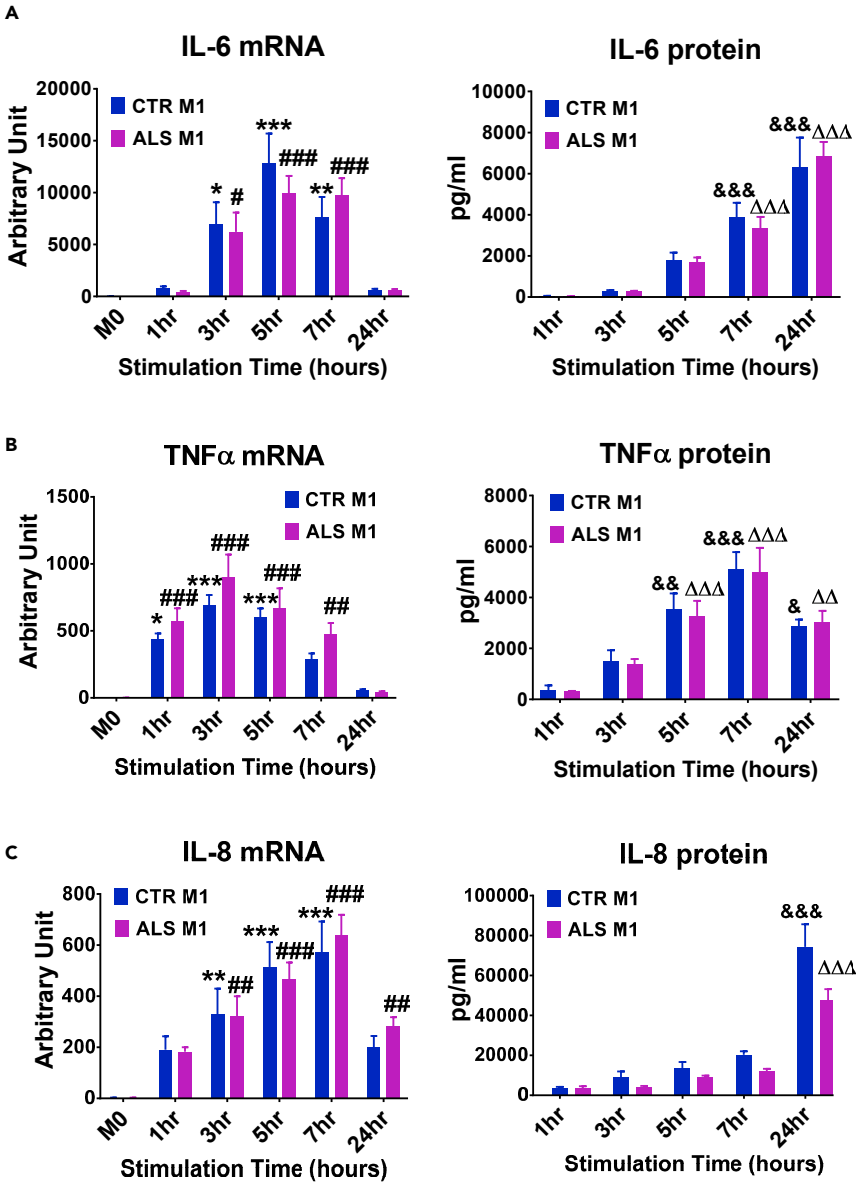


**Figure 2. M1 Macrophages Differentiated from iPSC-Derived Monocytes**

M0 macrophages were differentiated from monocytes derived from control (CTR) iPSCs (n = 5) and ALS iPSCs (n = 7) in the presence of GM-CSF (50ng/ml) for 7 days. To generate M1 macrophages, resting M0 cells were activated with LPS (1ng/ml) and IFN-γ (2 ng/ml) for different time periods as indicated. Both CTR and ALS M1 cells (1 x $10^5$/well) expressed up-regulated mRNA and protein of IL-6 (A), TNF-α (B), and IL-8 (C). The mRNA level of M0 were set as 1 arbitrary unit. *p < 0.05, **p < 0.01, ***p < 0.001 vs. CTR M0 for mRNA; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs. ALS M0 for mRNA; $^{\&}$p < 0.05, $^{\&\&}$p < 0.01, $^{\&\&\&}$p < 0.001 vs. CTR 1hr for protein; $^{\Delta\Delta}$p < 0.01, $^{\Delta\Delta\Delta}$p < 0.001 vs. ALS 1 h for protein.

enhanced proliferation of ALS Teffs at lower doses (Figure 5B). M2 cells from seven ALS iPSCs (including three ALS C9 iPSCs) were able to suppress ALS Teff proliferation as efficiently as M2 from four CTR iPSCs (Figure 5C).

## ALS iPSC-Derived M2 Macrophages Induce Functional Tregs from ALS Teffs

The expression of CD25 and Foxp3 (Tregs functional markers) were examined in ALS Teffs co-cultured with iPSC-derived M2 cells. The percentages of $CD4^+CD25^+Foxp3^+$ Tregs were increased in M2 and ALS Teffs co-cultures compared with cultures of ALS Teffs alone (Figure 6A), indicating that iPSC

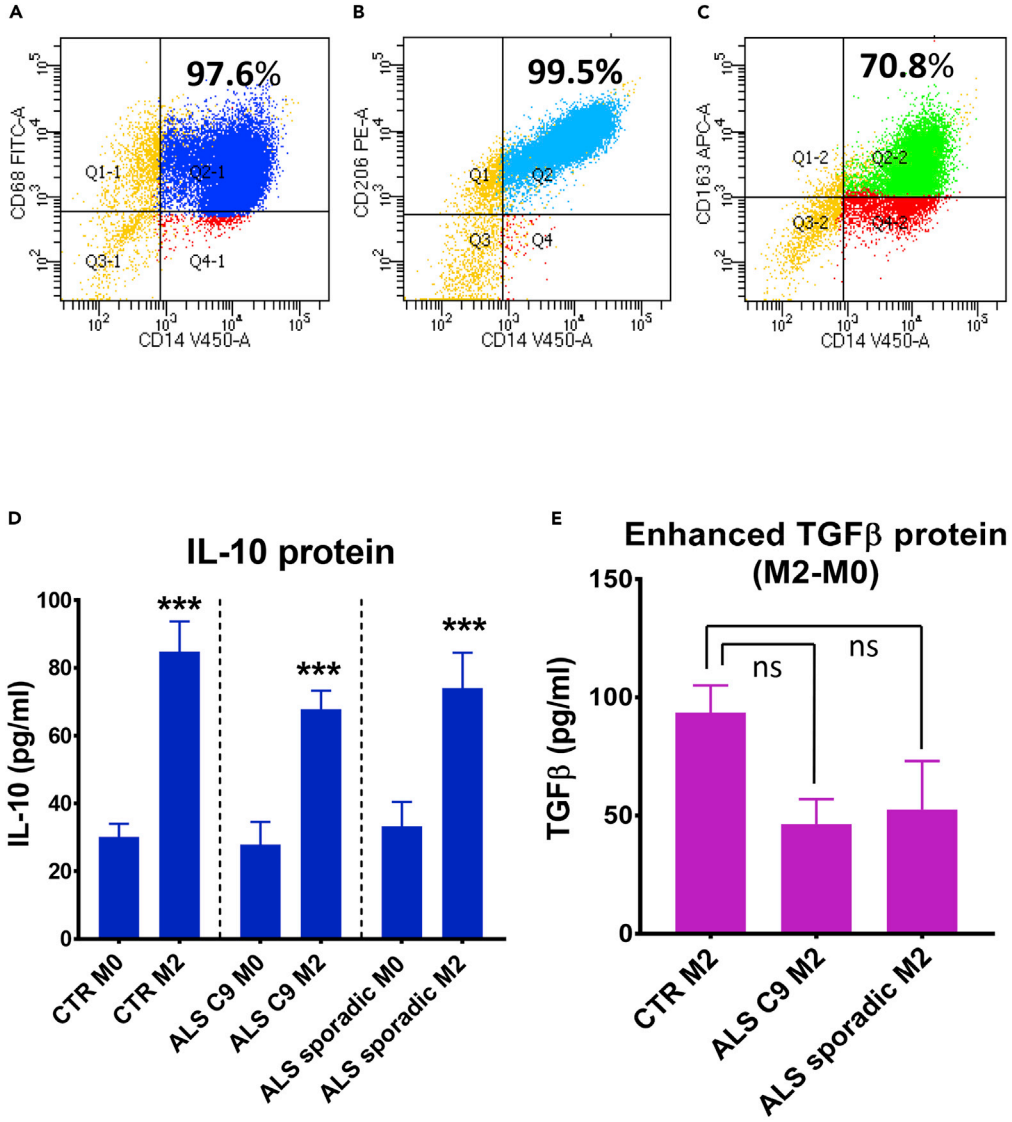


**Figure 3. M2 Macrophages Differentiated from iPSC-Derived Monocytes**

(A–C) To differentiate M2 cells, iPSC-derived monocytes were cultured with M-CSF (100ng/ml) for 7 days, then with the addition of IL-4, IL-10, and TGF-β (20ng/ml each) for 5-16 hours. M2 cells derived from iPSCs expressed CD68 (A), CD206 (B), and CD163 (C).

(D and E) M2 cells derived from control (CTR) iPSCs (n = 5), ALS C9orf72 (C9) iPSCs (n = 3), and sporadic ALS iPSCs (n = 4) released more IL-10 (D) and TGF-β (E) than their resting M0 cells. Enhanced TGF-β secreted by M2 cells were shown as subtracted levels from M0 cells cultured at the same time (M2-M0) to remove the amount of TGF-β existing in the culture serum. ***$p < 0.001$ vs. their corresponding M0. The levels of IL-10 and TGFβ were not different among CTR M2, ALS C9 M2 and ALS sporadic M2. ns = no significant difference.

M2 can induce Tregs from ALS Teffs. We then compared Treg induction ability of M2 with resting M0 and M1 cells derived from same ALS iPSCs. ALS M2 and M0 cells induced increased numbers of Tregs from ALS Teffs than ALS M1 cells or ALS Teffs alone; ALS M1 cells did not increase the percentage of Tregs compared with ALS Teffs alone (Figure 6B). M2 cells from three ALS C9 and four sporadic iPSCs had similar Treg induction capabilities as three CTR M2 cells (Figure 6C). In addition, M2-induced Tregs were functionally suppressive; Tregs induced by five ALS iPSC-derived M2 cells all suppressed Teff proliferation in a dose-dependent manner, as strongly as Tregs induced by three CTR M2 (Figure 6D).

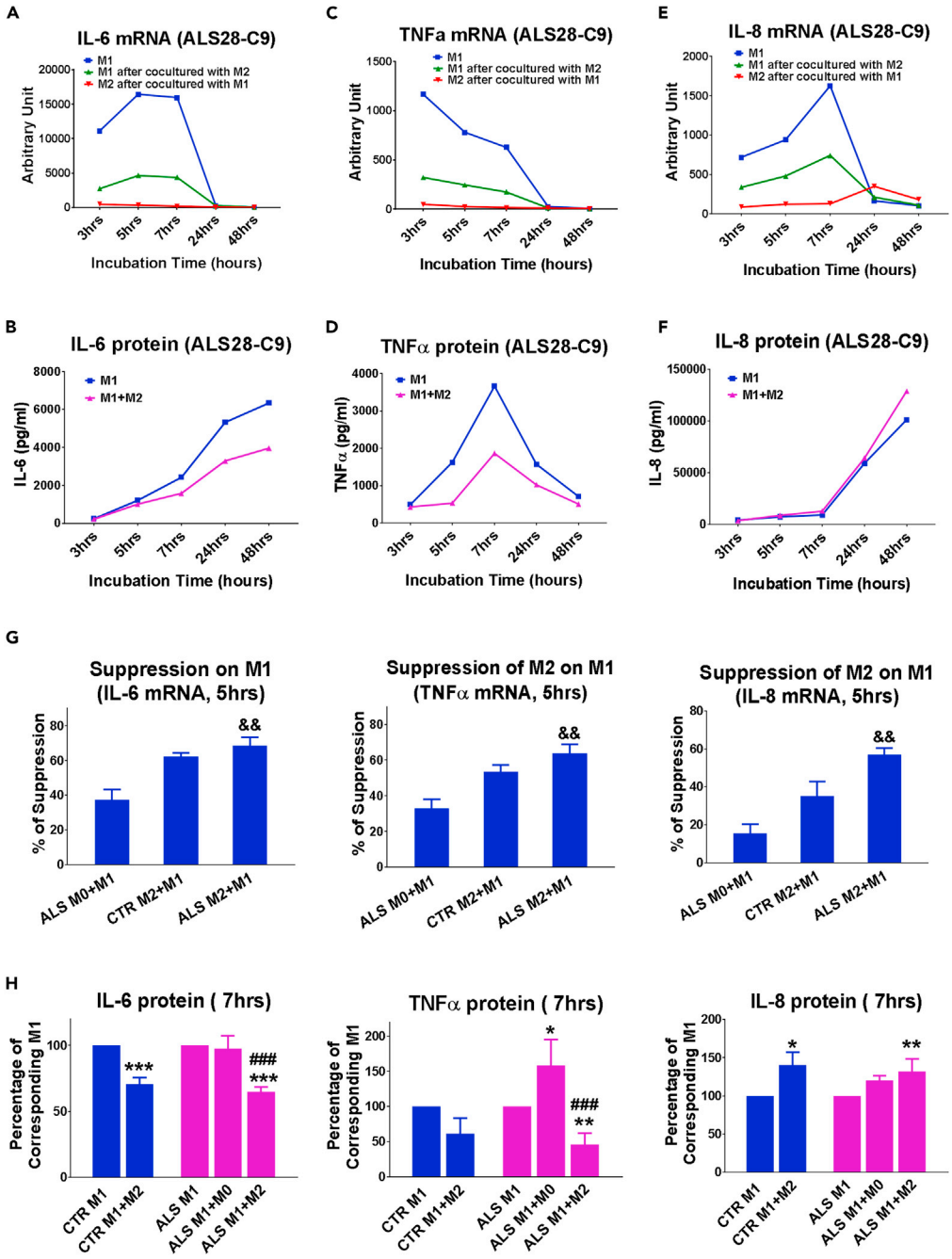


**Figure 4. ALS iPSC-Derived M2 Macrophages Inhibit M1 Activation**

(A–F) Representative time curves of M1 cells alone, M1 cells after being co-cultured with M2 cells, and M2 cells after being co-cultured with M1 cells. M1 and M2 cells in this graph were differentiated from a representative ALS28 C9 iPSC. IL-6 mRNA (A), TNFα mRNA (C) and IL-8 mRNA (E) were detected in M1 alone, or M1 or M2 cells after co-cultures. IL-6 protein (B), TNFα protein (D) and IL-8 protein (F) were measured in the supernatants of M1 alone, or M1+M2 co-cultures.

