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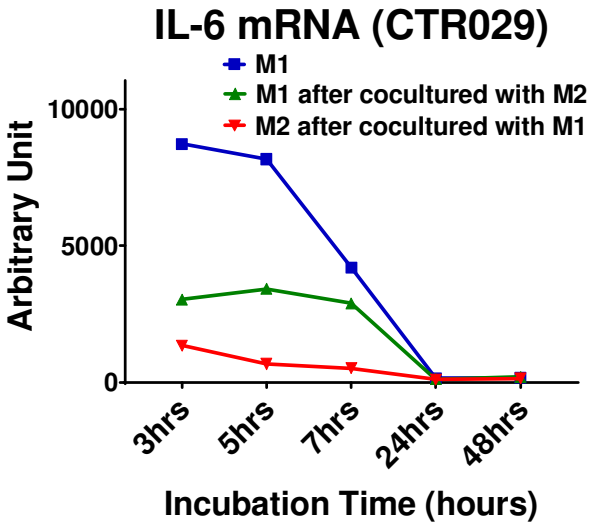
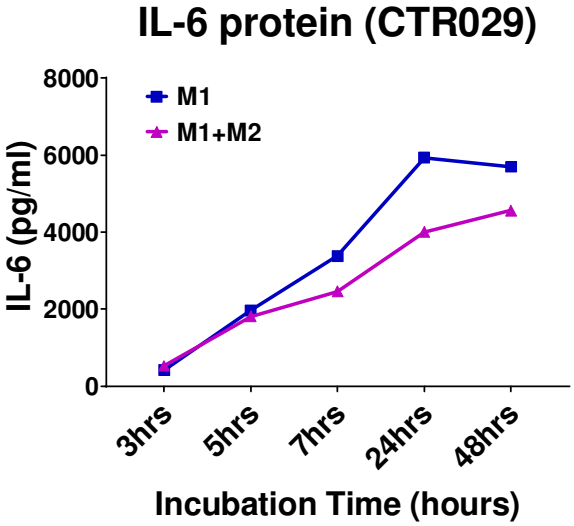
Supplemental Information

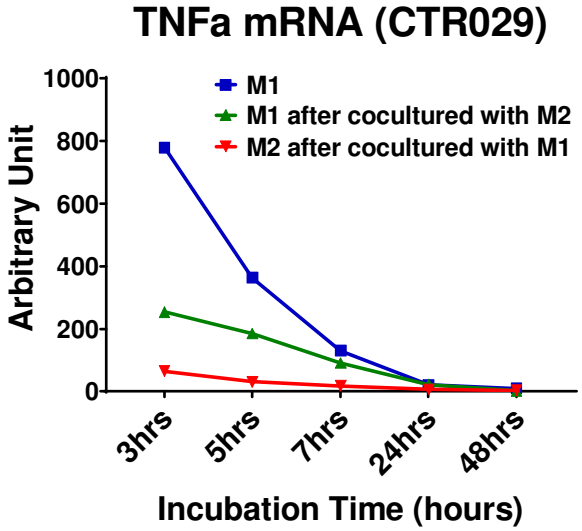
Immunosuppressive Functions of M2 Macrophages Derived from iPSCs of Patients with ALS and Healthy Controls

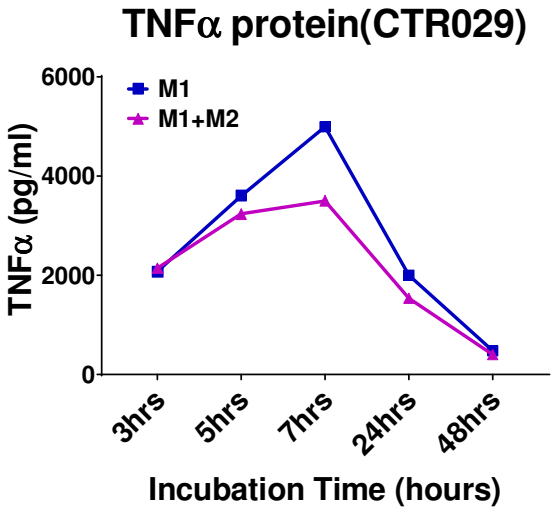
Weihua Zhao, David R. Beers, Jason R. Thonhoff, Aaron D. Thome, Alireza Faridar, Jinghong Wang, Shixiang Wen, Loren Ornelas, Dhruv Sareen, Helen S. Goodridge, Clive N. Svendsen, and Stanley H. Appel

