SUPPLEMENTARY INFORMATION

CRISPRi screens in human iPSC-derived astrocytes elucidate regulators of distinct inflammatory reactive states

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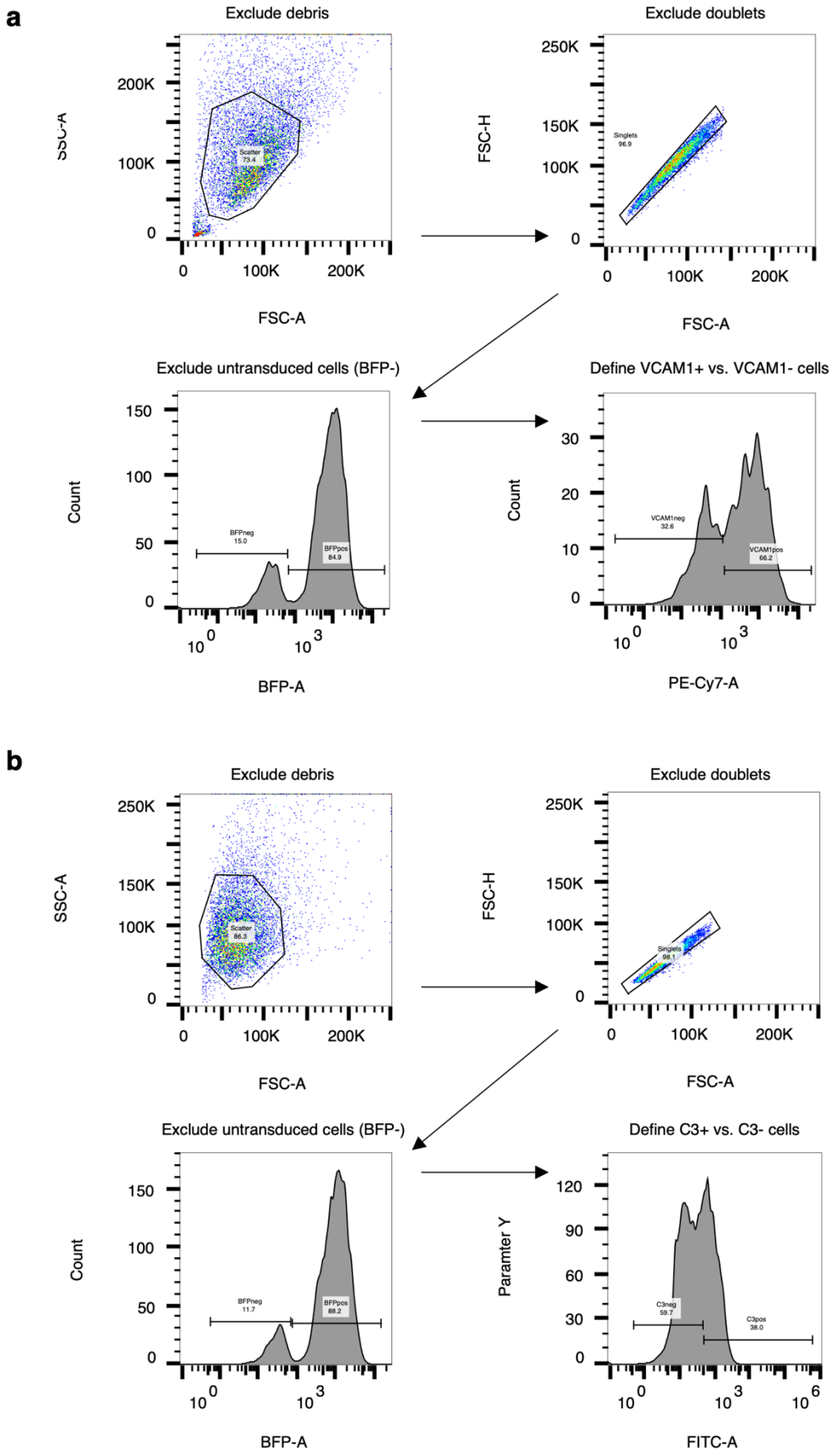
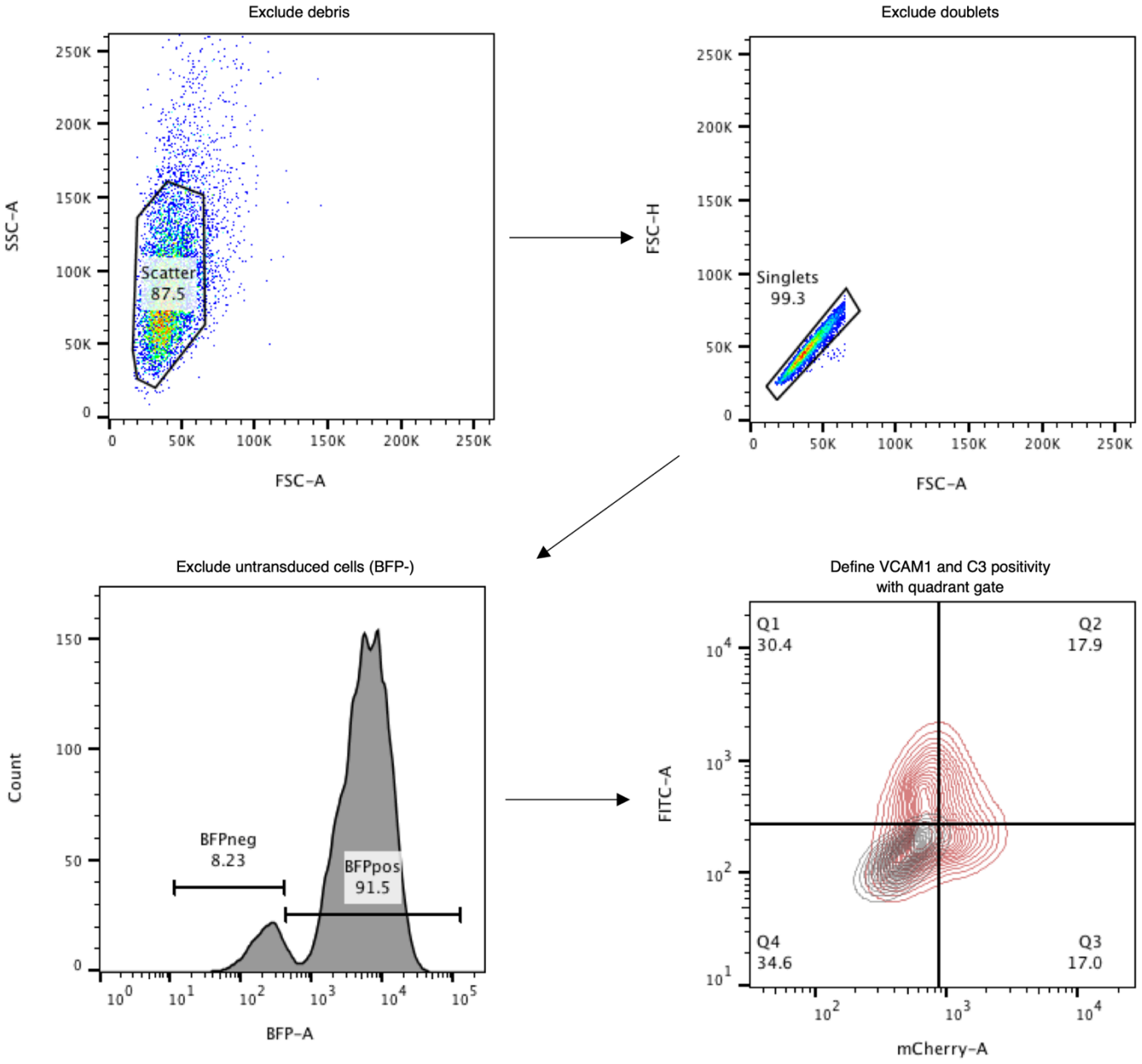
SUPPLEMENTARY FIGURES