(G) IL-6, TNF-α and IL-8 mRNA levels in M1 cells after being co-cultured with resting M0 cells from ALS iPSCs (n=3), M2 cells from CTR iPSCs (n=3), and M2 cells from ALS iPSCs (n=6) for 5hr. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs ALS M0+M1.

(H) IL-6, TNF-α and IL-8 proteins in CTR M1 cells or ALS M1 cells alone or co-cultures with CTR M2 cells, ALS M2 cells, or ALS resting M0 for 7hrs. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. their corresponding M1 cell alone cultures; $^{\#\#\#}p < 0.001$ vs. ALS M1+M0.

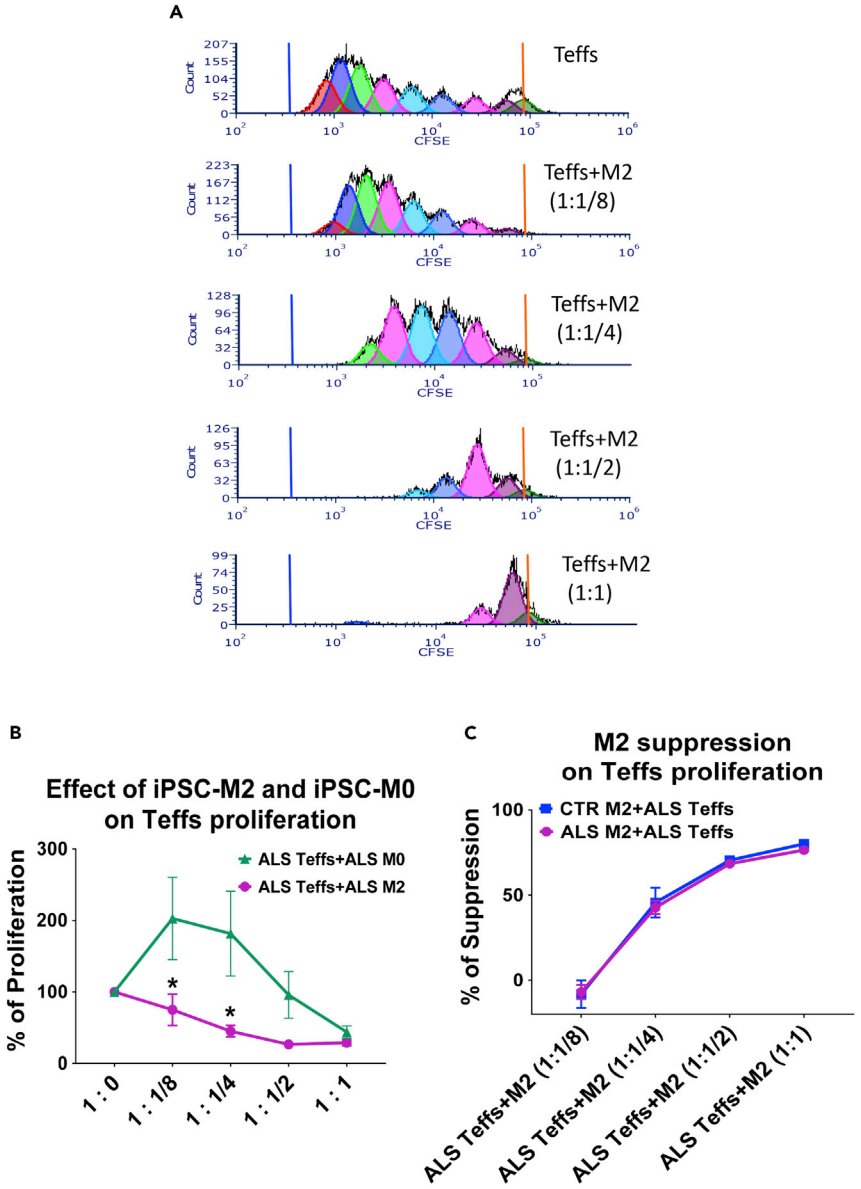


**Figure 5. ALS iPSC-Derived M2 Macrophages Suppress Proliferation of ALS Teffs**

(A) Representative proliferation plots examined by flow cytometry: ALS Teffs had fewer proliferative divisions after being co-cultured with an increased number of M2 cells derived from a representative ALS iPSC line. (B) Proliferation of ALS Teffs was lower when co-cultured with M2 cells from ALS iPSCs than co-cultured with resting M0 cells from same ALS iPSCs at 1:1/8 and 1:1/4 ratios of Teffs:M2/M0. *$p < 0.05$ vs. ALS Teffs+ALS M0 at same ratio. (C) M2 cells from CTR iPSCs ($n = 4$) and ALS iPSCs ($n = 7$) suppressed ALS Teffs' proliferation in a dose-dependent manner. The suppressive activity of ALS M2 cells on ALS Teffs was not different from CTR M2 cells.

## ALS iPSC-Derived M2 Macrophages Sustain CD25 and Foxp3 Expression and Suppressive Function of ALS Tregs

To determine the effects of iPSC-derived M2 cells on Tregs, M2 cells were co-cultured with Tregs isolated from the blood of patients with ALS. After culturing ALS Tregs alone for 2 days, the percentage of $CD4^+CD25^+Foxp3^+$ Tregs was decreased from 32.6% ± 0.9% to 11.8% ± 1.6%. When ALS Tregs were co-cultured with M2 cells derived from a representative ALS C9 iPSC, the percentage of $CD4^+CD25^+Foxp3^+$ Tregs increased in a dose-dependent manner (Figure 7A). M2 cells from both ALS C9 and sporadic ALS iPSCs had similar effects as CRT M2 cells and increased the percentage

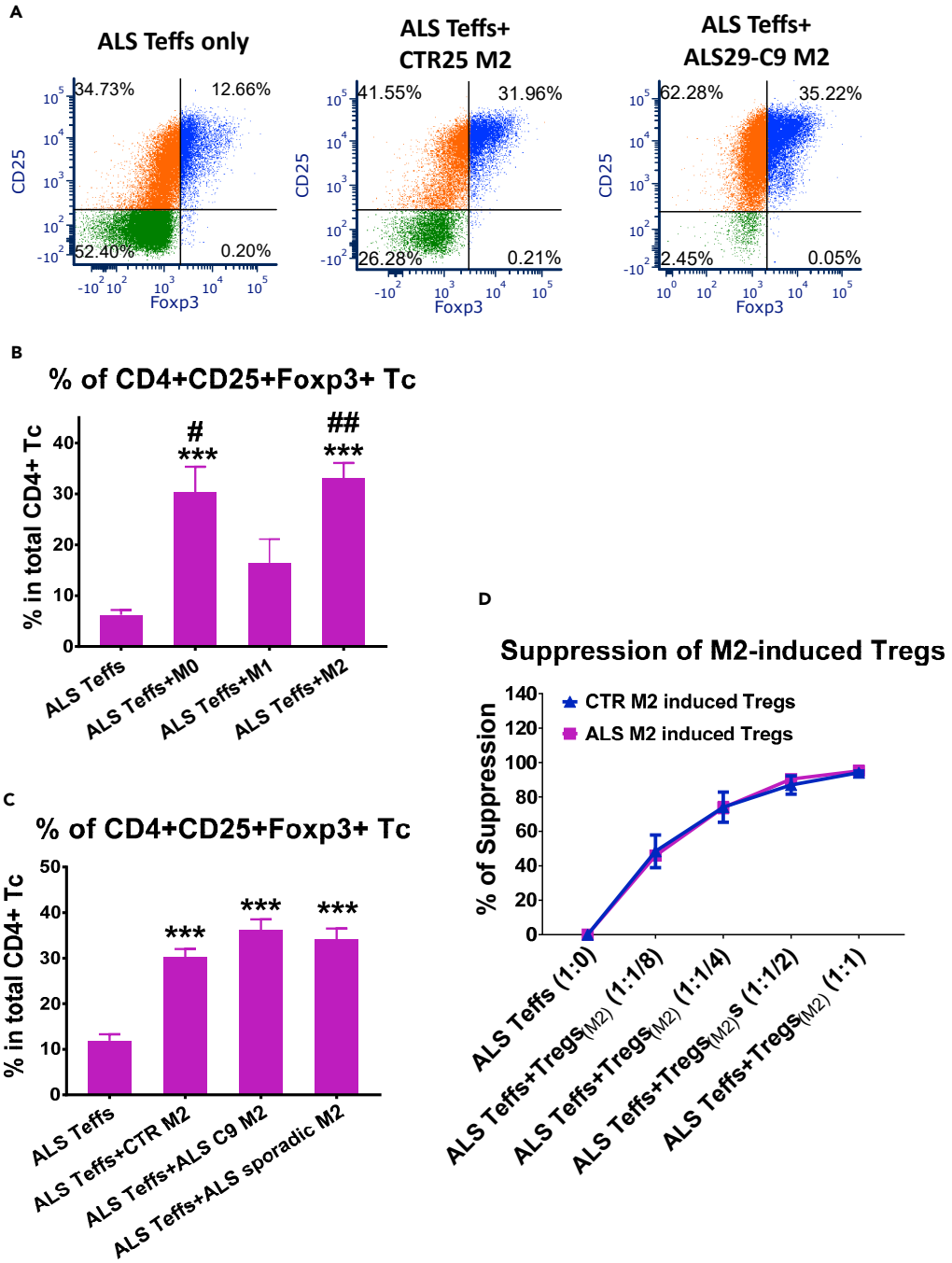


**Figure 6. ALS iPSC-Derived M2 Macrophages Induce Functional Tregs from ALS Teffs**

M2, M0, or M1 cells derived from iPSCs were co-cultured with ALS Teffs at a ratio of 1:1/4 (Teffs:M2) for 2 days. Cells were then subjected for flow cytometric analyses.

(A) Representative plots showed CD25 and Foxp3 expression of $CD4^+$ T-cells in ALS Teffs either cultured alone or co-cultured with M2 cells derived from one CTR25 iPSC, one ALS29 C9 iPSC.

(B) The percentages of $CD4^+CD25^+Foxp3^+$ Tregs in ALS M2+ALS Teffs (n = 3) and ALS M0+ALS Teffs (n = 3) were higher than ALS M1+ALS Teffs (n = 3) or ALS Teffs alone (n = 3). No differences were observed between ALS M1+ALS Teffs and ALS Teffs alone. ***$p<0.001$ vs. ALS Teffs alone; $^{\#\#}p<0.01$, $^{\#}p<0.05$ vs. ALS M1+ALS Teffs.

**Figure 6. *Continued***
(C) M2 cells from ALS C9 iPSCs (n = 3) and sporadic ALS iPSCs (n = 4) increased the percentage of $CD4^+CD25^+Foxp3^+$ Tregs from ALS Teffs compared with ALS Teffs alone cultures; Treg induction capacity of M2 cells from both ALS C9 and sporadic iPSCs were not different from CTR M2 cells (n = 3). ***$p<0.001$ vs. ALS Teffs alone.
(D) M2-induced $CD4^+CD25^+$ Tregs were isolated from M2+ALS Teffs co-cultures. These M2-induced Tregs ($iTregs_{(M2)}$) were then co-cultured with CFSE-stained responder Teffs from ALS patients for 5 days at different Teffs:$Tregs_{(M2)}$ ratio (1:1/8, 1:1/4, 1:1/2, 1:1). Tregs induced by CTR M2 (n = 3) and ALS iPSC M2 (n = 5) suppressed proliferation of ALS Teffs similarly in a dose-dependent manner.

of $CD4^+CD25^+Foxp3^+$ Tregs (Figure 7B). In addition, there were no differences in viability and $CD4^+$ T cell numbers among culture groups (Figure S10), which argues against the possibility that M2 cells selectively supported the survival of Tregs, or that the increased percentage of $CD4^+CD25^+Foxp3^+$ Tregs was due to loss of $CD4^+Foxp3^-$ T cells. M2-rescued Tregs were further isolated from ALS Tregs and ALS M2 co-cultures. As low numbers of Tregs were initially obtained from blood samples, fewer M2-rescued Tregs were isolated for their functional assay. Even at a ratio of 1:1/16 (responder Teffs:M2-rescued Tregs), significant reduction of Teff proliferation was noted (from 92.3% ± 0.42% in Teff alone to 50.9% ± 9.95% in Teffs + M2-rescued Tregs, Figures 7C and 7D).

## DISCUSSION

Accumulating evidence suggests that tissue inflammatory responses promote increased burden of disease and rate of progression in ALS (Appel et al., 2010, 2011; Beers et al., 2006, 2011b, 2017); ALS is now regarded as a disorder with immune alterations and dysregulation (Beers and Appel, 2019). The current study demonstrated for the first time that M2 cells derived from iPSCs of patients with ALS suppressed toxic M1 cell responses and inhibited ALS Tresp proliferation. Furthermore, these iPSC-derived M2 cells converted Teffs of patients with ALS into functionally suppressive Tregs and sustained the immunomodulatory functions of ALS Tregs. ALS iPSC-derived M2 cells were functionally different when compared with resting M0 cells or M1 cells derived from same ALS iPSCs, indicating that the effects of M2 cells are phenotype specific. This study also demonstrates that the immunomodulatory effects of ALS iPSC-derived M2 cells are as strong as those of control iPSC-derived M2 cells, suggesting that the capacity of ALS iPSCs to become functional M2 cells is not influenced by the ongoing disease process or by the expression of the C9 mutation in patients with ALS.

