Pathogenic TNF-α drives peripheral nerve inflammation in an Aire-deficient model of autoimmunity

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Immune cells infiltrate the peripheral nervous system (PNS) after injury and with autoimmunity, but their net effect is divergent. After injury, immune cells are reparative, while in inflammatory neuropathies (e.g., Guillain Barré Syndrome and chronic inflammatory demyelinating polyneuropathy), immune cells are proinflammatory and promote autoimmune demyelination. An understanding of immune cell phenotypes that distinguish these conditions may, therefore, reveal new therapeutic targets for switching immune cells from an inflammatory role to a reparative state. In an autoimmune regulator (Aire)-deficient mouse model of inflammatory neuropathy, we used single-cell RNA sequencing of sciatic nerves to discover a transcriptionally heterogeneous cellular landscape, including multiple myeloid, innate lymphoid, and lymphoid cell types. Analysis of cell–cell ligand–receptor interactions uncovered a macrophage-mediated tumor necrosis factor-α (TNF-α) signaling axis that is induced by interferon-γ and required for initiation of autoimmune demyelination. Developmental trajectory visualization suggested that TNF-α signaling is associated with metabolic reprogramming of macrophages and polarization of macrophages from a reparative state in injury to a pathogenic, inflammatory state in autoimmunity. Autocrine TNF-α signaling induced macrophage expression of multiple genes (*Clec4e*, *Marcksl1*, *Cxcl1*, and *Cxcl10*) important in immune cell activation and recruitment. Genetic and antibody-based blockade of TNF-α/TNF-α signaling ameliorated clinical neuropathy, peripheral nerve infiltration, and demyelination, which provides preclinical evidence that the TNF-α axis may be effectively targeted to resolve inflammatory neuropathies.

autoimmunity | peripheral nerve | TNF-α | macrophages | CIDP

Guillain-Barré syndrome (GBS) and chronic inflammatory demyelinating polyneuropathy (CIDP) are inflammatory diseases of the peripheral nervous system (PNS) that are characterized clinically by weakness, sensory loss, areflexia, and pain (1). GBS and CIDP result from autoimmune destruction of peripheral nerve myelin involving cellular and humoral factors, but the precise mechanisms that break immune tolerance to peripheral nerve myelin are not fully understood. Current treatment is limited to therapies with nonspecific mechanisms of action, including intravenous immunoglobulin, glucocorticoids, and therapeutic plasma exchange (2, 3). In nearly one-third of CIDP patients (4), treatment is ineffective, and 25% of GBS patients still require mechanical ventilation (5). Moreover, even with current therapies, GBS continues to be associated with a 3 to 5% mortality rate (5). A fuller understanding of precise mechanisms underlying disease pathogenesis and pathology is needed to develop more efficacious treatments that target specific autoimmune mechanisms critical for disease development.

Multiple lines of evidence support an important role for autoreactive T cells in inflammatory neuropathies. CIDP has been reported in rare patients with mutations in the autoimmune regulator (Aire) (6), a gene important in enforcing thymic negative selection of autoreactive T cells (7, 8). Mirroring this association in humans, we have reported that Aire-deficient mice develop spontaneous autoimmune peripheral polyneuropathy (SAPP) that recapitulates multiple features of GBS and CIDP (9, 10). Using Aire-deficient mice, we have shown that lack of Aire function leads to escape of PNS-reactive T cells from thymic negative selection, release of PNS-reactive T cells into the periphery and, ultimately, autoimmune destruction of peripheral nerves. However, the presence of autoreactive T cells in the periphery is insufficient to cause autoimmunity because autoreactive T cell clones are also found in healthy individuals due to incomplete thymic negative selection (11–13). Moreover, only a subset of Aire-deficient patients develops PNS autoimmunity, which suggests

Significance

GBS and CIDP are autoimmune disorders of the PNS that can be debilitating and even life threatening. Current therapies, which include corticosteroids and intravenous gammaglobulin, have poorly defined mechanisms of action and are ineffective in a fraction of patients. To identify more specific therapeutic targets, we used single-cell RNA sequencing to analyze immune cells in nerves during autoimmune attack. This analysis revealed a previously unappreciated TNFα cell–cell communication pathway that recruits and activates multiple immune cell types. Moreover, we show that TNF-α signaling is an essential feature of PNS autoimmunity, since ablating TNF-α signaling protects against disease. These findings suggest that anti–TNF-α agents, which are already used to treat other inflammatory diseases, should be considered for inflammatory neuropathies.

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that additional pathogenic events are needed that activate and recruit T cells into peripheral nerves.