Supplementary Fig. 1 | Flow cytometry gating strategy for single marker staining of VCAM1 or C3. a, Direct cell-surface VCAM1 staining using PE-Cy7-conjugated mouse anti-VCAM1 antibody (BioLegend) on live sgRNA-transduced iAstrocytes (BFP+) treated with IL-1α+TNF+C1q. **b**, Indirect intracellular C3 staining

using mouse anti-C3 primary (BioLegend) followed by AF488-conjugated goat anti mouse IgG secondary on fixed and permeabilized sgRNA-transduced iAstrocytes (BFP+) treated with IL-1α+TNF+C1q.



Supplementary Fig. 2 | Flow cytometry gating strategy for dual staining of VCAM1 and C3. Indirect staining of VCAM1 (Abcam rabbit anti-VCAM1) and C3 (BioLegend mouse anti-C3) with AF488-conjugated goat anti mouse IgG and AF568-conjugated goat anti rabbit IgG secondaries on fixed and permeabilized sgRNA-transduced iAstrocytes (BFP+) treated with IL-1α+TNF+C1q.

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RESULTS

Scalable generation of hiPSC-derived astrocytes (iAstrocytes)

To confirm that iAstrocytes were capable of performing typical astrocyte functions such as recycling glutamate[1] and phagocytosing synapses[2], we measured the ability of iAstrocytes to uptake glutamate or phagocytose pHrodo-labeled synaptosomes. We found that iAstrocytes had higher glutamate uptake activity (Fig. 1c) and synaptosome phagocytic activity (Fig. 1d) compared to TCW astrocytes. Furthermore, to see if iAstrocytes were capable of performing other canonical astrocyte functions such as maintaining the blood-brain barrier[3] or promoting neuronal synapse maturation[4], we co-cultured iAstrocytes with hiPSC-derived brain endothelial-like cells or neurons (see Methods). We found that brain endothelial-like cells co-cultured with iAstrocytes displayed improved barrier formation and integrity compared to mono-culture (Fig. 1e), demonstrating that iAstrocytes can promote the expected functional maturation of these cells. In addition, hiPSC-derived neurons (iNeurons) co-cultured with iAstrocytes developed synchronized calcium oscillations[5, 6] compared to iNeurons in mono-culture (Fig. 1f-h), demonstrating the ability of iAstrocytes to promote neuronal synapse maturation; the above is also consistent with the high expression of genes encoding synapse-promoting proteins such as GPC4 [7] and CADM1 [8] in iAstrocytes (Supplementary Table 1). Furthermore, we found that iAstrocytes co-cultured with iNeurons acquired more stellate morphology compared to iAstrocytes in mono-culture (Extended Data Fig. 1d).

Having validated that iAstrocytes performed typical astrocyte functions, we next tested whether iAstrocytes could be used to model inflammatory astrocyte reactivity. It has been shown that the cytokines IL-1α+TNF+C1q induce a form of inflammatory reactivity associated with neurotoxicity and loss of homeostatic functions in both primary mouse astrocytes[9] and human cerebral organoid-derived astrocytes[10]. We found that treatment of iAstrocytes generated from multiple hiPSC lines with IL-1α+TNF+C1q recapitulated previously reported *in vitro* phenotypes such as decreased phagocytosis of CNS substrates, decreased support of neuronal synapse maturation, and neurotoxicity[9, 10]. Specifically, iAstrocytes displayed decreased phagocytosis of pHrodo-labeled synaptosomes after treatment with IL-1α+TNF+C1q (Fig. 1i-j, Extended Data Fig. 2c). In addition, IL-1α+TNF+C1q treatment of iAstrocytes co-cultured with iNeurons abolished the development of synchronized neuronal calcium oscillations (Fig. 1f-h), demonstrating decreased support of neuronal synapse maturation by iAstrocytes. Finally, we found that the conditioned media from IL-1α+TNF+C1q-treated iAstrocytes was toxic to iNeurons, whereas the conditioned media from vehicle control-treated iAstrocytes was modestly protective compared to unconditioned media (Fig. 1k, Extended Data Fig. 2e).