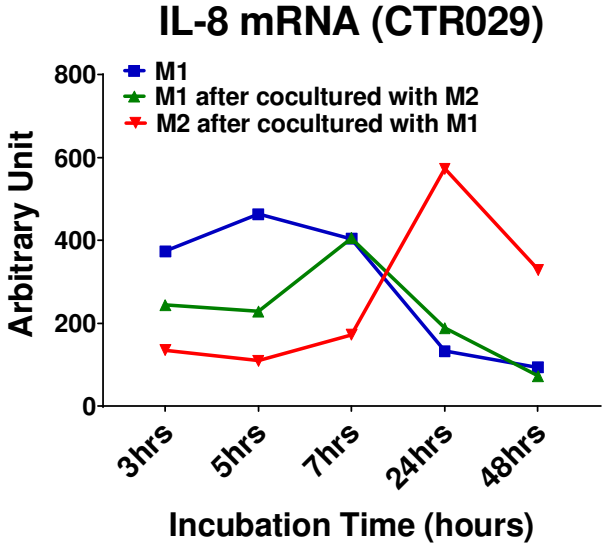
Supplemental Figure 1

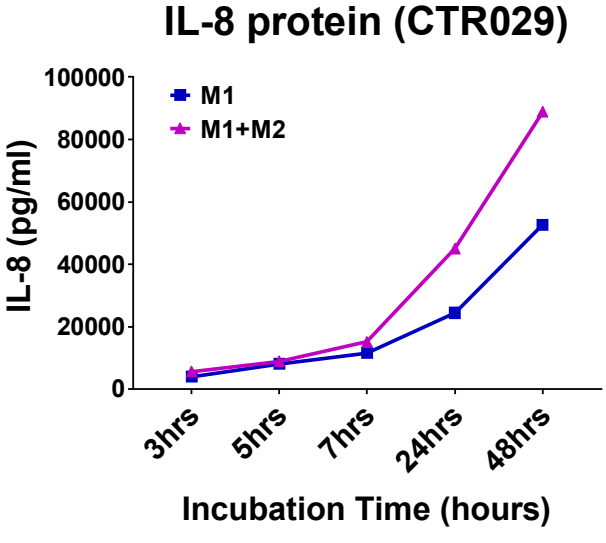












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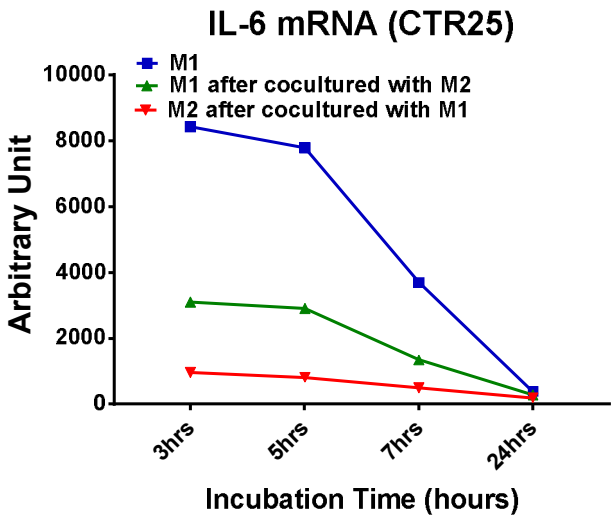
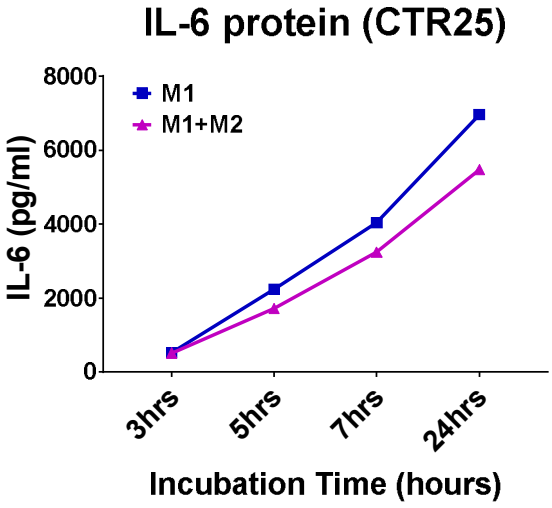
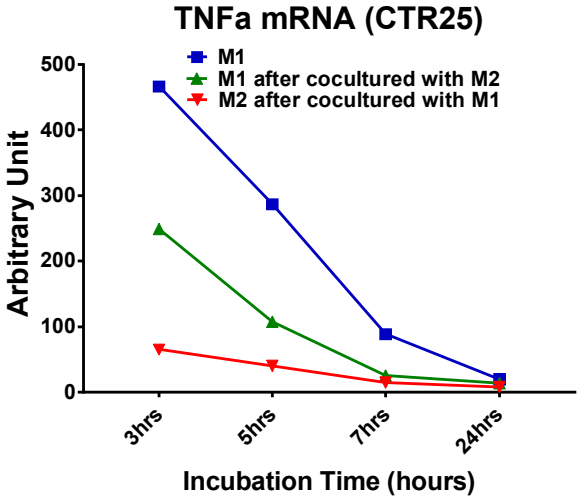
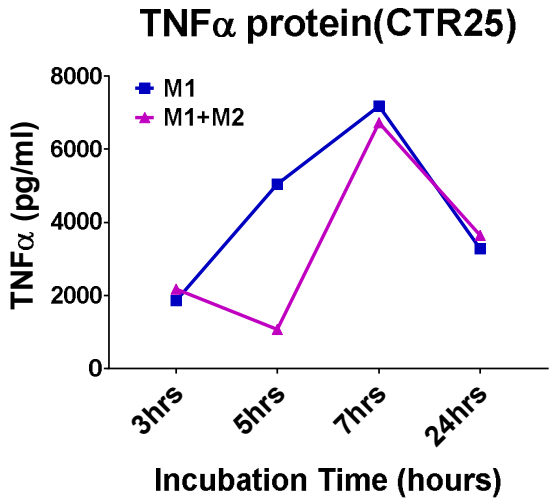
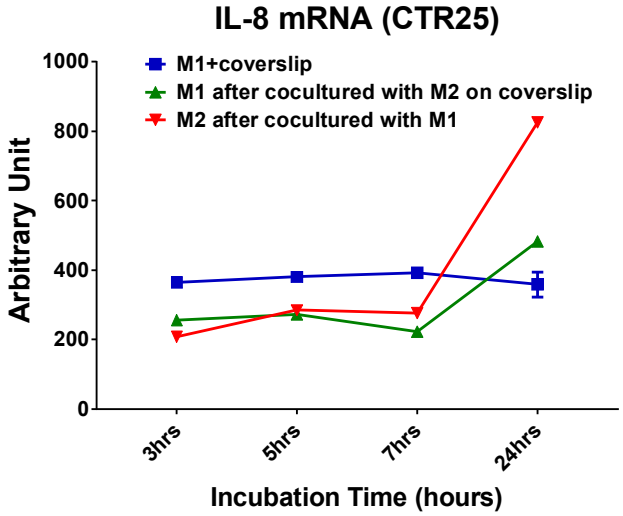
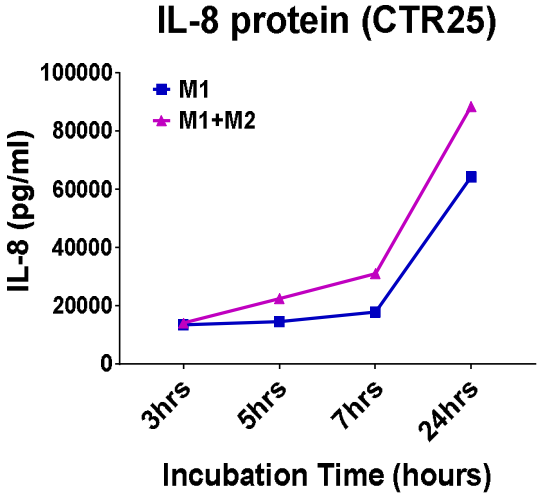