A recent meta-analysis reported that cytokine levels of IL-6, IL-1β, and TNF-α were increased in the blood of patients with ALS, implicating a peripheral systemic pro-inflammatory immune response (Hu et al., 2017). These cytokines not only contribute to the innate immune system's pro-inflammatory milieu in patients with ALS but also contribute to the alteration/education of the adaptive immune system in these patients. IL-6 has been shown to induce differentiation of pathogenic Th17 and inhibit Tregs generation induced by TGF-β (Bettelli et al., 2006). TNF-α is not only known as a modulator of inflammation and is upregulated in patients with ALS (Brohawn et al., 2016) but also inhibits Treg-suppressive function by down-regulating Foxp3 expression and phosphorylation, and their adaptive immune response (Nie et al., 2013; Valencia et al., 2006). Thus, both the innate and adaptive immune systems play a pivotal and intertwined role in the ALS disease process; as disease burden accumulates and progression rates accelerates, the initial supportive anti-inflammatory M2/Tregs immune response is transformed into an injurious pro-inflammatory M1/Th1 response (Beers et al., 2011a; Henkel et al., 2013; Liao et al., 2012). The current study demonstrated that M2 cells derived from ALS iPSC lines suppressed M1 cell inflammatory responses by reducing IL-6 and TNF-α production, inhibited the proliferation of ALS Teffs, and induced and rescued ALS Tregs. These data suggest that iPSC-derived M2 cells may reverse the imbalance between Tregs/M2 and Th1/M1 reactivity and reduce the pro-inflammatory milieu promoted by M1 and Th1.

iPSC-derived M2 cells expressed low levels of IL-6 and TNF-α in the M1 and M2 co-cultures and suppressed IL-6 and TNF-α produced by M1 cells. However, M2 cells upregulated IL-8 expression 24 h after being co-cultured with M1 cells, and IL-8 protein levels in M1 + M2 co-cultures were higher than M1 cultures alone. IL-8 is a chemokine responsible for attracting neutrophils and T cells to inflamed sites. A previous study demonstrated that IL-8 expression was upregulated in peripheral monocytes of patients with ALS (Zhao et al., 2017). Another study reported that IL-8 stimulated the M2 polarization of tumor-associated macrophages (Xiao et al., 2018); IL-8 mediated migration of $CD4^+$ T cells including Tregs (Eikawa et al., 2010). These cumulative data suggest that IL-8 may attract pro-inflammatory as well as anti-inflammatory cells to lesions. Thus the role of IL-8 in ALS remains unclear, and further investigations are required.

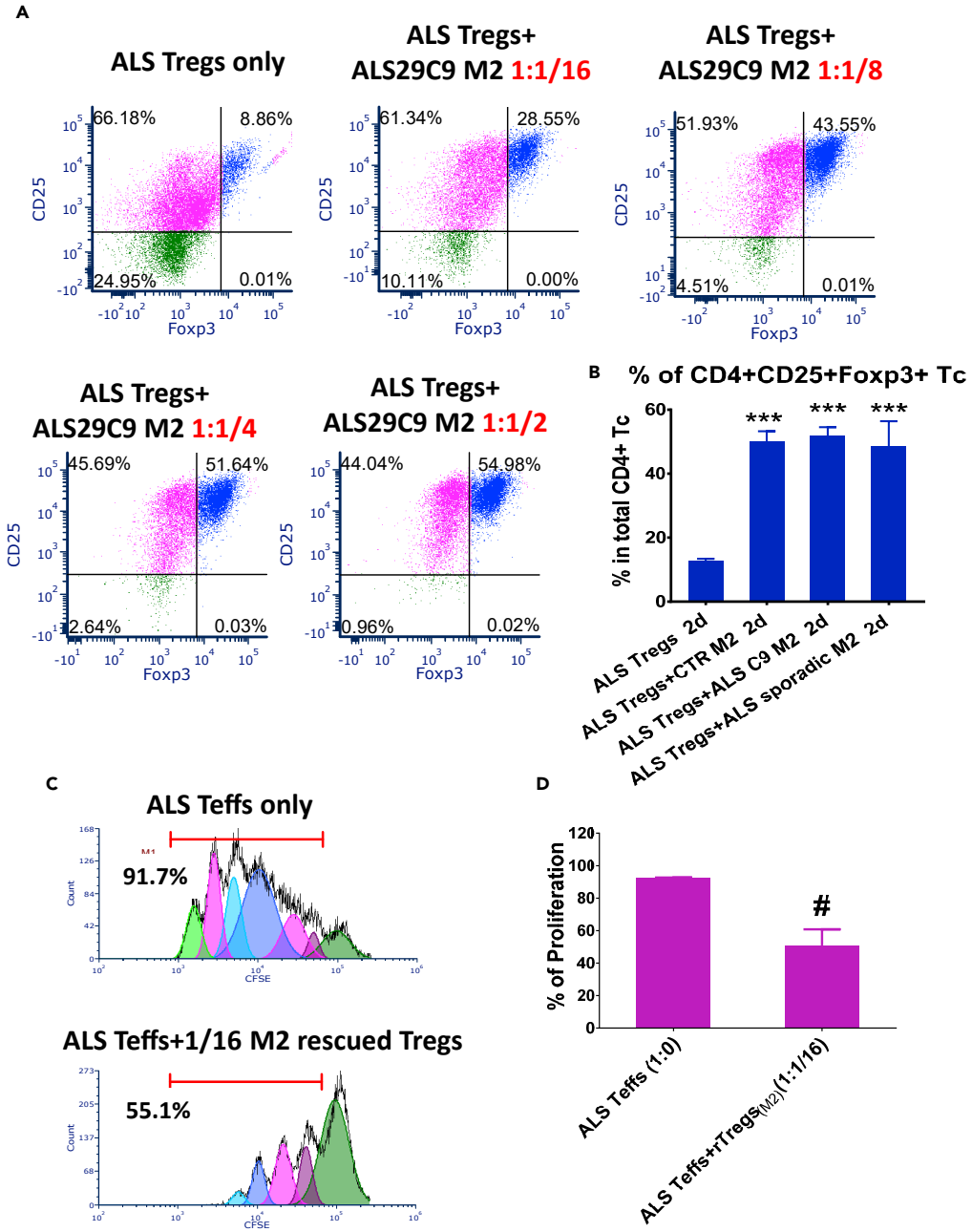


**Figure 7. ALS iPSC-Derived M2 Macrophages Sustain the Expression of Treg Functional Markers CD25 and Foxp3**

Tregs purified from blood of ALS patients were either cultured alone or co-cultured with iPSC-derived M2 cells at a ratio of 1:1/2 (Tregs:M2). After 2 days, T-cells were collected for flow cytometric analyses.

(A) Representative plots showed that the percentage of $CD4^+CD25^+Foxp3^+$ Tregs increased in a dose-dependent fashion after being co-cultured with M2 cells derived from a representative ALS C9 iPSC.

(B) Similar to CRT M2 cells (n = 3), M2 cells from ALS C9 iPSCs (n = 3) and sporadic ALS iPSCs (n = 4) increased the percentage of $CD4^+CD25^+Foxp3^+$ Tregs. ***$p < 0.001$ vs. ALS Tregs alone.

(C and D) M2-rescued $CD4^+CD25^+$ Tregs were isolated from M2 and ALS Tregs co-cultures. These M2-rescued Tregs ($rTregs_{(M2)}$) were then co-cultured with CFSE-stained responder Teffs from ALS patients for 5 days at a ratio of 1:1/16 (Tresp : M2-rescued Tregs). After co-cultured with Tregs rescued by ALS M2 cells, proliferation of ALS Tresp was lower than ALS Tresp alone (D) ($^{\#}p < 0.05$, n = 3). Representative proliferation plots were shown in C.

The current data also suggest that iPSC-derived M2 cells enhanced ALS Tregs by sustaining their number and converting Teffs into Tregs. Tregs are potent modulators of the immune system, suppressing injurious pro-inflammatory responses; Tregs can directly suppress the toxic properties of microglia (Zhao et al., 2012). The passive transfer of Tregs prolonged the slowly progressing phase of disease in ALS mice, augmented survival, increased M2 markers, and decreased M1 markers and their pro-inflammatory cytokines (Beers et al., 2011a). Furthermore, *in vivo* expansion of Tregs using an IL-2/IL-2Ab complex prolonged survival of ALS mice with reduced microglial immunoreactivity (Sheean et al., 2018). In patients with ALS, both number and function of Tregs were decreased as disease advanced from a slowly progressing stage to a rapidly progressing disease (Beers et al., 2017; Henkel et al., 2013). More importantly, autologous infusions of expanded Treg cells in patients with ALS were shown to be safe and slowed the patient's disease progression (Thonhoff et al., 2018). Thus, the demonstration that iPSC-derived M2 cells induce and rescue suppressive Tregs provides a potentially novel approach to maintain suppressive and anti-inflammatory effects of Treg-related therapy; M2 and Tregs may have a synergistic potential to quench the pro-inflammatory milieu.

The mechanism of how iPSC-derived M2 cells suppress cytokine production and secretion from M1 cells is unclear. TGF-β and IL-10 have been reported to regulate microglia, macrophages, and Teffs (Butovsky et al., 2014; Fiorentino et al., 1991; Kelly et al., 2018; Zhao et al., 2012; Zheng et al., 2007) and play an important role in the induction of Tregs (Hsu et al., 2015; Zheng et al., 2007). However, in our experiments, blocking antibodies (abs) against IL-10 and TGF-β did not reverse the effects of iPSC-derived M2 cell on M1 cell activation, Teff proliferation, and induction of Tregs from Teffs (data not shown). We could not document that either IL-10 or TGF-β contributed to the suppressive mechanisms *in vitro*. Further studies are clearly required to define the specific suppressive mechanisms.

The blood-brain barrier (BBB) is impaired in patients with ALS and in ALS animal models (Garbuzova-Davis et al., 2012; Henkel et al., 2009; Nicaise et al., 2009; Winkler et al., 2013; Zhong et al., 2008). As a result, peripheral monocytes can invade the spinal cord parenchyma (Zondler et al., 2016). Monocyte-derived macrophages have a distinct transcriptional profile, and when they enter the CNS, they maintain their transcriptional profile, which differs from the resident microglial transcriptional profile (Cronk et al., 2018). However, it has also been reported that when monocytes enter the CNS in pathological conditions and become macrophages, many of the genes expressed in macrophages are similar to the ones expressed in microglia (Friedman et al., 2018; Grassivaro et al., 2020). Further studies are needed to determine whether activated ALS macrophages remain distinct from activated microglia.

Blood-born macrophages have been shown to contribute to disease progression in ALS mice (Butovsky et al., 2012) and could similarly induce either neuroinflammation or neuroprotection in ALS. It is clear that extensive neuro-immune cross talk occurs between the brain and peripheral immune systems, especially in the presence of an altered BBB. Thus, peripheral myeloid M2 cells could modulate disease progression by suppressing M1-mediated inflammation in the periphery or in the CNS. The demonstration that iPSC-derived M2 cells were able to suppress the activation of M1 cells suggests that iPSC-derived M2 cells have the functional ability to protect motor neurons from injury mediated by M1 cells, and thereby slow ALS disease progression.

Our data indicate that C9 mutation and some ALS-related changes in iPSCs did not compromise their *in vitro* development to M2 cells with strong immunosuppressive function. However, we could not rule out the fact that ALS iPSCs-derived M2 may differ from control iPSC-derived M2 cells in other functions (such as phagocytosis) or following diverse differentiation conditions. It may also be possible that microglia/macrophages in patients with ALS contain genetic and disease-specific changes, which could promote M1 activation of ALS macrophages *in vivo*. In our recent study, monocytes from patients with ALS were more readily differentiated to a pro-inflammatory M1 phenotype than healthy control monocytes (Du et al., 2020). We had too few iPSC specimens to document whether different regimens that promote the M1 phenotype from iPSC cells could differentiate ALS M1 from control M1 cells. It is also possible that exposure to *in vivo* ALS-related signals as well as genetic differences may yield different responses in ALS and healthy control monocytes. More studies are clearly required to explore the underlining mechanisms.

Our previous studies demonstrated that M2 microglia in spinal cords of ALS mice lost their markers and a shift of M2 to M1 phenotype occurred during rapid progressing stage (Beers et al., 2011a; Liao et al., 2012).

Macrophages infiltrating to CNS or along peripheral nerves may undergo this shift from M2 to M1 cells in a similar way. However, it is still unknown which exact signals in ALS drive this M2 to M1 transition. It is also unclear whether iPSC-derived M2 cells would maintain their phenotype after transfer *in vivo*. We used M-CSF and IL-4/IL-10/TGF-β to differentiate M2 macrophages. This optimized protocol has been shown to induce human M2 cells to release more anti-inflammatory cytokines with higher suppressive function, especially more resistant to pro-inflammatory stimulation than other M2 differentiation protocols (Mia et al., 2014).

Although the *in vivo* protective anti-inflammatory functions of M2 have not been reported in ALS, mouse M2 cells have been shown to have protective effects in mouse models of diabetes and nephrosis; transferred M2 macrophages were shown to maintain their anti-inflammatory phenotypes *in vivo* in these models (Cao et al., 2010; Parsa et al., 2012). One single injection of mouse M2 cells before disease onset protected >80% of treated mice against type 1 diabetes for at least 3 months and suppressed T cell proliferation in pancreatic lymph nodes of treated mice; injected M2 cells were found to migrate predominantly into the inflamed pancreas and associated lymph nodes (Parsa et al., 2012). M2 transfer also reduced renal inflammation in murine adriamycin nephrosis; accumulation of injected M2 was also observed in inflamed kidney and renal draining lymph nodes after adriamycin administration (Cao et al., 2010). Thus, these reports indicate that M2 macrophages have *in vivo* immunosuppressive properties, are able to enter inflamed lesion sites, and provide clinical benefit.