Complex interactions occur between autoreactive T cells and other immune cell types within target tissues. Using histological approaches, flow cytometry, and electron microscopy, we have previously reported that $CD4^+$ T cells, $CD8^+$ T cells, and macrophages are present in the infiltrated sciatic nerves of Aire-deficient mice (10, 14). These studies, however, are limited by the use of known markers and phenotypic features to identify immune cell types. Moreover, little is known about the communication between immune cell types within peripheral nerves undergoing autoimmune attack. Better definition of the cellular landscape and cell–cell communication pathways in peripheral nerves targeted by autoimmunity may therefore be key to understanding the basis of PNS autoimmunity. A recent study employed single-cell RNA sequencing (scRNA-seq) to provide a broad characterization of mouse peripheral nerve cells during early autoimmunity (15). This study, however, focused solely on nerves prior to neuropathy onset. Thus, the full range of immune cell heterogeneity and cell–cell communication during active, symptomatic disease has not yet been addressed.

In addition to autoimmunity, immune cells also infiltrate peripheral nerves during mechanical injury. While immune cells are pathogenic in autoimmune disease, injury-associated immune cells are largely reparative and promote wound-healing (16, 17). Comparisons of immune cell composition and signaling pathways in autoimmunity vs. injury may therefore provide insight into how a proinflammatory immune response may be switched to a restorative one. In this study, we performed single-cell transcriptomics of sciatic nerve immune cells isolated from an Aire-deficient mouse model of inflammatory neuropathy with active, clinically apparent disease. These analyses revealed a prominent macrophage-mediated tumor necrosis factor-α (TNF-α) signaling network that is highly up-regulated in immune cells of sciatic nerves affected by autoimmunity. Autoimmunity-associated T cells produced interferon-γ (IFN-γ), which up-regulated TNF-α expression in macrophages. In comparison, TNF-α expression and signaling was significantly lower in peripheral nerve macrophages after injury, suggesting that minimizing TNF-α activity may be key to enforcing a reparative macrophage state. Indeed, genetic ablation of TNF-α signaling, as well antibody-based blockade of TNF-α, protected from autoimmune neuropathy development. Together, these studies show that increased TNF-α signaling is a defining feature of PNS autoimmunity and may be targeted for therapeutic benefit.

Results

Heterogeneous Immune Cells in Sciatic Nerves of Neuropathic *NOD.Aire$^{GW/+}$* Mice Up-Regulate Multiple Cytokine Pathways. On the nonobese diabetic (NOD) genetic background, mice harboring a dominant loss-of-function Aire G228W mutation (*NOD.Aire$^{GW/+}$* mice) develop SAPP characterized by clinical neuropathy, histological evidence of immune infiltration in peripheral nerves, and findings on nerve conduction studies characteristic of demyelination (10, 18). Due to the multiple shared features between SAPP and human inflammatory neuropathies, *NOD.Aire$^{GW/+}$* mice have been a useful model for understanding pathogenic mechanisms underlying inflammatory neuropathies. SAPP begins to develop in *NOD.Aire$^{GW/+}$* female mice at 15 wk of age. By 22 wk of age, ∼80% of female *NOD.Aire$^{GW/+}$* mice will display the neuropathy phenotype, which begins as bilateral weakness in the hind limbs (10, 18).

To better understand the immune composition of infiltrated sciatic nerves, we performed scRNA-seq on immune cells from sciatic nerves of neuropathic *NOD.Aire$^{GW/+}$* mice (Fig. 1*A*). Live, $CD45^+$ cells were sorted by flow cytometry from dissociated sciatic nerves and subjected to 10X Genomics pipeline library preparation and sequencing (n = 3 mice, 11,640 total cells). Using a Seurat single-cell transcriptome analysis pipeline (19), 11 heterogeneous immune cell populations were identified (Fig. 1*B*). Immune cell groups consisted of $CD4^+$ T cells (Cd4: *Cd3e*, *Cd4*); $CD8^+$ T cells (Cd8: *Cd3e*, *Cd8a*); regulatory T cells (Treg: *Cd3e*, *Cd4*, *Foxp3*, *Ctla4*); γδ T cells, natural killer cells, and natural killer T cells (γδ/NK/NKT: *Tcrd*, *Ncr1*, *Ccr5*), B cells (B: *CD79a*, *CD19*); plasma cells (Plasma: *Cd79a*, *Xbp1*); macrophages (Mac: *Cd68*, *Cd14*, *Lyz2*, *Fcgr1*, *Adgre1*); conventional dendritic cells (cDC: *Fcgr1*, *Cd68*, *Bst2*); plasmacytoid DCs (pDC: *Siglech*); and a mixed population of innate lymphoid cells and γδ T cells (mixed: CD3e, *Tcrd*, *Il7r*, *Rorc*, *Rora Cxcr6*, *Tmem176a*, *IL17a*) (Fig.1 *C* and *D*, *SI Appendix*, Fig. 1*A*, and Dataset S1). T cells (including Cd4, Cd8, and Treg) constituted the major group of the infiltrating immune cells in sciatic nerves, followed by B cells, gd/NK/NKT cells, macrophages, cDC, pDCs, plasma cells, and mixed (γδ T and ILC) cells. (*SI Appendix*, Fig. 1*B*). The presence of major sciatic nerve-infiltrating immune cell types was verified by conventional flow cytometry (T cells [$CD3^+$], macrophages [$F4/80^+$], B cells [$CD3^-$ $F4/80^-$ $B220^+$], pDCs [$CD3^-$ $F4/80^-$ $B220^+$ $CD11c^+$], and cDCs [$CD3^-$ $F4/80^-$ $B220^-$ $CD11c^+$])] (Fig. 1*E*). This diversity of immune cell populations suggests the dynamic interplay of multiple immune cell types in the autoimmune peripheral nerve.