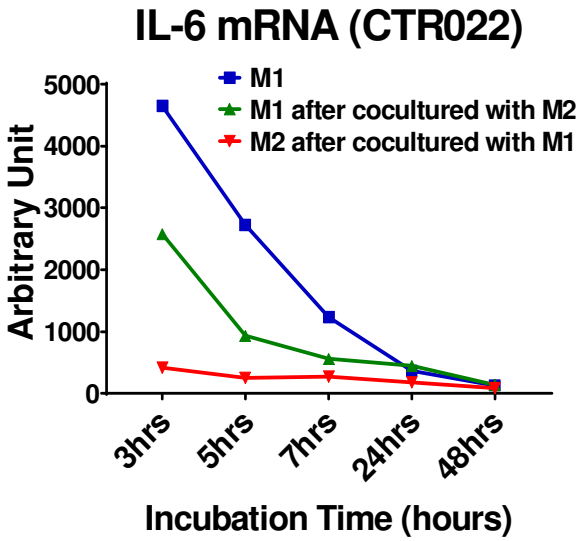


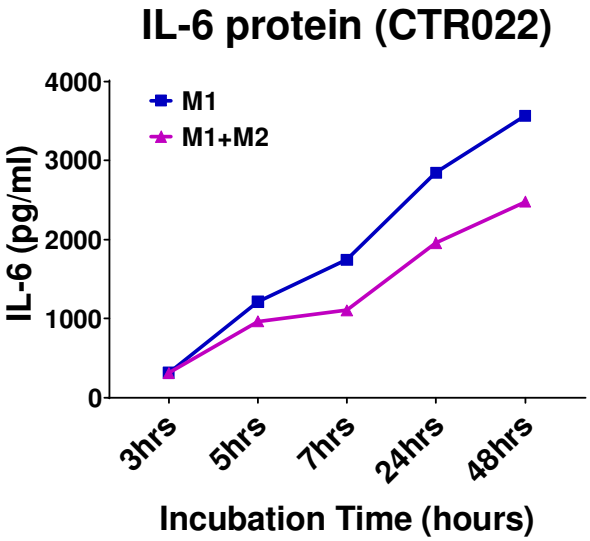


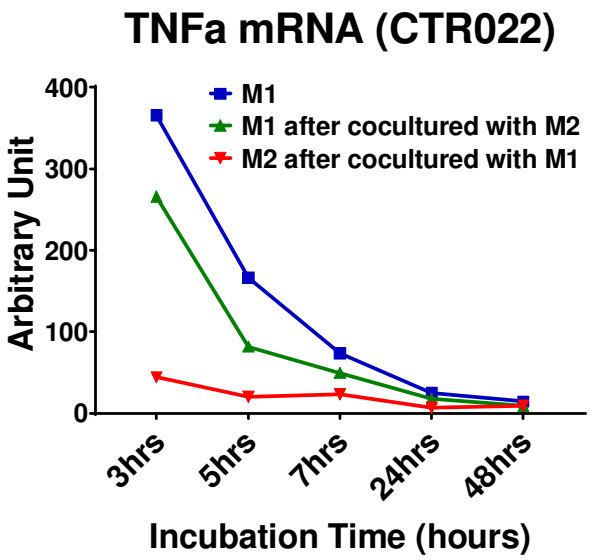


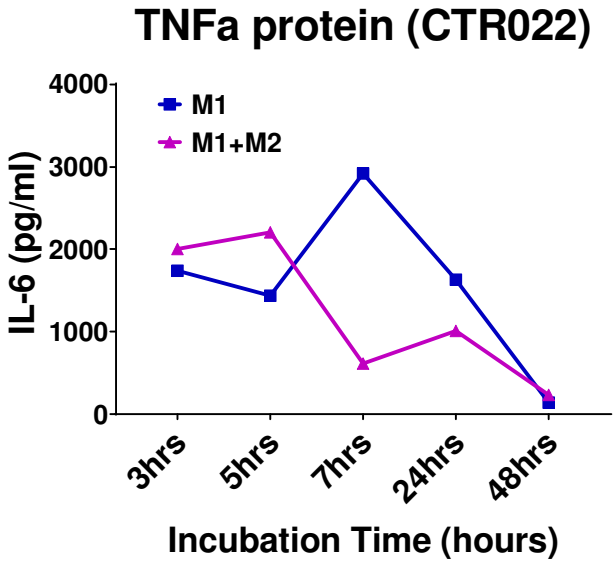


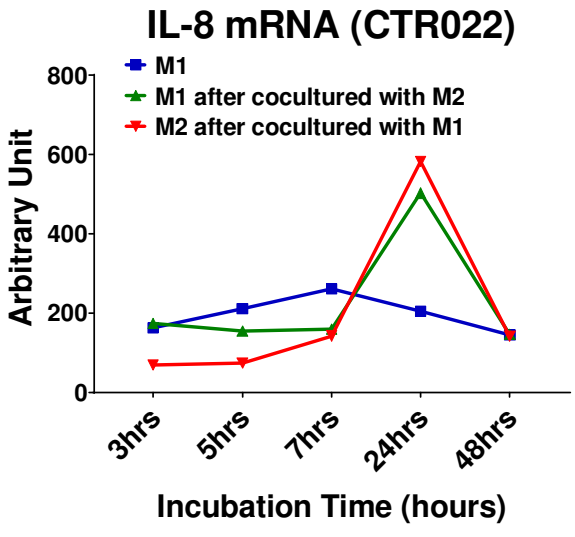
Supplemental Figure 3

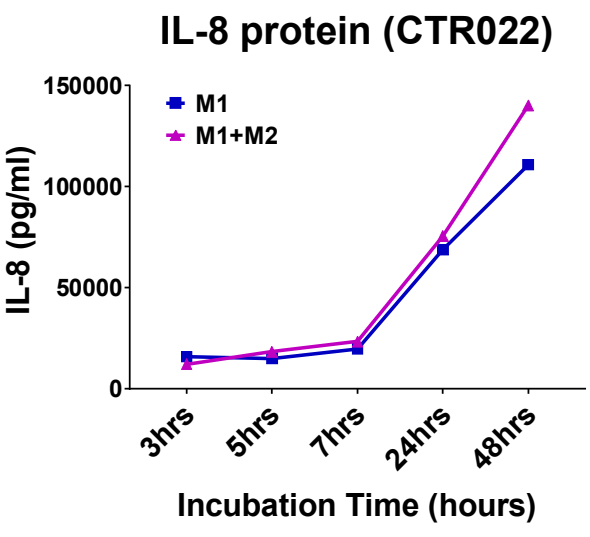