The current study demonstrates that ALS does not cause deficits in iPSC-derived M2 cells; M2 cells differentiated from iPSCs of patients with ALS suppress inflammation as strongly as M2 derived from healthy controls' iPSCs. Our data provide critical evidence for the autologous treatment of patients with ALS using their own iPSC-derived M2 cells. It remains to be determined whether iPSC-derived M2 cells can effectively ameliorate the pathogenesis of disease in ALS.

### Limitations of the Study

The first limitation of this study is that therapeutic mechanisms of suppressive action and induction of Tregs from Teffs mediated by iPSC-derived M2 macrophages are presently unclear. Second, we do not have definitive evidence that the *ex vivo* immunomodulatory activity of iPSC-derived M2 macrophages will be translated into *in vivo* therapy. Third, how to maintain the suppressive function of M2 cells and prevent them from shifting to M1 cells in pro-inflammatory environment is not addressed in this study. The answer to these questions would be valuable for further *in vivo* therapeutic investigation.

### Resource Availability

#### *Lead Contact*

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Stanley H. Appel (sappel@houstonmethodist.org).

#### *Materials Availability*

iPSC lines generated in this study will be made available upon request for a minimal fee to cover production of iPSC Cryovials after completing a Materials Transfer Agreement with the Cedars-Sinai Biomanufacturing Center (CBC). The iPSC lines are readily accessible from the CBC Cell Line Catalog. This study did not generate any other unique reagents.

#### *Data and Code Availability*

This study did not generate datasets or codes. There is no any unpublished custom code, software, or algorithm that is central to supporting the main claims of the paper.

## METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101192.

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## AUTHOR CONTRIBUTIONS

W.Z., J.W., and S.W. performed the research and collected data. L.O. and D.S. generated iPSC lines. W.Z. analyzed data. S.H.A. and W.Z. designed the project and interpreted the data. S.H.A. and C.N.S. organized patient recruitment and provide clinical information. C.N.S., H.S.G., D.R.B., J.R.T., A.D.T., and A.F. conceptualized the study, performed further data interpretation, and provided critical experimental suggestions. W.Z., S.H.A., D.R.B., and A.F. wrote and revised the manuscript.

## DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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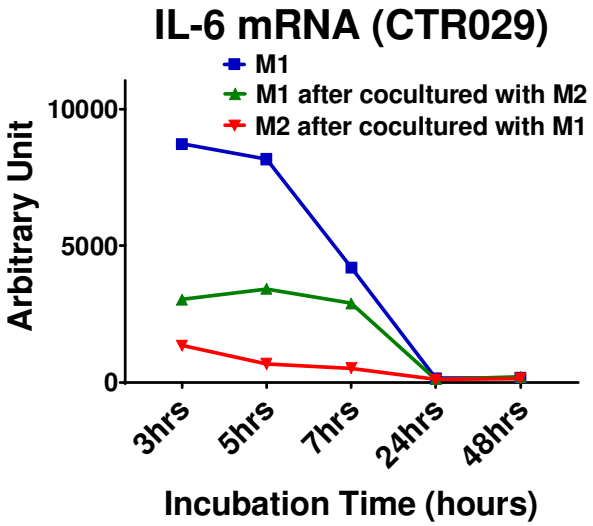
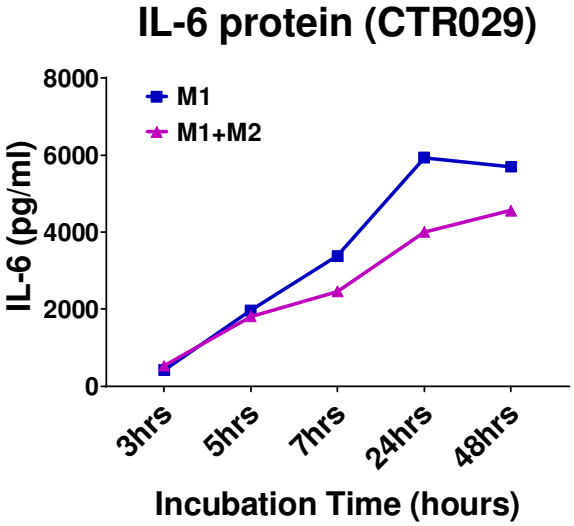
## Supplemental Information

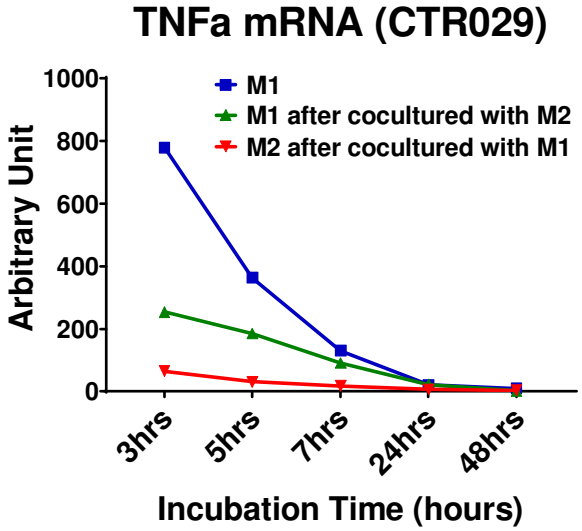
# Immunosuppressive Functions of M2 Macrophages Derived from iPSCs of Patients with ALS and Healthy Controls

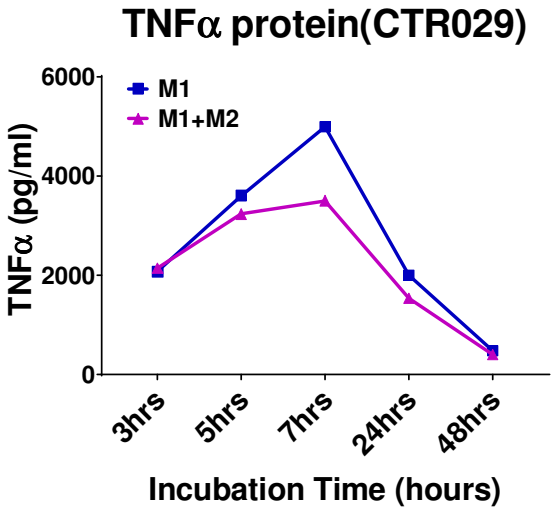
**Weihua Zhao, David R. Beers, Jason R. Thonhoff, Aaron D. Thome, Alireza Faridar, Jinghong Wang, Shixiang Wen, Loren Ornelas, Dhruv Sareen, Helen S. Goodridge, Clive N. Svendsen, and Stanley H. Appel**