To probe the variable biological properties that distinguish distinct immune cell groups, we utilized VISION, a tool that identifies sources of variation in scRNA-seq data (20). Among the top pathways identified by autocorrelation score were cytokine signaling pathways (*SI Appendix*, Fig. 1*C*). Interestingly, the "IFN-γ response" and "TNF-α signaling via NF-κB" pathways were prominent in myeloid groups (cDC and macrophages) (Fig. 1 *F* and *G*). "TNF-α signaling via NF-κB" pathway genes that were highly expressed by macrophages included *Nfkbia*, *Cxcl1*, *Nfkb1*, *Nfkb2*, *Il1a*, *Tnf*, *Il7r*, *Csf1*, *Stat5a*, *Il12b*, and *Il6st* (Fig. 1*H*). These genes have been shown to promote recruitment and development of immune cells, immune cell activation, and production of inflammatory signals (21, 22). We and others have previously reported that IFN-γ is highly expressed in neuropathic *NOD.Aire$^{GW/+}$* sciatic nerves and that IFN-γ plays an essential role in SAPP development (23, 24). However, TNF-α and TNF-α signaling have not previously been noted in SAPP (25). Thus, while corroborating published studies that IFN-γ plays a prominent role in SAPP development, these data identify myeloid cells as prime targets of IFN-γ signaling. Furthermore, these data reveal prominent TNF-α–mediated signaling in myeloid cells which may play an essential role in SAPP development.

IFN-γ Is Expressed by T Cells and Induces TNF-α Expression in *NOD.Aire$^{GW/+}$* Macrophages. Given this evidence of prominent cytokine signaling networks, we used CellChat to identify ligand–receptor interactions and predict cell–cell communication inputs and outputs (26). Consistent with VISION analysis, "IFN-II (also known as IFN-γ) signaling pathway network" (Fig. 2 *A* and *B*) and "TNF signaling pathway network" (Fig. 2 *C* and *D*) were identified as significant signaling pathways. Network centrality analysis of the inferred IFN-γ pathway identified $CD4^+$ and $CD8^+$ T cells as the main IFN-γ sources (Fig. 2 *A* and *B*), consistent with previously published work (10, 23). Comparison of IFN-γ transcript expression in cell groups corroborated that IFN-γ is most highly expressed by $CD4^+$ and $CD8^+$ T cells (Fig. 2*C*). Moreover, myeloid populations (macrophages, and to a lesser extent, cDCs) were identified as the main IFN-γ targets (Fig. 2 *A* and *B*), consistent with our VISION analysis (Fig. 1*F*). Thus, IFN-γ–mediated cell–cell communication among immune cells in autoimmune sciatic nerves was shown to be largely paracrine, with T cells as the IFN-γ source and myeloid cells as the IFN-γ responders.