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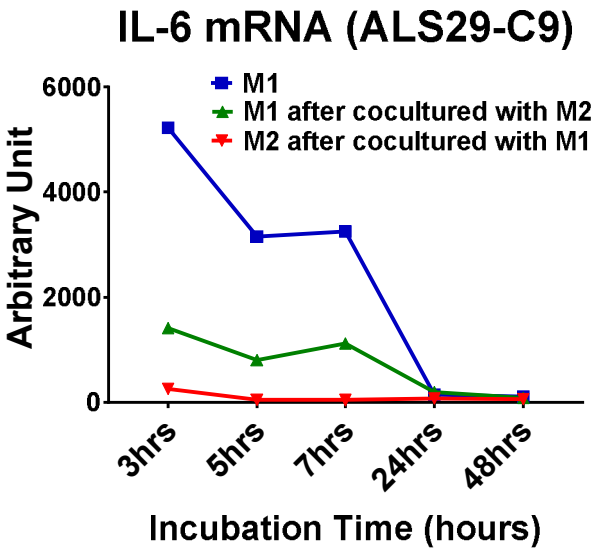
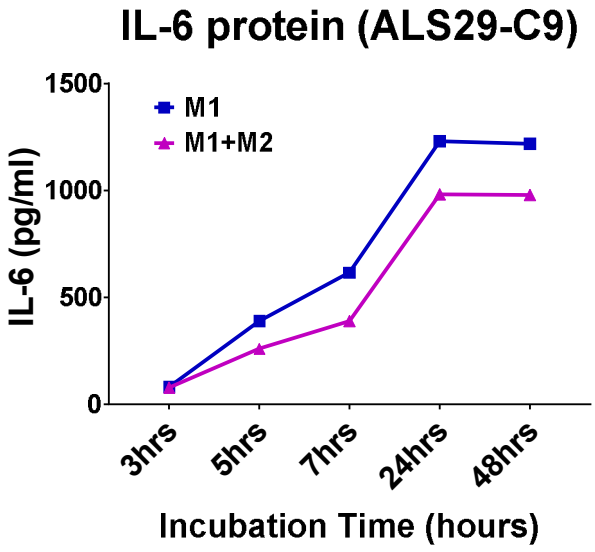
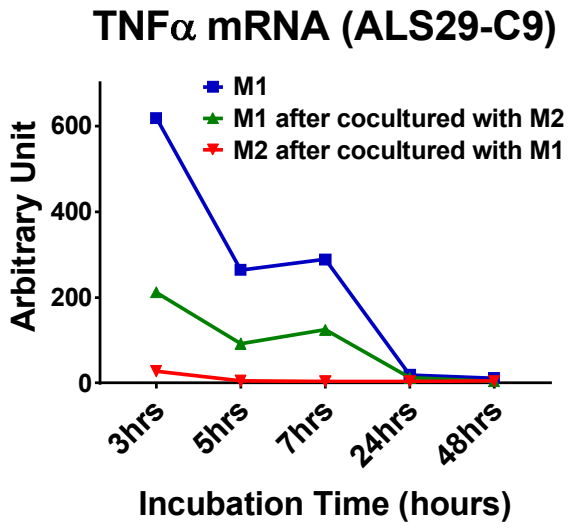
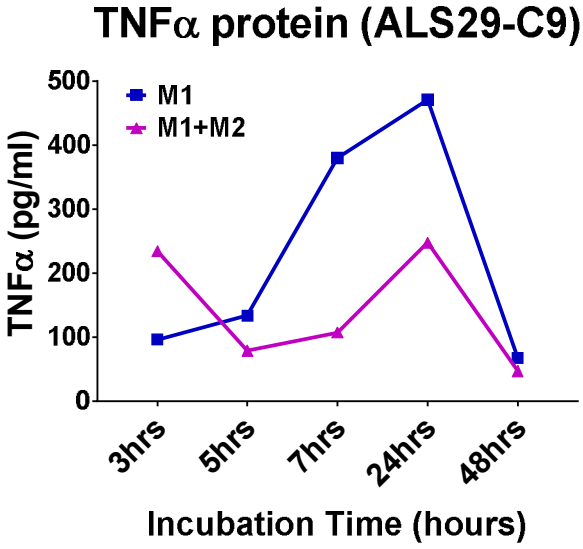
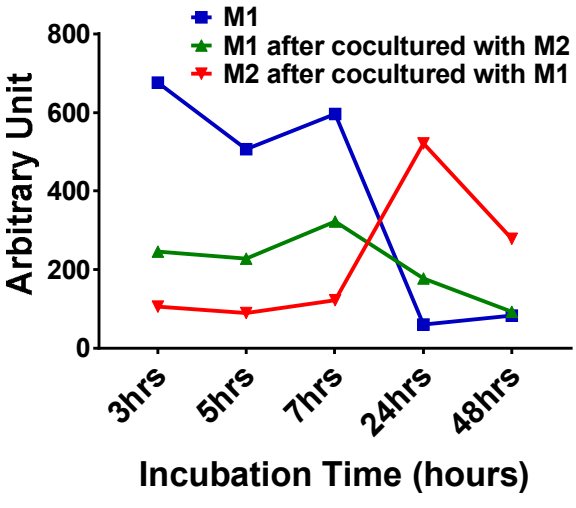
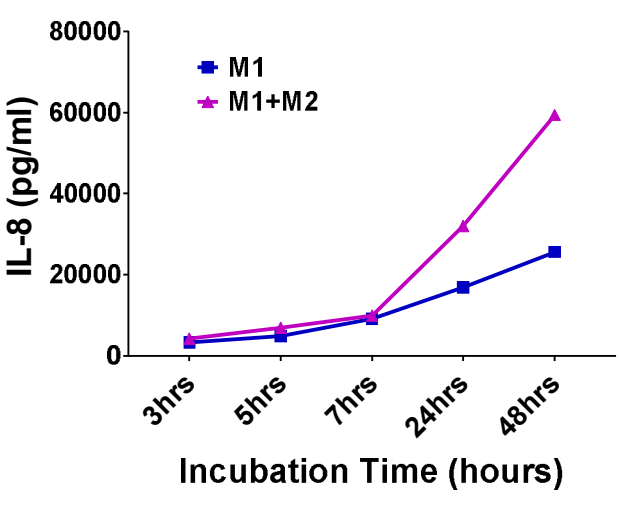