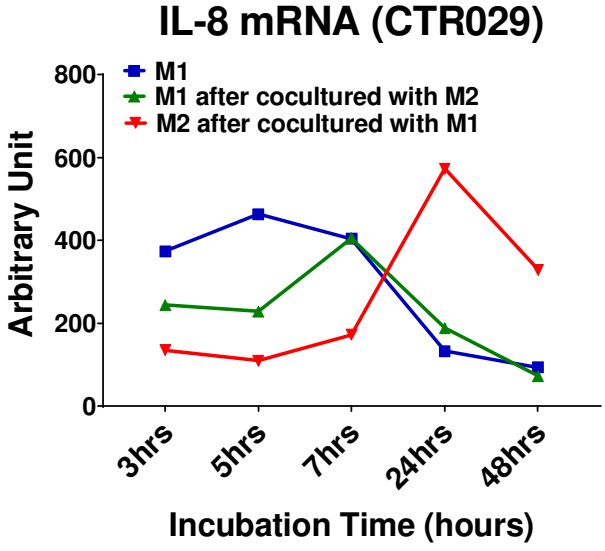
# Supplemental Figure 1

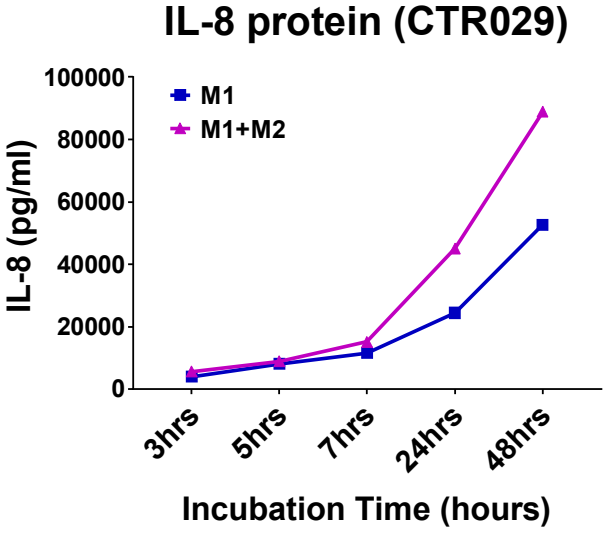


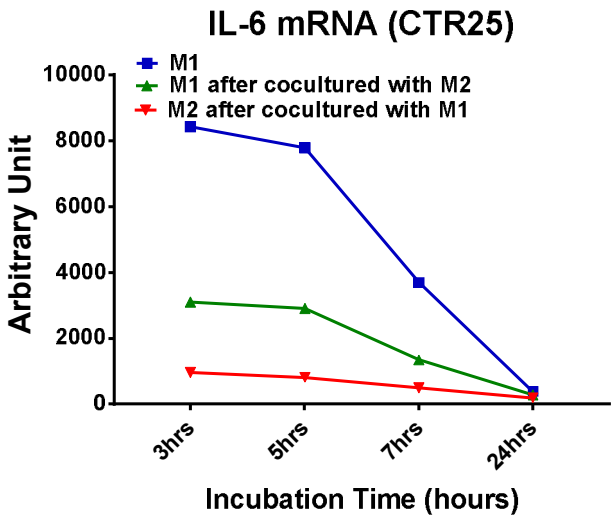



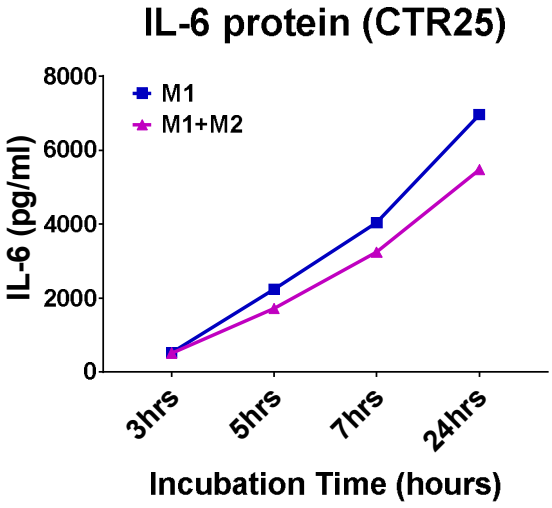



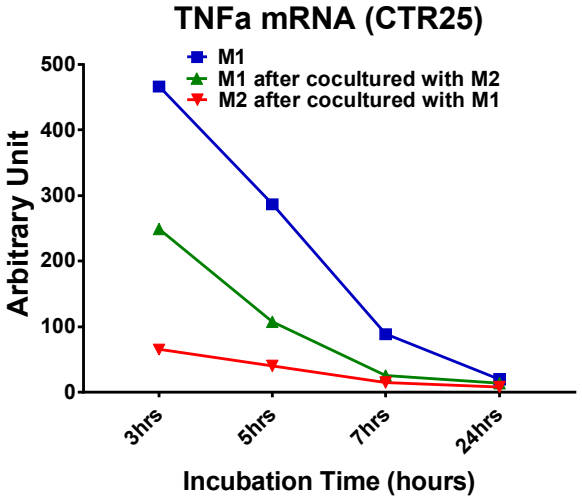



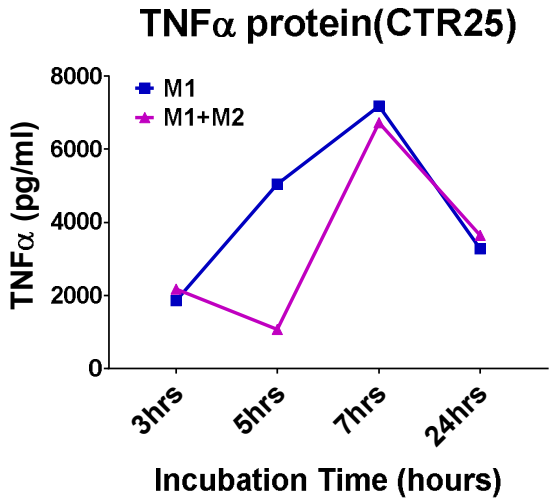



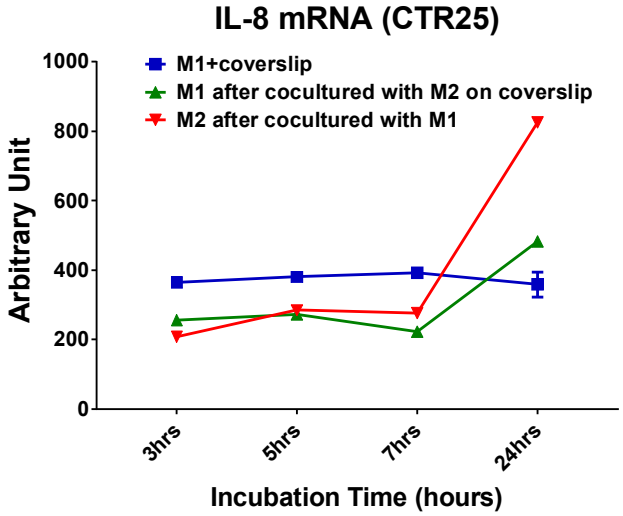
# Supplemental Figure 2

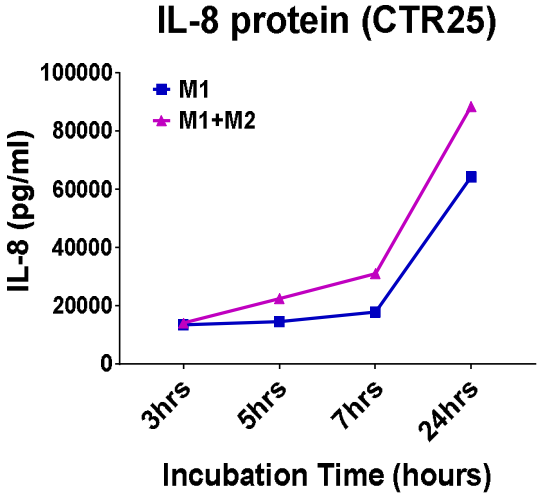


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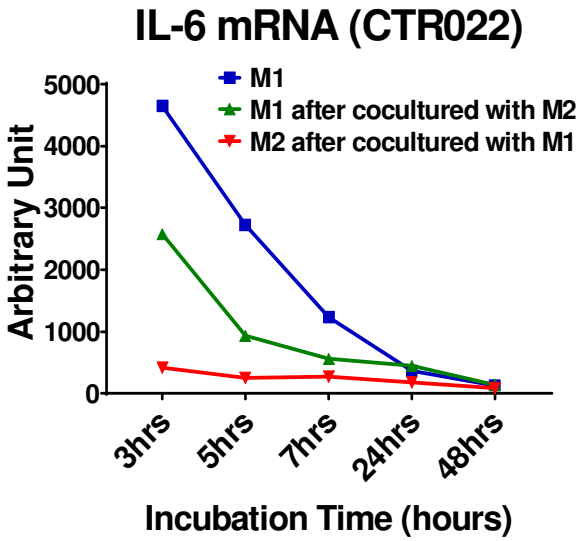
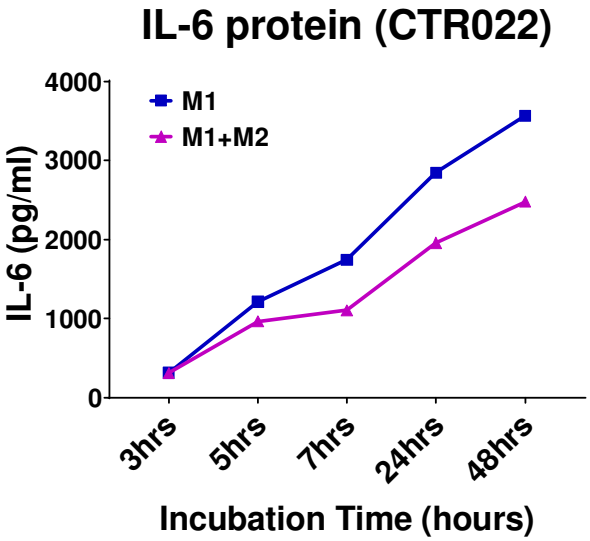
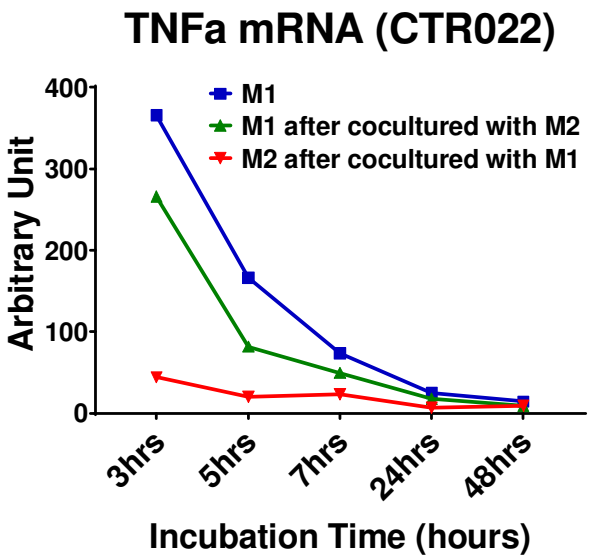
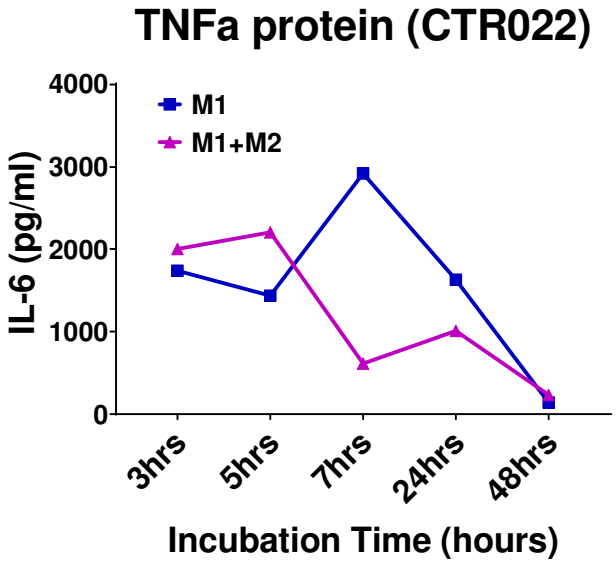
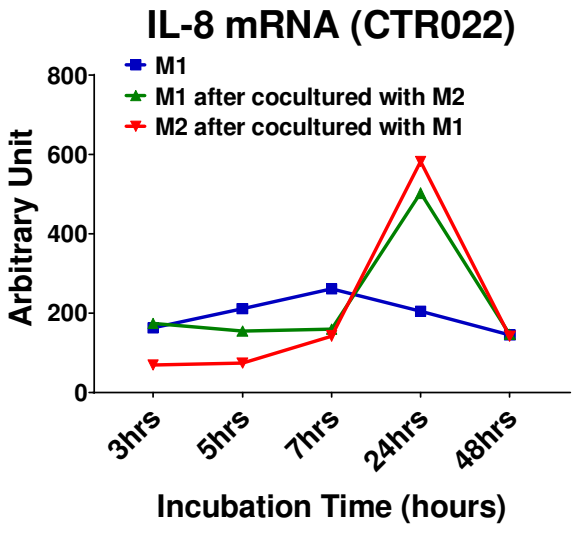
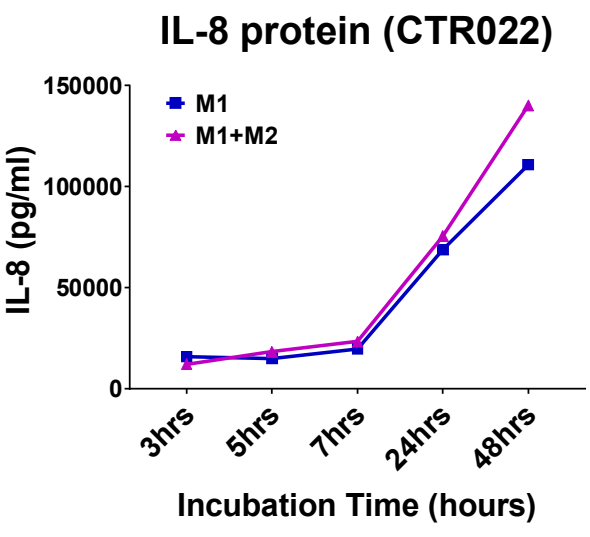