Regarding the transcriptomic profile of hiPSC-derived astrocytes treated with IL-1α+TNF+C1q, we noted that the differentially expressed genes (DEGs) induced by IL-1α+TNF+C1q in hiPSC-derived astrocytes were not restricted to the previously defined "pan-reactive" and "A1" reactive categories (Extended Data Fig. 3c), which were derived from transcriptomic characterization of astrocytes from LPS-treated mice[9, 11]. This finding mirrored similar observations from Barbar *et al.*[10]. The discrepancy in behavior between hiPSC-derived astrocytes and mouse astrocytes could be due to differences in the experimental conditions or phylogenetic differences between human and mouse astrocytes[12].

CRISPRi screening and computational master regulator analysis

For pooled CRISPRi screening, we used the same experimental timeline as described in Fig. 2b. We conducted screens based on two cell-autonomous phenotypes induced by IL-1α+TNF+C1q that can be analyzed by fluorescence activated cell sorting (FACS): decreased phagocytosis of pHrodo-labeled synaptosomes (Fig. 1i-j)

and upregulation of cell-surface of VCAM1 (Fig. 1m-n). For synaptosome phagocytosis, we performed the screens in both vehicle control-treated and IL-1α+TNF+C1q-treated iAstrocytes to find genes whose knockdown could rescue or exacerbate the decrease in synaptosome phagocytosis induced by IL-1α+TNF+C1q (Fig. 3a). For upregulation of cell-surface VCAM1, we performed the screens in only IL-1α+TNF+C1q-treated iAstrocytes (Fig. 3a) given that in the absence of cytokine treatment, very few iAstrocytes expressed cell-surface VCAM1 (Fig. 1m). To ensure that the results from the screens are generalizable, we validated that IL-1α+TNF+C1q also caused decreased synaptosome phagocytosis and upregulation of cell-surface VCAM1 in hiPSC-derived astrocytes generated using alternative protocols (Extended Data Fig. 3f-h), as well as confirming the upregulation of cell-surface VCAM1 in primary mouse astrocytes (Extended Data Fig. h-i).

For MRA, the workflow consisted of first reconstructing a gene regulatory network between upstream regulators (e.g. transcription factors, or kinases and phosphatases) and target genes in human astrocytes based on coexpression, which required integrating a large number of previously published human astrocyte expression profiles collected under diverse experimental conditions[13] (see Methods); upstream regulators that control inflammatory reactivity were then predicted by examining the overlap between the regulons of upstream regulators with the DEGs induced by IL-1α+TNF+C1q in iAstrocytes (Fig. 3b).

Regarding genes related the canonical NF-κB pathway in the CRISPRi screens, the directionality of their screening phenotypes or MRA activity scores was consistent with activation of the canonical NF-kB pathway being required for the induction of inflammatory reactivity. For example, knockdown of *RELA*, which encodes the p65 subunit of the NF-κB transcription factor, blocked the induction of cell-surface VCAM1 (Fig. 3e) and rescued the decrease in phagocytosis (Fig. 3d) induced by IL-1α+TNF+C1q (also see Extended Data Fig. 4b). In addition, the activity score of *RELA* from MRA was positive, consistent with the positive average log-fold change of DEGs under the control of *RELA* (Supplementary Tables 2-3). For the case of *NFKB2*, knockdown of which actually increased VCAM1 induction by IL-1α+TNF+C1q (Fig. 3e), it is possible that the encoded protein, p100, may act to inhibit *RELA*-dependent transcription through cytoplasmic sequestration of p65 [14].

Regarding the role of *STAT3*, *CEBPB*, and *CEBPD* in the CNS, these transcription factors control similar responses in reactive astrocytes as in systemic acute phase inflammation, e.g. the production of acute phase proteins C3 and α1-antitrypsin (*SERPINA1*) by astrocytes challenged with IL-1 and TNF[15] (also see Fig. 1l and Supplementary Table 1). Furthermore, *STAT3* is required for the full induction of astrocyte reactivity caused by spinal cord injury[16-18], and *CEBPB* has been implicated in the pathogenesis of Alzheimer's disease[19, 20].