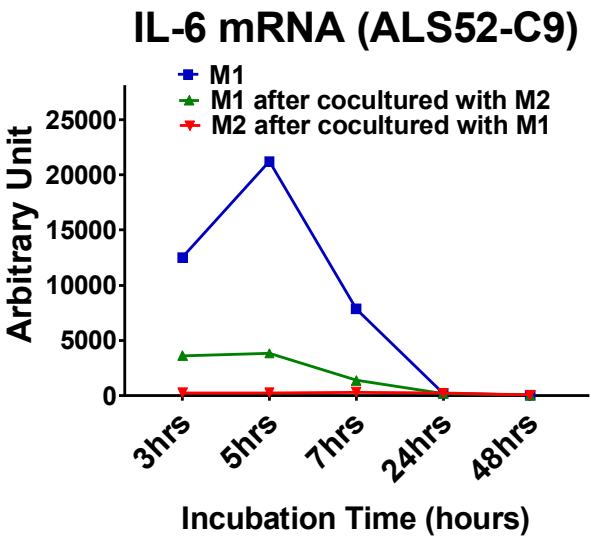


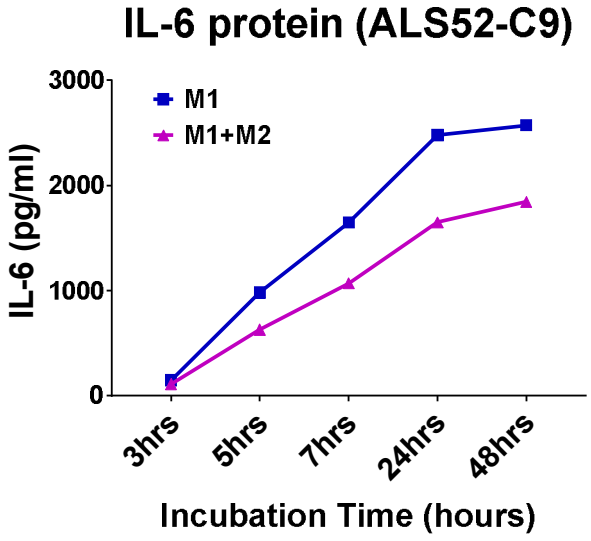


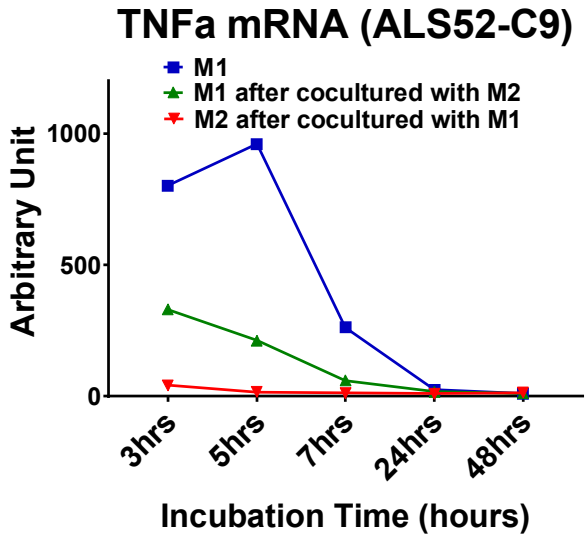


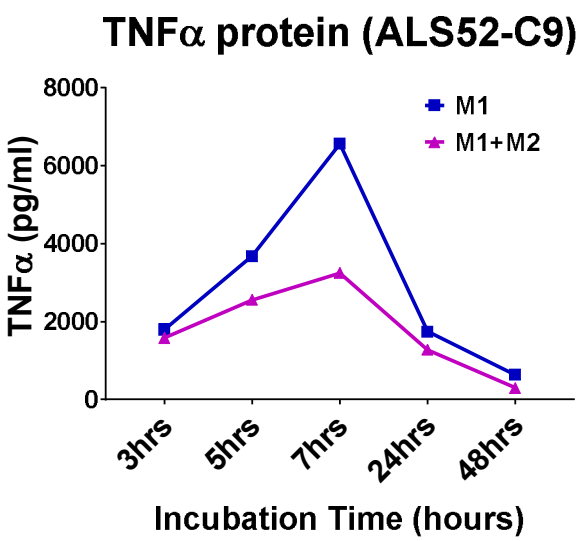


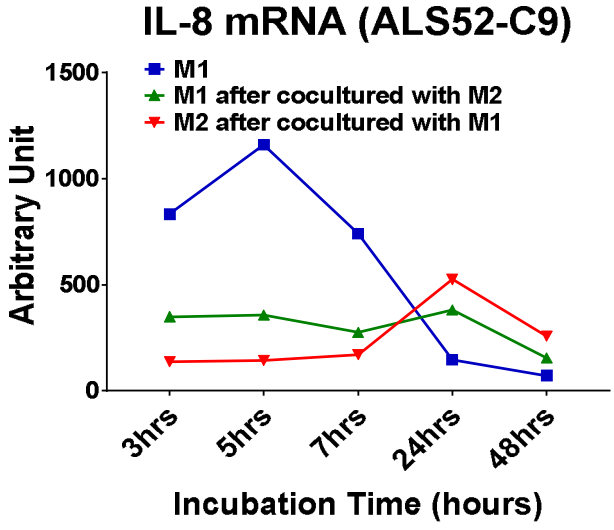
Supplemental Figure 5

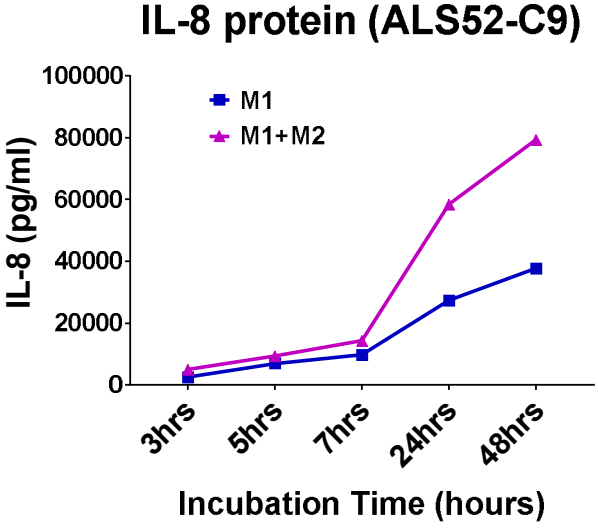