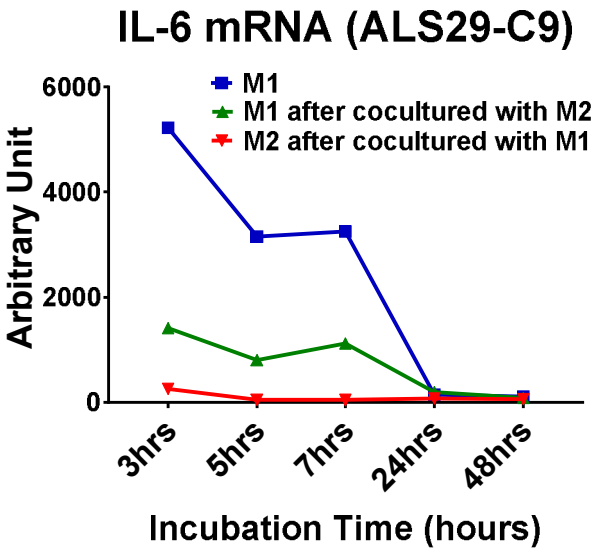



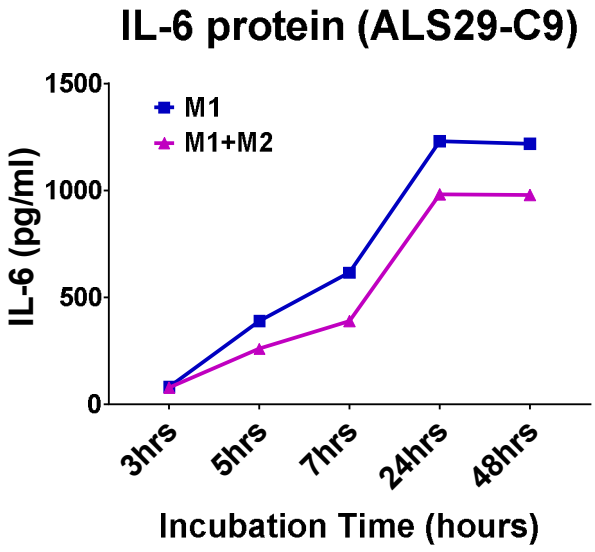



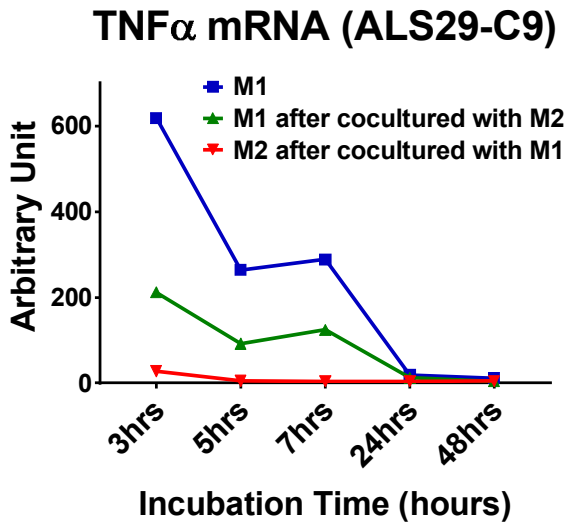



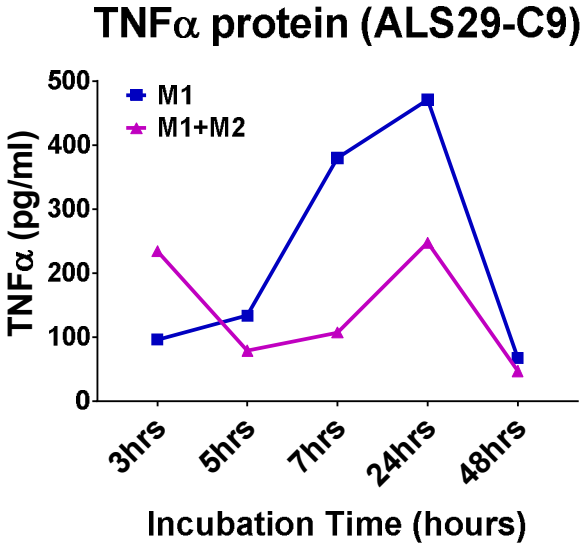



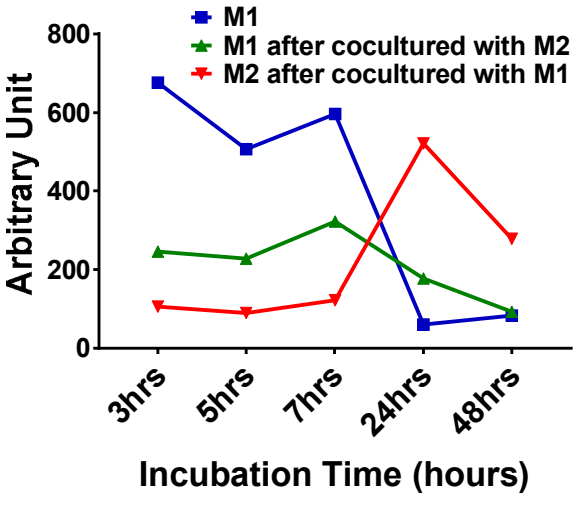
# Supplemental Figure 4

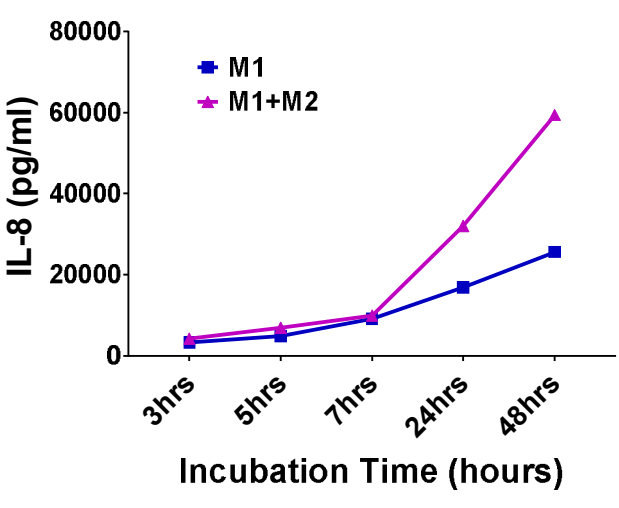


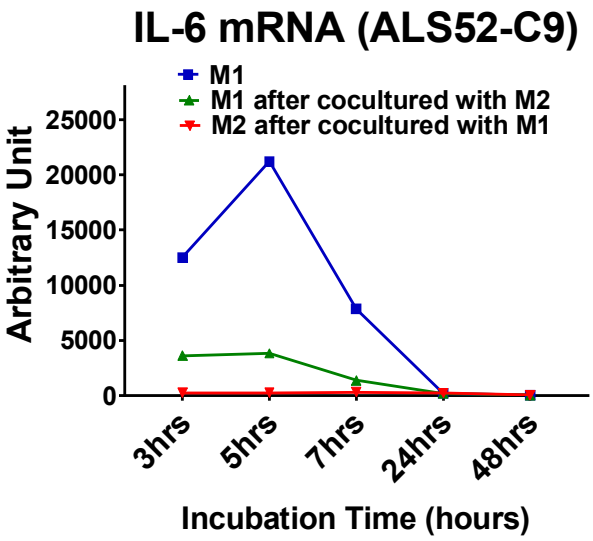



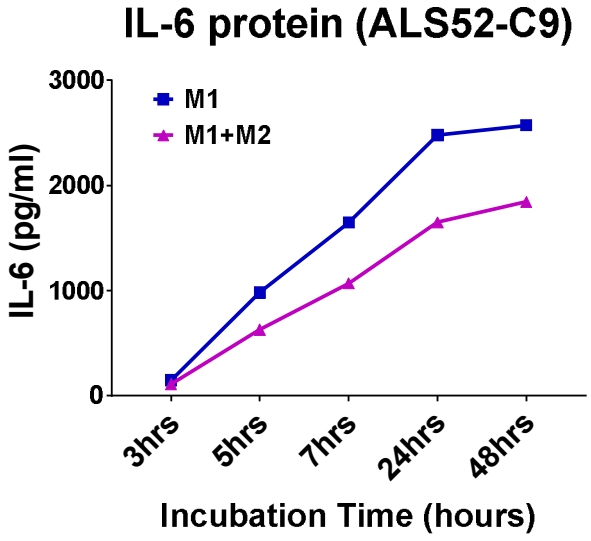



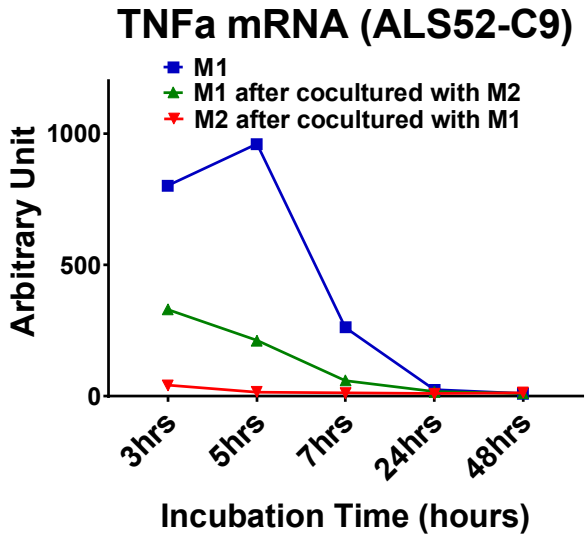



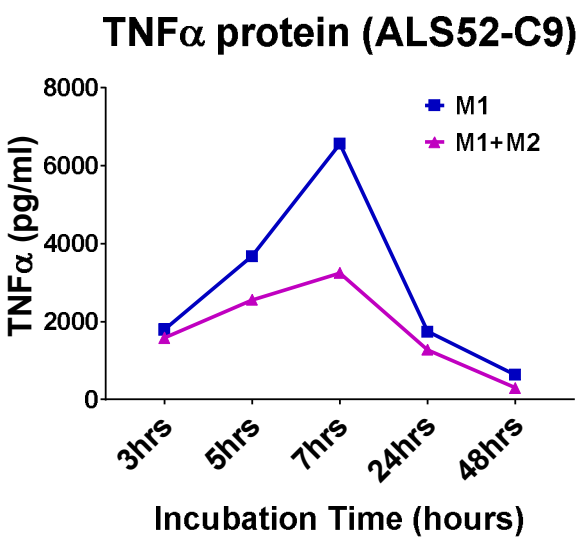



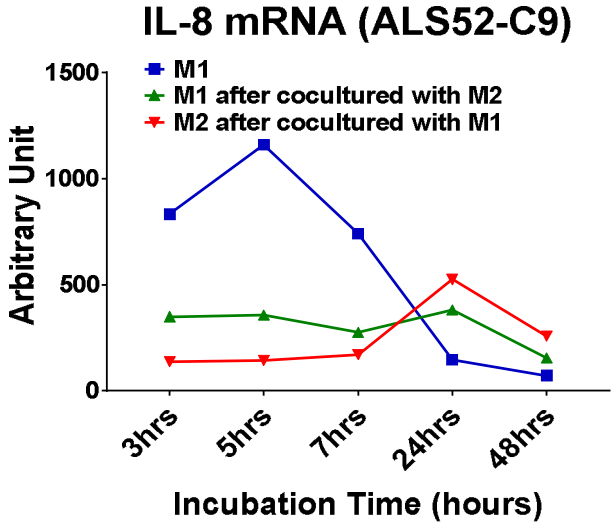
# Supplemental Figure 5

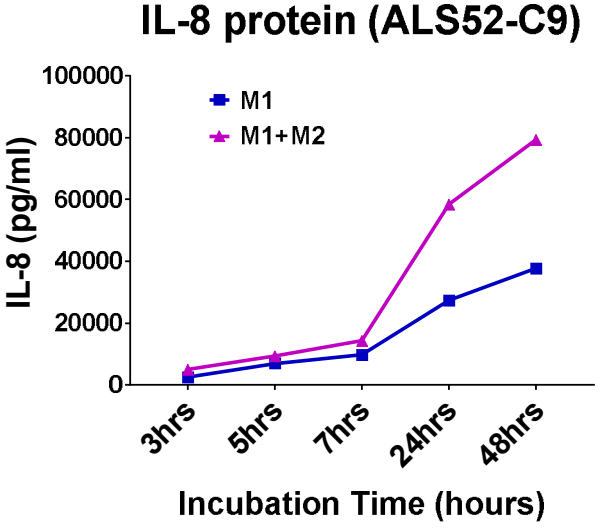


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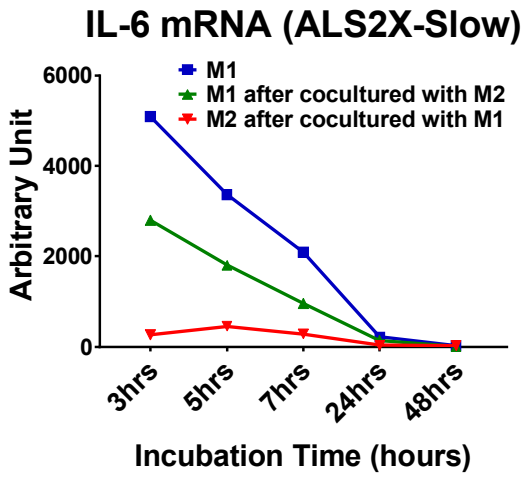
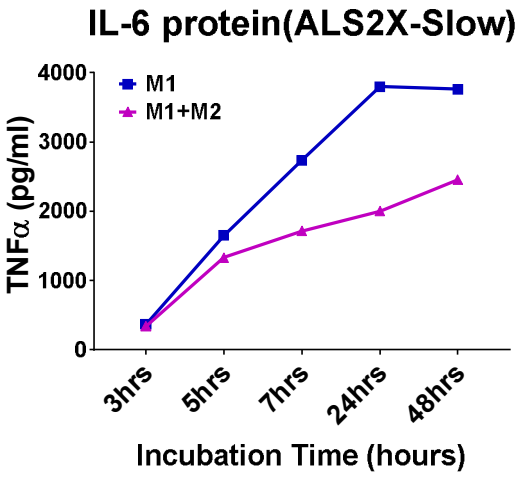
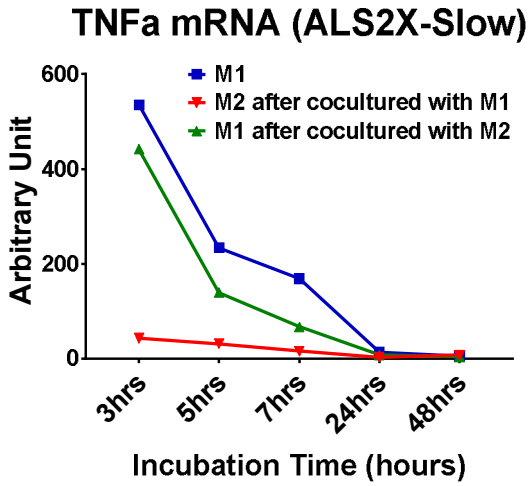
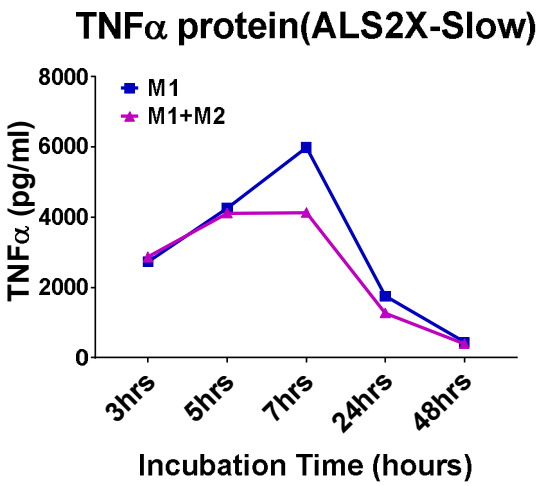
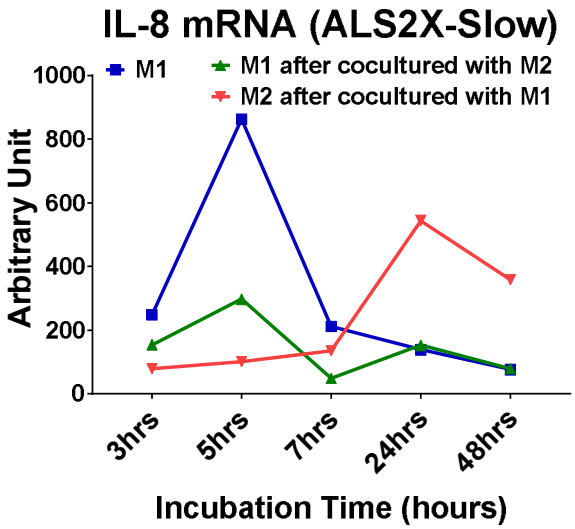
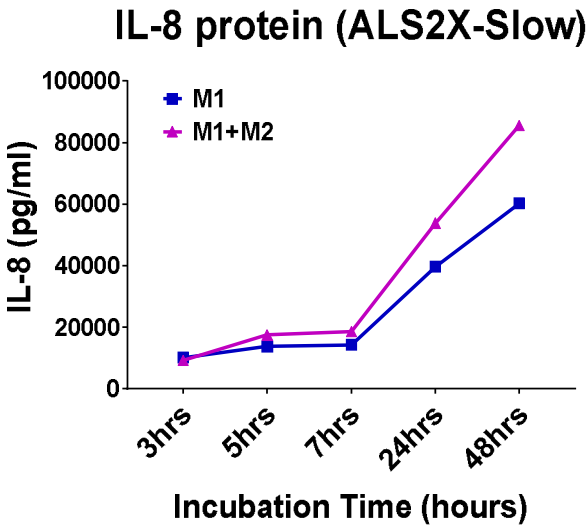