IRAS1 and IRAS2 are induced by autocrine-paracrine IL-6 and interferons

To better understand the behavior of VCAM1+/C3+ iAstrocytes, we treated iAstrocytes with all possible combinations of IL-1α, TNF, and C1q, with or without concurrent addition of exogenous IFN-β or IL-6 (in the form of an IL-6/IL-6R chimera; see Methods). We found that additional IL-6 boosted the induction of VCAM1–/C3+ iAstrocytes while inhibiting the induction of VCAM1+/C3– and VCAM1+/C3+ iAstrocytes, whereas additional IFN-β inhibited the induction of VCAM1–/C3+ iAstrocytes while boosting the induction of VCAM1+/C3– and VCAM1+/C3+ iAstrocytes (Fig. 6b). Given that IL-6 and IFN-β had similar effects on VCAM1+/C3– and VCAM1+/C3+ iAstrocytes, we mapped VCAM1+/C3+ iAstrocytes to IRAS2. This is also consistent with the fact that in our single-cell data, a subset of IRAS2 iAstrocytes had high transcript levels of both *VCAM1* and *C3* (Fig. 4b).

Upon examining the activity of IL-1α, TNF, and C1q alone or in combination, we found that IL-1α by itself induced VCAM1–/C3+ iAstrocytes, whereas TNF by itself induced VCAM1+/C3– and VCAM1+/C3+ iAstrocytes (Fig. 6b). Interestingly, IL-1α+TNF decreased the induction of VCAM1+/C3– astrocytes compared to TNF by itself (Fig. 6b). To validate these findings, we performed immunostaining against C3 and IFIT3, an alternative IRAS2 marker (Extended Data Fig. 7a-b), in iAstrocytes derived from three different hiPSC lines

and observed similar results (Extended Data Fig. 7c). In terms of cytokine production (Extended Data Fig. 7d), IL-1α by itself, but not TNF by itself induced IL-6 and GM-CSF, whereas TNF by itself induced higher levels of CXCL10 compared to IL-1α by itself. Also, IL-1α+TNF decreased CXCL10 production compared to TNF by itself. These findings were similar between iAstrocytes derived from two different hiPSC lines (Extended Data Fig. 7d).

Taking stock of both VCAM1/C3 induction and cytokine production, the inhibitory effect of IL-1α on the induction of CXCL10 and VCAM1+/C3– iAstrocytes by TNF likely reflects the antagonistic effect of IL-1 on the activity of autocrine-paracrine interferon signaling[21], which can be induced by both IL-1α and TNF (Extended Data Fig. 7d). As for the activity of C1q, C1q by itself did not induce any cytokine production or any inflammatory reactive astrocytes marked by VCAM1/C3; C1q also had little additional effect in combination with IL-1α, TNF, or IL-1α+TNF (Fig. 6b; Extended Data Fig. 7c-d). However, C1q may exert effects not detectable by the assays used above, e.g. at the transcriptional level.

Regarding small molecules known to modulate STAT3 or STAT1/2 activity, we found that napabucasin, which is known to inhibit STAT3-dependent transcription[22], reduced STAT3 Y705 phosphorylation in a dose-dependent manner (Extended Data Fig. 9b) and at doses above 1.25 μM abrogated the induction of VCAM1–/C3+, VCAM1+/C3–, and VCAM1+/C3+ in IL-1α+TNF+C1q-treated iAstrocytes . The different effect of napabucasin vs. *STAT3* knockdown is not surprising given that the mechanism of action of napabucasin is not well understood and likely involves multiple cellular pathways[23]. For modulation of STAT1/2 activity, we found that RGFP966, a HDAC3 inhibitor known to reduce STAT1 activity[24], boosted the induction of VCAM1–/C3+ iAstrocytes while inhibiting the induction of VCAM1+/C3– and VCAM1+/C3+ iAstrocytes (Extended Data Fig. 9d). The different effect of RGFP966 vs. *STAT1/2* knockdown could be attributed to effects of HDAC3 inhibition beyond reducing STAT1 activity.