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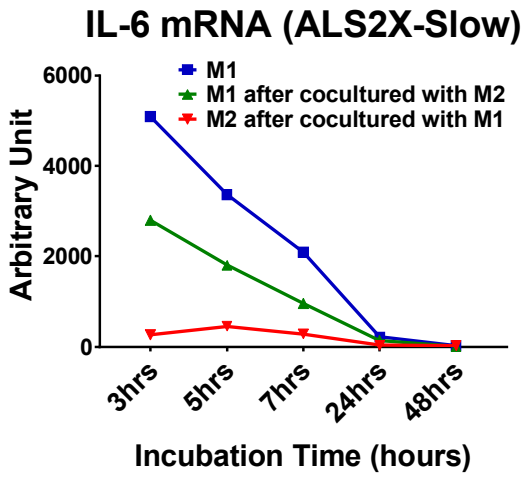
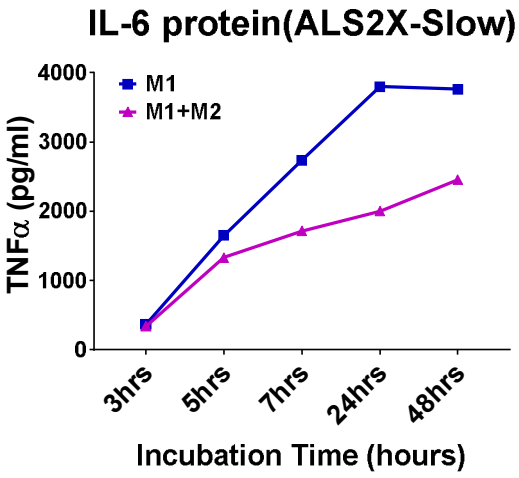
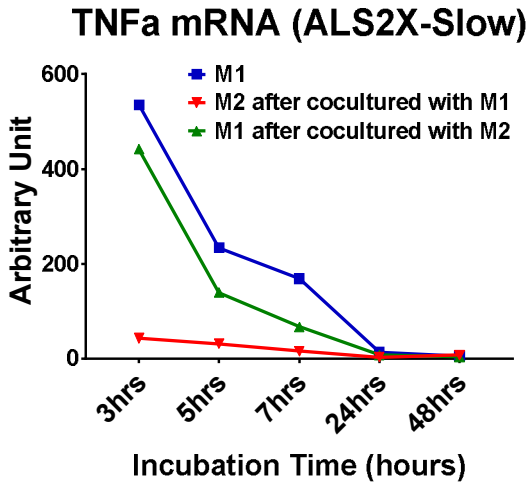
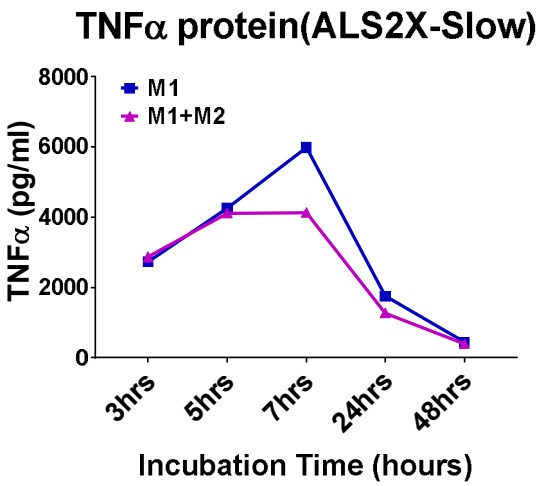
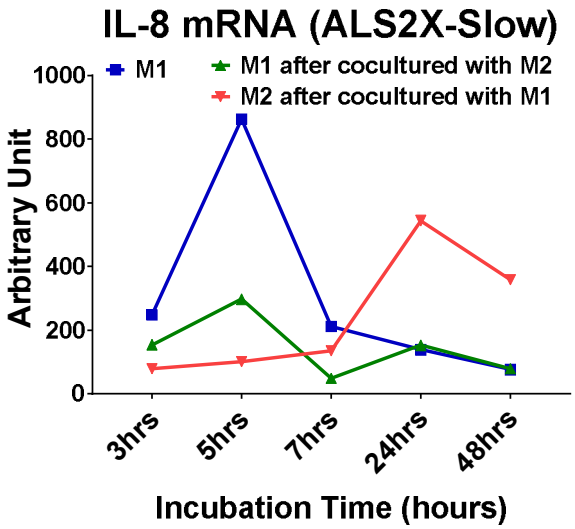
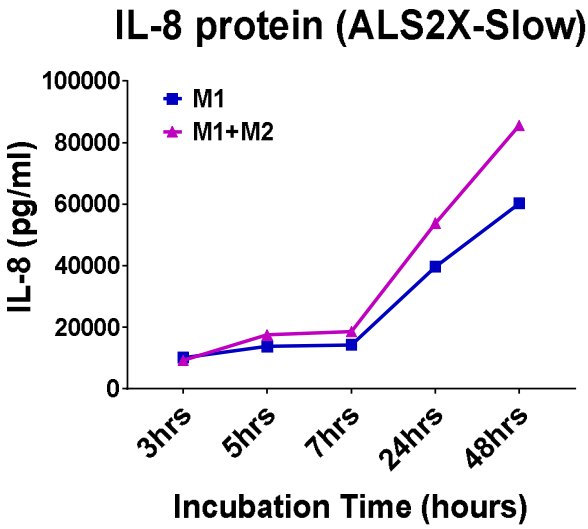