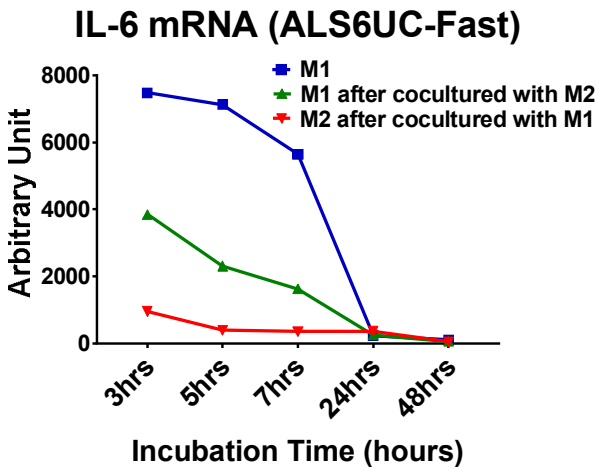



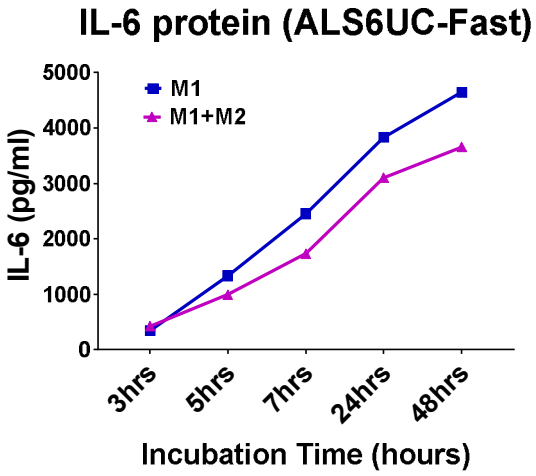



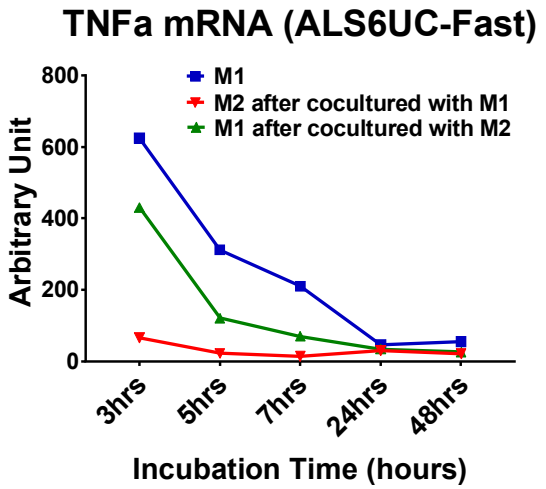



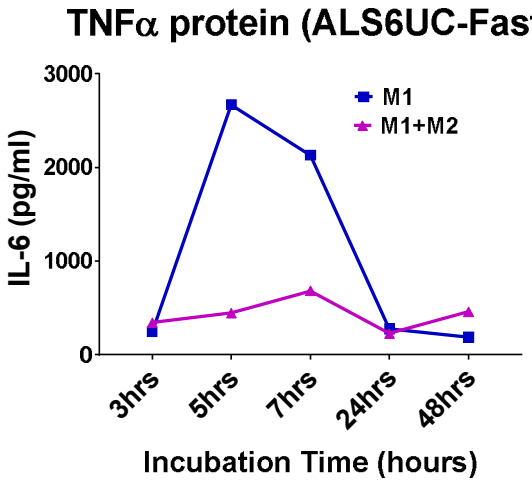



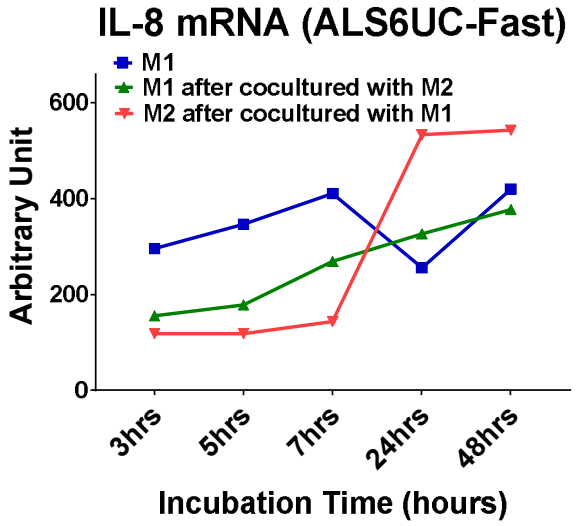
# Supplemental Figure 7

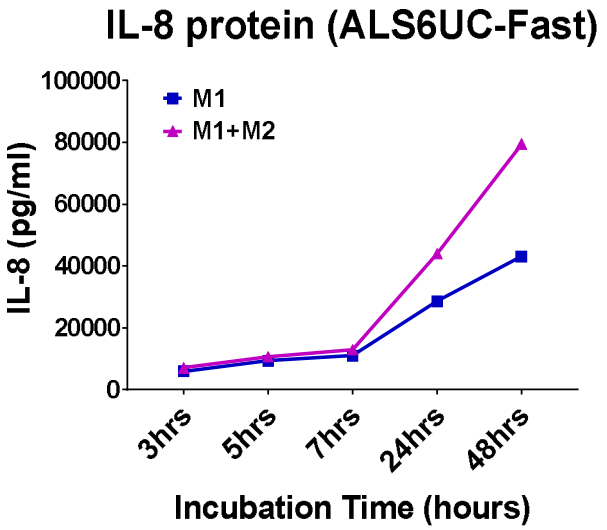


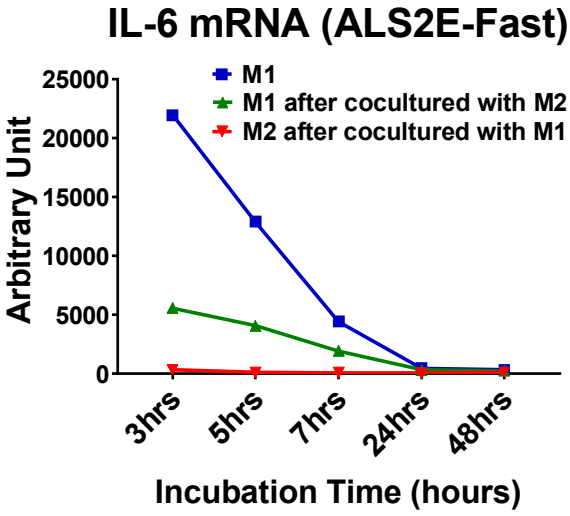



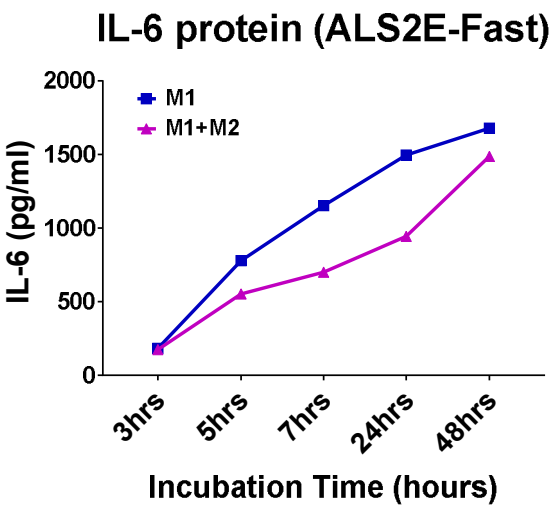



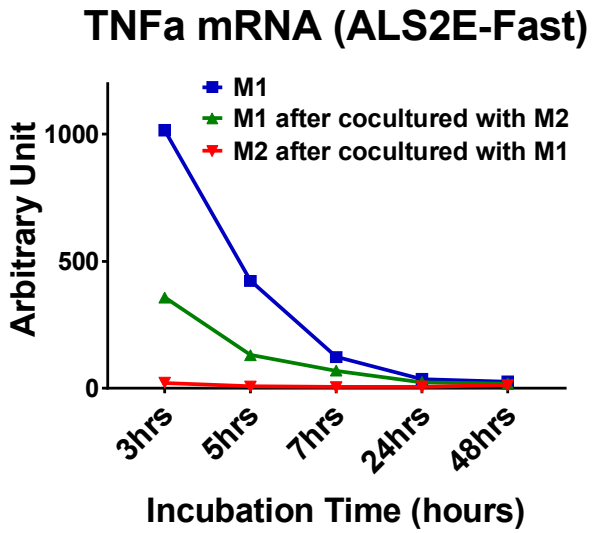



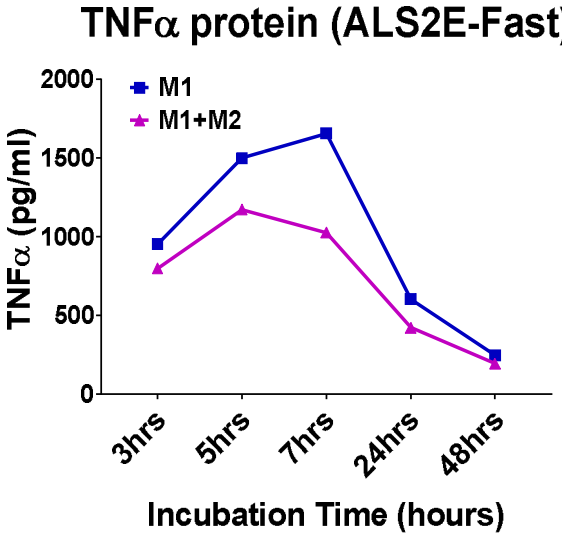



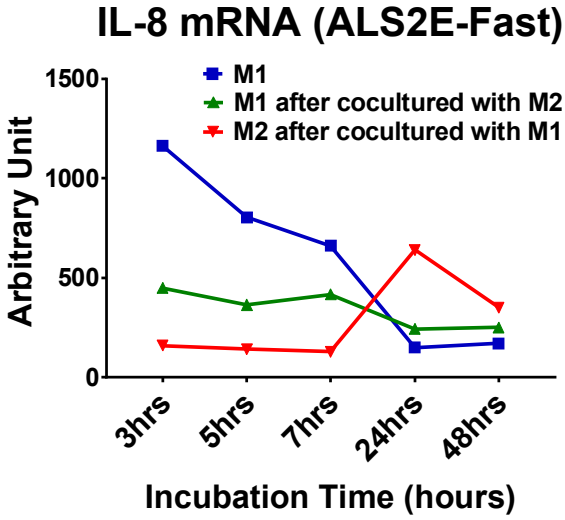
# Supplemental Figure 8

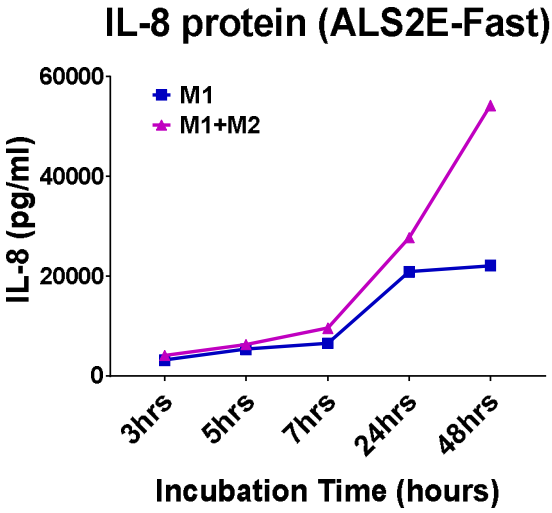


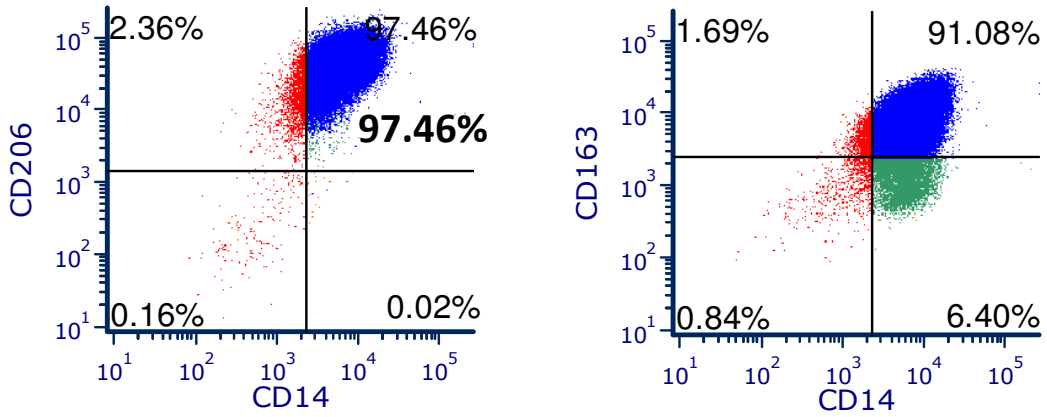



# Supplemental Figure 9

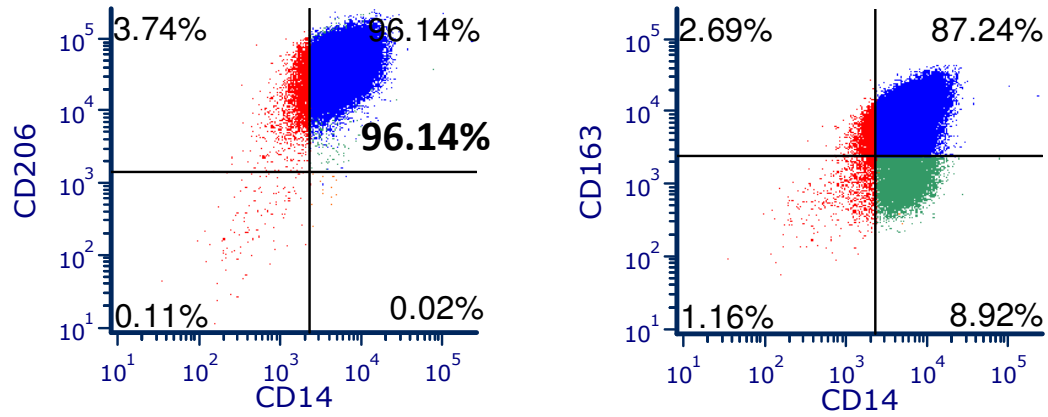
**A**

## M2 derived from ALS 52 C9 iPSC

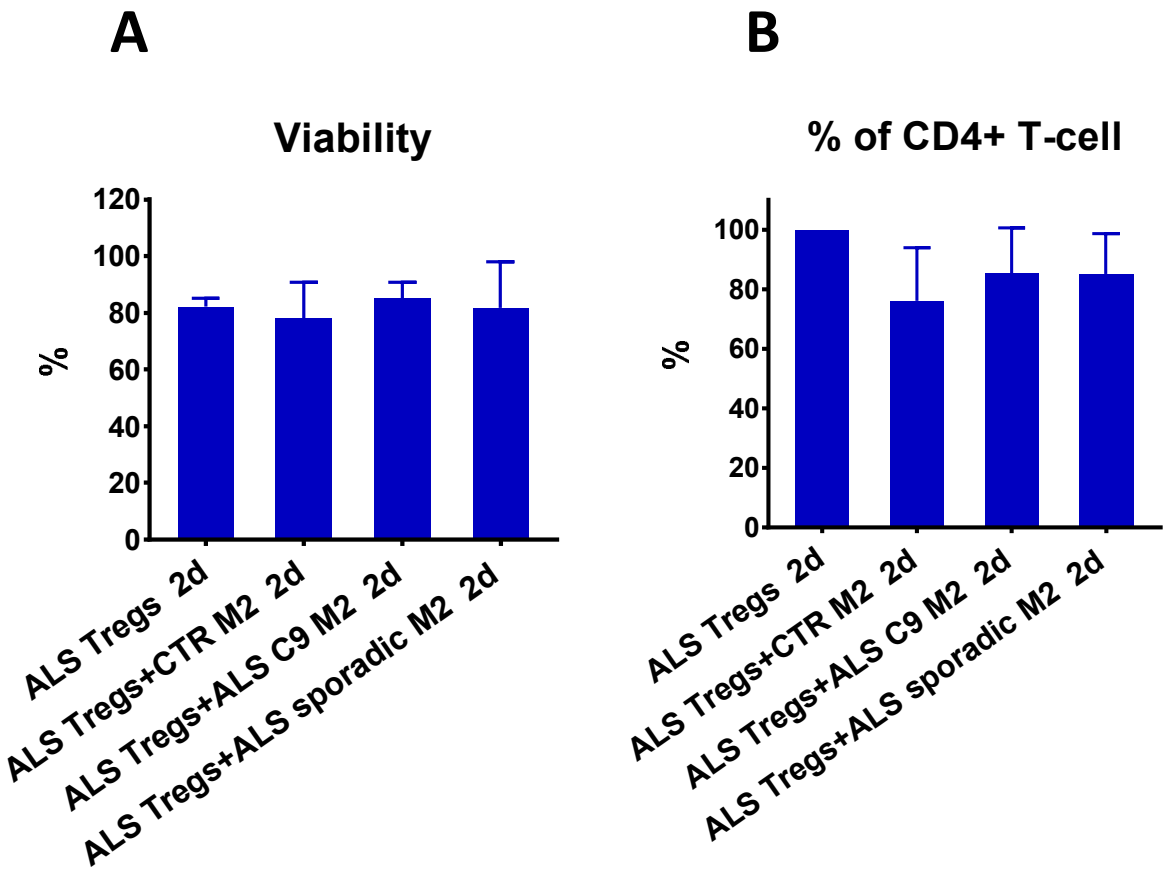



**B**

## M2 derived from ALS 29 C9 iPSC



# Supplemental Figure 10



**Supplemental Figure 1-8.** Time-response curves of each iPSC-derived M1 alone, M1 or M2 after co-culture, related to Figure 4. M1 and M2 cells were differentiated from 3 control iPSCs (Fig. S1-S3), 2 ALS C9 iPSCs (Fig. S4-S5), and 3 sporadic ALS iPSCs of one slowly progressing patient (Fig. S6) and 2 rapidly progressing patients (Fig. S7-S8). IL-6, TNF-α, and IL-8 mRNA levels were detected in M1 or M2 cells. IL-6, TNF-α, and IL-8 protein production were measured in culture supernatants.

**Supplemental Figure 9.** CD14, CD206 and CD163 surface expression on M2 cells derived from ALS52 C9 iPSC (A) and ALS29 C9 iPSC (B), related to Figure 3.

**Supplemental Figure 10.** No changes were observed on viability and $CD4^+$ T-cell numbers in ALS Tregs alone or co-cultured with iPSC-derived M2 cells, related to Figure 7. Tregs isolated from blood of ALS patients were either cultured alone or co-cultured with iPSC-derived M2 cells at a ratio of 1:1/2 (Tregs:M2). After 2 days, dead cells were stained with blue-fluorescent reactive dye to determine viability (A). (B): The number of $CD4^+$ T-cells were counted by flow cytometry after being stained with anti-human CD4-V500 antibody. $CD4^+$ T-cell numbers were shown as percentage of $CD4^+$ T-cells in ALS Treg alone cultures in each experiment.

Supplemental Table 1: iPSC lines and their source cells used in this study, and demographic and clinical information of donors, related to Figure 1-7.

| iPSC line ID | Source Cells | Sex of Donors | Donor Age at time of Collection | Site of ALS Disease Onset of Donors |
|---|---|---|---|---|
| CS25iCTR-18n2 | Fibroblast | Male | 76 | N/A |
| EDi029-A | PBMC | Male | 80 | N/A |
| EDi022-A | PBMC | Male | 79 | N/A |
| CS28iALS-C9n2 (with C9 mutation) | Fibroblast | Male | 47 | Left Lower Extremity |
| CS29iALS-C9n1 (with C9 mutation) | Fibroblast | Male | 47 | Left Lower Extremity |
| CS52iALS-C9n6 (with C9 mutation) | Fibroblast | Male | 49 | Left Upper Extremity |
| CS6ULBiALS-n2 (slow) | PBMC | Male | 51 | Axial,Trunk |
| CS2XWCiALS-n1 (slow) | PBMC | Male | 37 | Limb Upper Left, Arm |
| CS2EVPiALS-n2 (fast) | PBMC | Male | 69 | Bulbar |
| CS6UC9iALS-n1 (fast) | PBMC | Male | 54 | Bulbar |

## TRANSPARENT METHODS

### Generation of iPSC lines

Human peripheral blood mononuclear cells (PBMCs) were derived from multiple individuals and cryopreserved in CryoStor CS10. Approximately, PBMCs (5 x $10^6$) were nucleofected with a plasmid mixture using 0.83 µg per plasmid - pEP4 E02S ET2K, pCXLE-hOCT3/4-shp53-F, pCXLE-hUL, pCXLE-hSK, and pCXLE-EBNA1, all plasmids obtained from Addgene. The program V-024 on the Amaxa Nucleofector 2D Device was utilized along with the Amaxa Human T-cell Nucleofector® Kit. Cells (1 x $10^6$) were then seeded into 3 wells of a 6-well plate covered with a mitomycin treated mouse embryonic fibroblast (MEF) layer. Post nucleofection, cells were plated in 2 mL of αMEM supplemented with 10% FBS, 10ng/ml IL-3, 10ng/ml IL-6, 10ng/ml G-CSF and 10ng/ml GM-CSF. Two days after nucleofection, an equal amount of Primate ESC medium (ReproCell) containing 5 ng/ml bFGF was added to the wells without aspirating the previous medium. Beginning on day four, the medium was gently aspirated from each well and 2ml of fresh reprogramming media was added to each well. Human dermal fibroblast were expanded in DMEM supplemented with 15% FBS, 1% NEAA, 1% GlutaMAX (Gibco), 1% Anti-Anti (Gibco) and 4 ng/ml bFGF. Approximately 300,000 cells were nucleofected with a plasmid mixture using 2.5 µg per plasmid - pCXLE-hOCT3/4-shp53-F, pCXLE-hUL and pCXLE-hSK all plasmids obtained from Addgene. The program U-023 on the Amaxa Nucleofector 2D Device was utilized along with the NHDF Nucleofector Kit (Lonza). Approximately 100,000 cells per well were seeded into 3 wells of a BD Matrigel™ growth factor-reduced Matrix (Corning) coated 6-well plate. For both PBMC and fibroblast-originated cultures, medium was replaced every other day. At 18 days post nucleofection, individual colonies were observed. At 25 days (for PBMC-derived colonies) or 20 days (for fibroblast-derived colonies) post nucleofection, individual colonies were isolated and sub-cloned into 1 well of a BD Matrigel™ growth factor-reduced Matrix (Corning) coated 12-well plate containing 1ml of mTeSR1 (Stemcell Technologies). Subclones were monitored and were mechanically isolated and transferred onto Matrigel coated 6-well plates and

maintained in mTeSR®1 medium. All cultures were maintained at 20% O2 during the reprogramming process except cultures from fibroblasts were maintained at 5% O2 before day 20. iPSC lines and their source cells, demographic and clinical information of donors are provided in Supplemental Table 1.