Reactive astrocyte signatures overlap with those from previous datasets

In Barbar *et al.*[10], mixed cultures of neurons and glia derived from dissociation of human cerebral organoids were treated with vehicle control or IL-1α+TNF+C1q. After isolating astrocytes from the Barbar *et al.* dataset and integrating them with NTC iAstrocytes from our study (see Methods), we found that a subset of IL-1α+TNF+C1q-treated astrocytes from Barbar *et al.* co-clustered with IRAS1 (IL-1/IL-6 responsive) and IRAS2 (TNF/IFN-responsive) iAstrocytes (Fig. 7a-c). While Barbar *et al.* astrocytes co-clustering with IRAS1 iAstrocytes expressed IL-1/IL-6-responsive markers such as *IL6*, *CXCL2*, and *CXCL5*, those co-clustering with IRAS2 iAstrocytes expressed TNF/IFN-responsive markers such as *CXCL10*, *IFIT3*, and *ISG15* (Fig. 7d; Extended Data Fig. 10e; Supplementary Table 8). Furthermore, the DEGs between IRAS2 vs. IRAS1 iAstrocytes overlapped strongly with the DEGs between the corresponding co-clustering Barbar *et al.* astrocytes (Extended Data Fig. 10a).

In Wheeler *et al.*[25], astrocytes were isolated by FACS from mice induced with experimental autoimmune encephalomyelitis (EAE), a widely used model of multiple sclerosis (MS), at different stages of EAE progression (naïve, acute, and remitting). After removing low-quality astrocytes from the Wheeler *et al.* dataset and integrating the remaining astrocytes with NTC iAstrocytes from our study (see Methods), we found that the vast majority of Wheeler *et al.* astrocytes from the acute stage of EAE co-clustered with IRAS1 and IRAS2 iAstrocytes (Fig. 7e-g) and also correspondingly expressed IL-1/IL-6-responsive markers such as *Srgn*, *Cxcl2*, and *Il1b* or TNF/IFN-responsive markers such as *Cxcl10*, *Ifit3*, and *Isg15*, respectively (Fig. 7h; Extended Data Fig. 10f; Supplementary Table 8). Overlap analysis of the DEGs between IRAS2 vs. IRAS1 iAstrocytes and the DEGs between the corresponding co-clustering astrocytes from Wheeler *et al.* also showed good agreement (Extended Data Fig. 10b), although the degree of overlap (Jaccard index) was lower compared to the integration with Barbar *et al* likely due to the difference in species.

Lastly, in Hasel *et al.*[26], astrocytes were isolated by FACS from mice injected peripherally with saline or LPS, which induces acute neuroinflammation similar to that caused by sepsis. After removing non-astrocyte cells from the Hasel *et al.* dataset, integration of the Hasel *et al.* astrocytes with NTC iAstrocytes from our study showed that a subset of Hasel *et al.* astrocytes from LPS-injected mice co-clustered with IRAS1and IRAS2 iAstrocytes (Fig. 7i-k) and also correspondingly expressed IL-1/IL-6 responsive markers such as *C3*, *Cxcl5*, and *Tnfaip2* or TNF/IFN-responsive markers such as *Cxcl10*, *Ifit3*, and *Isg15* (Fig. 7l; Extended Data Fig. 10g; Supplementary Table 8). Overlap analysis of the DEGs between IRAS2 vs. IRAS1 iAstrocytes and the DEGs between the corresponding co-clustering astrocytes from Hasel *et al.* further supported this correspondence (Extended Data Fig. 10c).