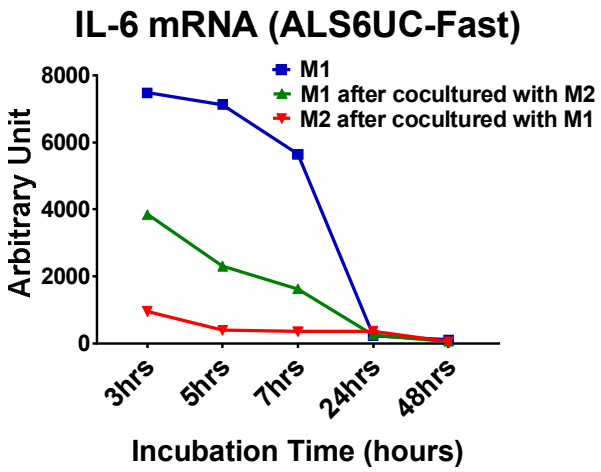


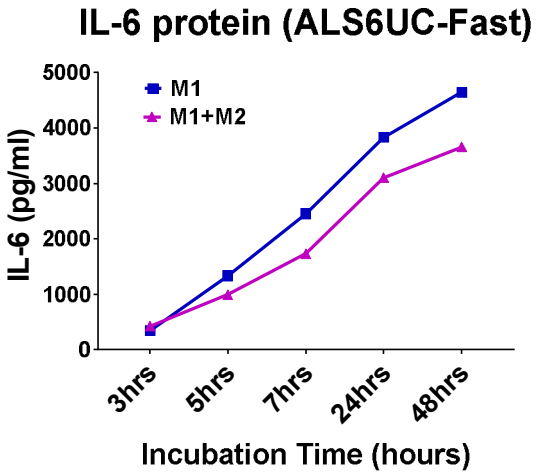


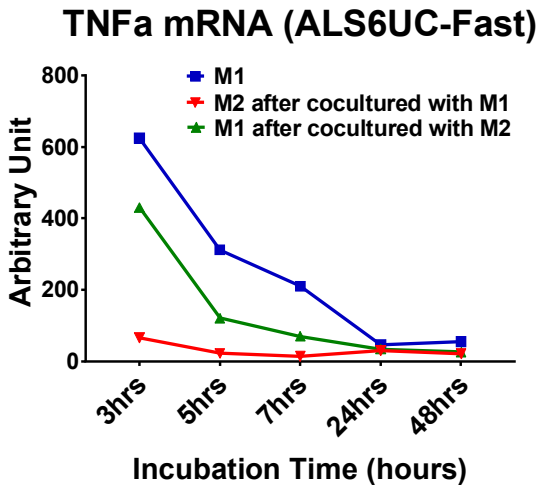


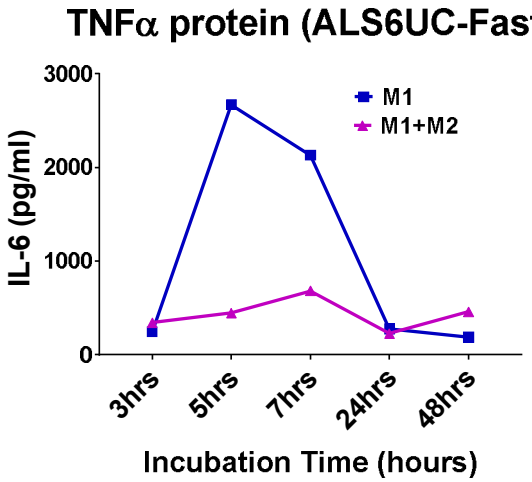


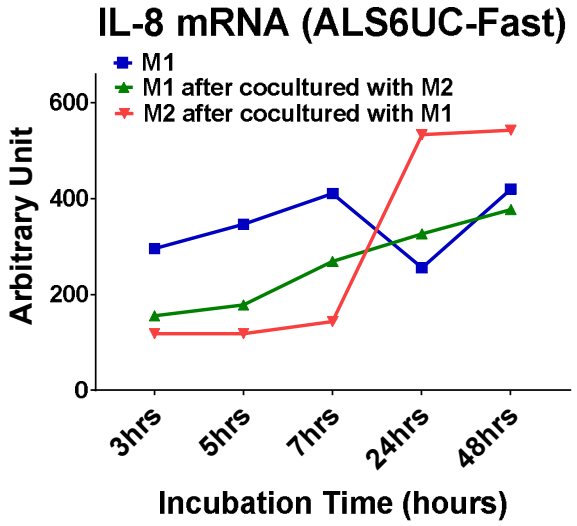
Supplemental Figure 7

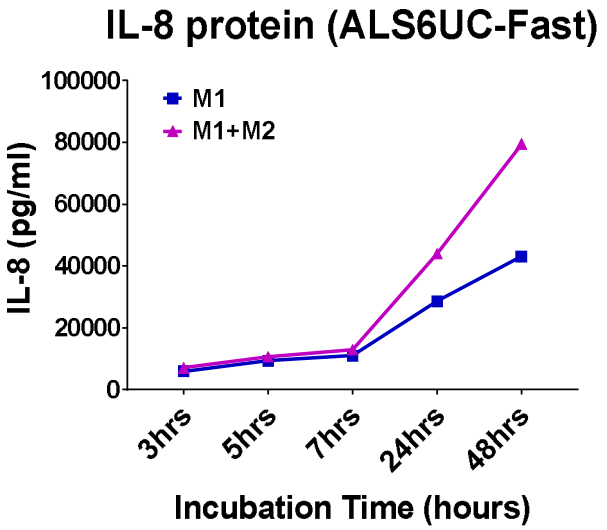












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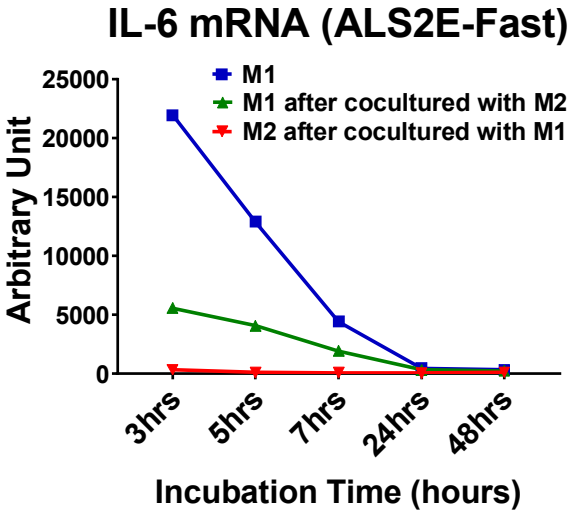
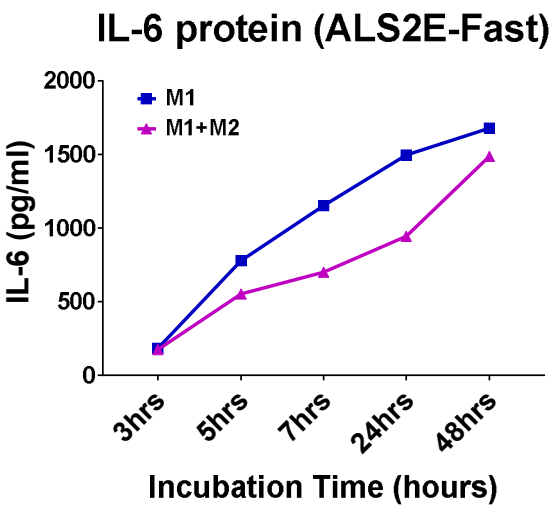
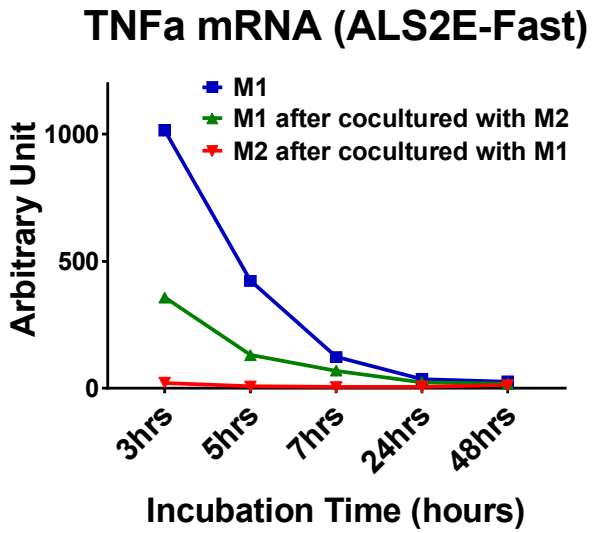
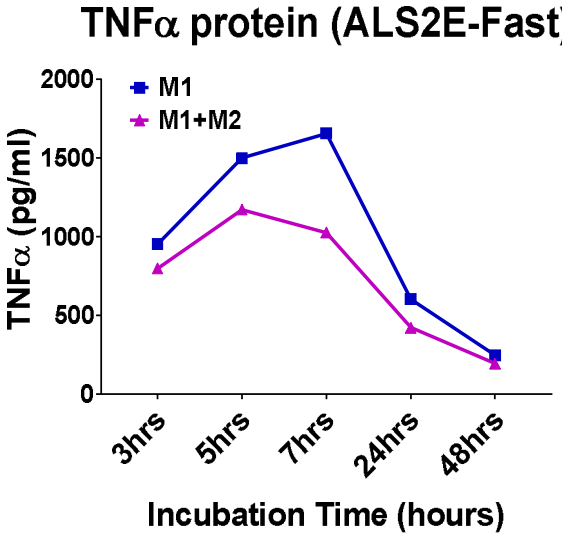
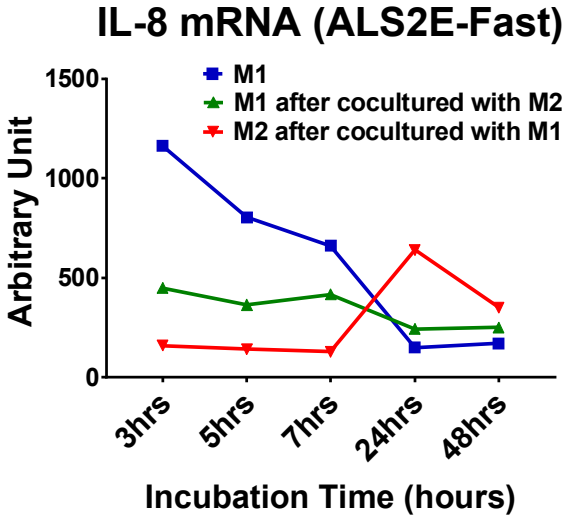
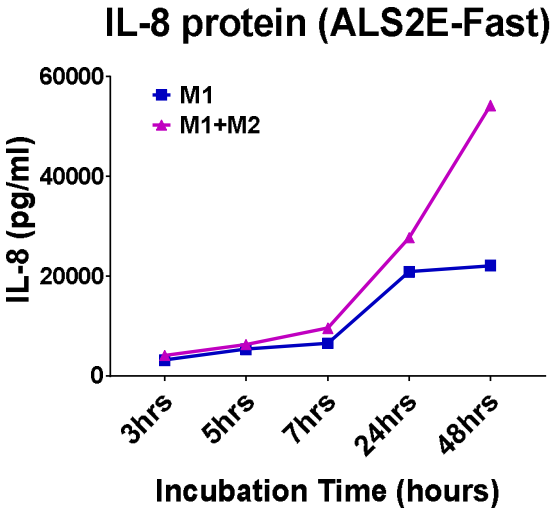