## Monocytes and M1/M2 differentiation from iPSCs

Monocytes were differentiated from iPSCs of 7 ALS patients and 6 healthy controls under serum-free, feeder cell-free defined conditions (Yanagimachi et al., 2013). After 4-step differentiation procedure, monocytes were purified from floating cells in cultures twice a week using CD14 microbeads (Miltenyi Biotec) according to manufacturer's instructions. For M1 cell differentiation, iPSC-derived monocytes were cultured in RPMI medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 1mM sodium pyruvate, 1×nonessential amino acids, 55 μM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin (complete RPMI medium) in the presence of GM-CSF (50ng/ml, R & D Systems) for 7 days. Media were changed at day 4, and then LPS (1ng/ml, Sigma) and IFNγ (2ng/ml, Invitrogen) were added to the cultures for different periods as indicated. For M2 cell differentiation, M-CSF (100ng/ml, R & D Systems) were used to treat monocytes in complete RPMI medium for 7 days with medium change at day 4, and human IL-4 (20ng/ml, R & D Systems), human IL-10 (20ng/ml, R & D Systems) and TGF-β (20ng/ml, R & D Systems) were added to the cultures to promote M2 macrophages on day 7. After incubation with IL-4/IL-10/TGF-β for 5hrs, M2 macrophages ($5x10^5$/well) were extensively washed in order to remove the exogenous IL-4, IL-10, and TGF-β. M2 cells were then cultured for 24hrs before supernatants were collected for detecting IL-10 and TGFβ production from M2 cells by ELISA assay.

## M1 and M2 co-cultures

M1 cells ($1x10^5$/well) derived from iPSCs were cultured in a 24-well plate. M2 or M0 cells ($2x10^5$/well) derived from same iPSCs were cultured on coverslips in a 24-well plate. Coverslips with M2 or M0 cells were placed into wells containing M1 cells 1hr after adding LPS/IFNγ to M1 cells. Coverslips without cells were added to M1 cells alone wells at the same time as controls. M2 or M0 cells or coverslips were taken out at different time periods as indicated, and then the M1 and M0/M2 cells were assayed separately after co-culture. M1 and M0/M2 cells were lysed in Trizol separately for mRNA expression analyses. Culture supernatants were collected for detecting protein levels by ELISA.

### Teffs/Tregs isolation and co-cultures with M0/M2 cells

$CD4^+CD25^-$ Teffs and $CD4^+CD25^+$ Tregs were purified from blood of ALS patients using Human $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotec). After staining with CFSE (3 μM, Invitrogen), Teffs ($5x10^4$) were co-cultured with M0/M2 cells at different ratio (Teffs: M2= 1:1/8, 1:1/4, 1:1/2, 1:1) for 6 days in the presence of CD3 Ab (1μg/ml, OKT3, eBiosciences) and CD28 Ab (1μg/ml, BioLegend). Proliferation of Teffs was then determined by flow cytometry. Teffs or Tregs ($5x10^4$) were co-cultured with M0/M2 cells for 2 days, and then stained with anti-human CD4-V500 Ab (BD Bioscience), CD25-PerCP-Cyc5.5 Ab (eBiosciences), and Foxp3-PE Ab (BioLegend) for flow cytometry analyses. To examine the suppressive function of M2-induced or M2-rescued Tregs, $CD4^+CD25^+$ T-cells (induced or rescued Tregs) were purified after co-culturing ALS Teffs or ALS Tregs with M2 cells for 2 days. Purified Tregs were then co-cultured with CFSE-stained responder ALS Teffs at different ratio (Teffs: M2= 1:1/16, 1:1/8, 1:1/4, 1:1/2, 1:1). After 4 days, proliferation of Teffs was assayed by flow cytometry. The suppression of induced or rescued Tregs were determined by comparing Teffs proliferation in Teffs+Tregs co-cultures with Teffs proliferation in Teffs alone cultures.

### Flow cytometry for M2 phenotype

Floating cells in iPSC differentiation cultures were stained with anti-human CD14-V450 Ab, anti-human CD115-PE Ab, and anti-human HLA-DR-PerCP-cyc5.5 Ab (Thermo Fisher Scientific). Differentiated M2 cells were stained with anti-human CD14-V450 Ab, anti-human CD68 FITC Ab, anti-human CD206-PE Ab, and anti-human CD163-APC Ab (Thermo Fisher Scientific). All cells were treated with human Fc Receptor Binding Inhibitor (Thermo Fisher Scientific) before surface antibodies were added. Dead cells were stained by LIVE/DEAD® Fixable Blue Dead Cell Stain Kit (Life Technology). Cells stained by Isotype IgGs were used as controls. The cells were detected on BDTM LSRII-16 colors flow cytometer configured with 488 nm, 633 nm, 405 nm, and UV lasers (BD Biosciences). Flow cytometry data were analyzed by FACSDiva (BD Biosciences) and FCS Express 6 (De Novo Software).

## Quantitative RT-PCR

RNA samples were extracted and purified from M1 or M2 cell cultures using Direct-zol™ RNA MiniPrep Kit (Zymo Research) according to manufacturer's recommendations. Quantitative RT-PCR was performed using one-step RT-PCR kit with SYBR Green (Bio-Rad Laboratories) and the iQ5 Multicolor Real-time PCR detection System (Bio-Rad Laboratories) according to manufacturer's recommendations. The conditions of PCR were as follows: for IL-6, the primers: 5'-AAA TTC GGT ACA TCC TCG ACG G-3' and 5'-GGA AGG TTC AGG TTG TTT TCT GC-3', Tm=60°C; for TNF-α, the primers: 5'-GAG GCC AAG CCC TGG TAT G-3' and 5'-CGG GCC GAT TGA TCT CAG C-3', Tm=60°C; for IL-8, the primers: 5'-AGC TCT GTG TGA AGG TGC AGT -3' and 5'- AAT TTC TGT GTT GGC GCA GTG -3', Tm=55.4°C; for β-actin, the primers: 5'-GCA TCC ACG AAA CTA CCT TCA -3' and 5'- GCA GTG ATC TCC TTC TGC ATC -3', Tm=60°C. Primer efficiency was assessed by analyzing a serial dilution of RNA. The relative expression level of each mRNA was calculated using the ΔΔCt method normalizing to β-actin and relative to the control samples. The presence of one product of the correct size was verified

by both 2% agarose gel electrophoresis and melt curve analyses containing a single melt curve peak.

## ELISA

Human ELISA Ready-SET-Go kits (Invitrogen) were used to determine the concentration of IL-10, TGF-β, IL-6, IL-8 and TNF-α protein levels in the supernatants of cultures according to manufacturer's instructions.

## Statistics

The statistical analyses were done by using ANOVA for more than 2 groups or Student's t-test for two groups. Data are expressed as mean±SE and *p* values less than 0.05 were considered significant.