In addition to single-cell RNA-seq datasets, we also reanalyzed bulk RNA-seq data from Anderson *et al.* [17], where astrocyte-specific RNA was purified from wild-type or astrocyte-specific *Stat3* conditional knockout (cKO) mice subject to spinal cord injury (SCI). We found that Stat3-dependent genes (i.e. genes with lower expression in *Stat3* cKO SCI vs. WT SCI; e.g. *C3*) tended to be highly expressed by IRAS1 (IL-1/IL-6-responsive) iAstrocytes (Extended Data Fig. 10h), whereas Stat3-repressed genes (i.e. genes with higher expression in *Stat3* cKO SCI vs. WT SCI; e.g. *Iigp1*) were enriched for genes involved in the response to interferons (Extended Data Fig. 10i), overlapping with IRAS2 (TNF/IFN-responsive) markers (see Supplementary Table 9 for *Stat3* cKO-related DEGs from Anderson *et al.*). Thus, the results from Anderson *et al.* further corroborate the regulatory role of STAT3 in promoting IL-1/IL-6-responsive reactivity and inhibiting TNF/IFN-responsive reactivity that we have proposed.

DISCUSSION

In our analysis of the results from CRISPRi screens and computational master regulator analysis (MRA), we gave more weight to the results from the CRISPRi screens, which demonstrate causality, than those from MRA, which are correlational and likely to contain more false positive results. However, given that the CRISPRi screens are more likely to be limited by false negative results (e.g. due to sgRNA dropout), further exploration of the hits from MRA is warranted in future studies. We believe that the gene regulatory network we generated from MRA will be useful for identifying potential master regulators of diverse astrocyte phenotypes for which transcriptomic data is available.

Regarding the functional outputs associated with the reactive astrocyte signatures identified in our work, it is likely that IL-1/IL-6-responsive and TNF/IFN-responsive reactive astrocytes have distinct functional outputs. Several studies already point towards potential functional outputs of IL-1/IL-6-responsive reactivity, which is dependent upon STAT3. For example, in Anderson *et al.* [17], astrocyte-specific deletion of *Stat3* was shown to prevent proper axon regeneration after spinal cord injury, suggesting that IL-1/IL-6-responsive reactivity may promote axon regeneration. In addition, in Kim *et al.*[27], inhibition of STAT3 in hiPSC-derived astrocytes co-cultured with hiPSC-derived brain endothelial-like cells rescued the decrease in endothelial barrier integrity caused by treatment with TNF, suggesting that IL-1/IL-6-responsive reactivity may perturb blood-brain barrier and cerebrovascular function under inflammatory conditions[28].

On the other hand, interferon-responsive reactivity may play an important role in EAE and MS. For example, Rothhammer *et al.*[29] showed that inhibiting type I interferon signaling in astrocytes exacerbated the severity of EAE. Similarly, Hindinger *et al.*[30] showed that inhibiting type II interferon in in astrocytes exacerbated the severity of clinical symptoms during peak disease. Furthermore, given that interferon-responsive reactive astrocytes upregulate VCAM1 and were shown to be adjacent to vasculature in Hasel *et al.*[26], they may be important for controlling the trafficking of peripheral immune cells into the CNS parenchyma.

With regard to AD, literature on mouse models of AD supports a role for both IL-1/IL-6-responsive reactivity and TNF/IFN-responsive reactivity. Reichenbach *et al.* demonstrated that astrocyte-specific deletion of Stat3, which would be expected to reduce IL-1/IL-6-responsive reactivity, decreased the burden of amyloid plaques

and dystrophic neurites, lowered inflammatory cytokine levels, and ameliorated cognitive deficits in the APP/PS1 mouse model of AD[31]. Roy *et al.* detected interferon-responsive astrocytes that were expanded in a amyloid pathology-dependent in the 5XFAD mouse model of AD[32]. Although we did not observe an increase in the percentage of VCAM1+ astrocytes in our examination of human AD brain tissue, this does not rule out the presence of interferon-responsive reactive astrocytes in AD, since VCAM1 may not be an optimal marker of interferon-responsive reactive astrocytes in all circumstances. Interestingly, Sadick *et al.* detected a strong interferon-responsive signature in astrocytes profiled by single-nucleus RNA-seq from a donor with vascular dementia[33].

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