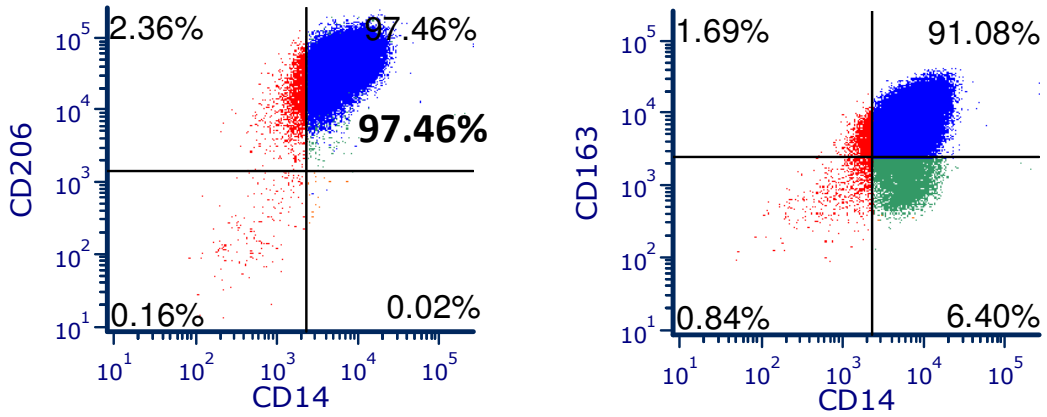




Supplemental Figure 9

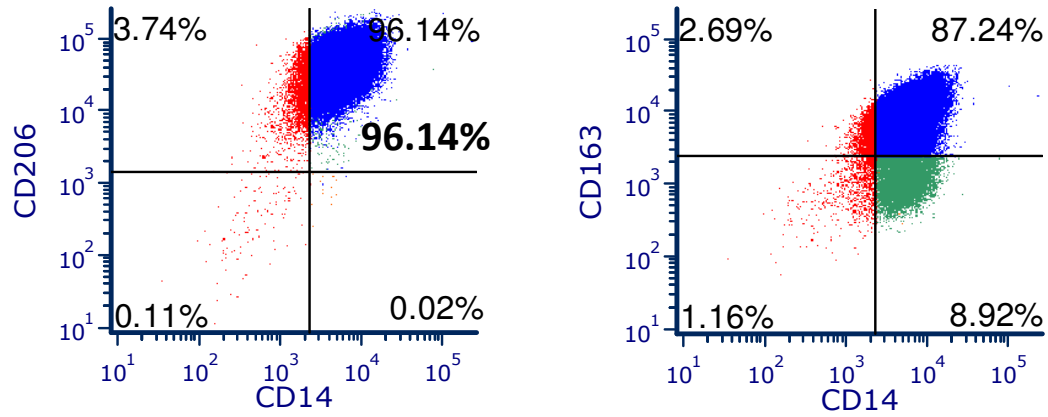
A

M2 derived from ALS 52 C9 iPSC

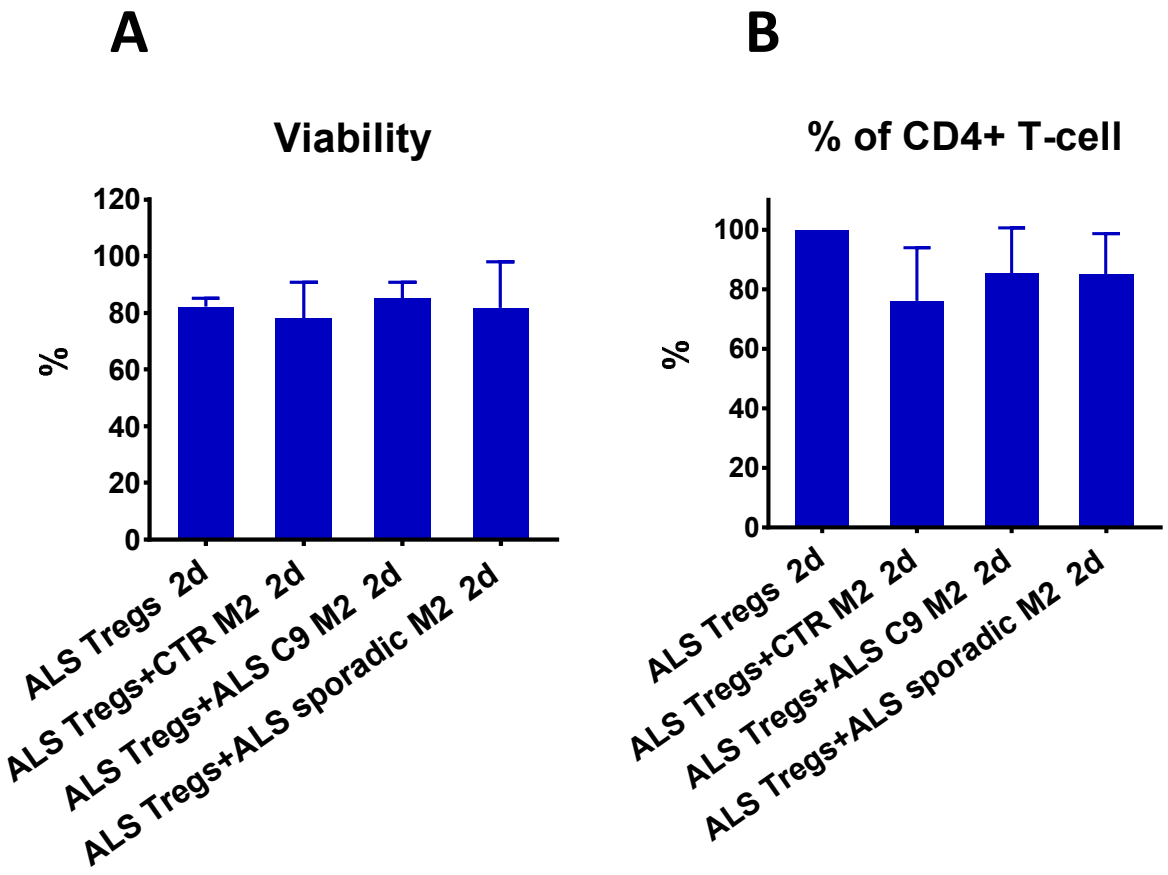


B

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.