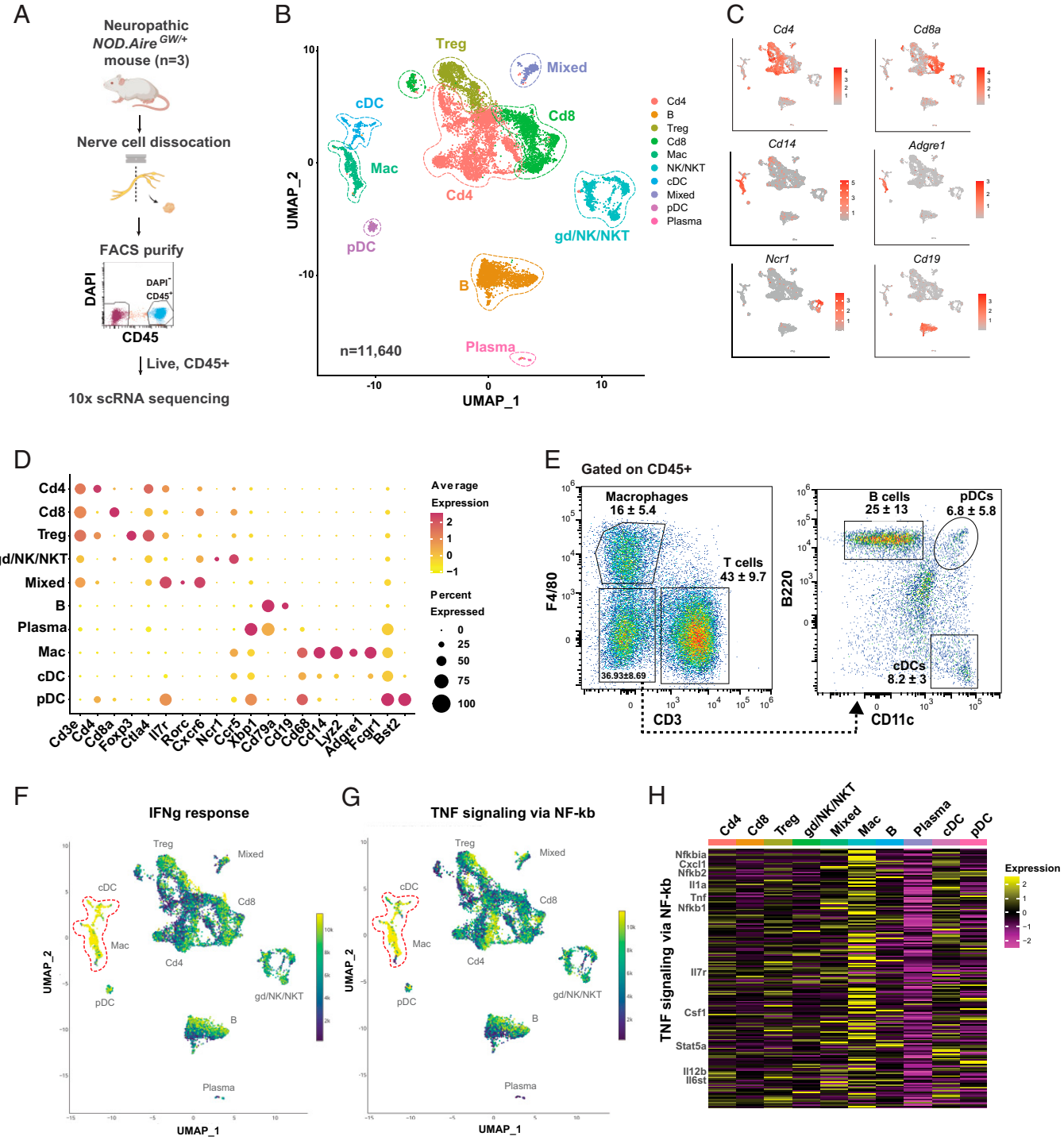

Fig. 1. Sciatic nerves undergoing active autoimmunity are infiltrated by diverse immune cells that up-regulate TNF-α signaling. (*A*) Schematic of workflow in which sciatic nerve samples from neuropathic *NOD.Aire*$^{GW/+}$ mice were dissociated and FACS sorted on DAPI^{-} CD45^{+} cells and processed using 10X Genomics scRNA-seq. (*B*) UMAP plot showing clusters of DAPI^{-} CD45^{+} cells (n = 11,640) from integrated peripheral nerve samples of *NOD.Aire*$^{GW/+}$ mice (n = 3). Dimension reduction was performed by principal component analysis using the Seurat scRNA-seq analysis package. Clusters are indicated by color and labels were manually assigned after analysis of highly expressed canonical markers for each cell type. B, B cell; Cd4, CD4^{+} T cells; Cd8, CD8^{+} T cells; Mac, macrophage; Mixed, mixed population. (*C*) Feature plots of scRNA-seq data showing expression of canonical markers for a subset of immune cells. (*D*) Dot plot of scRNA-seq data showing the canonical markers for each cluster. (*E*) Flow cytometry identification of major lymphoid and myeloid cell populations in sciatic nerves of 20- to 25-wk-old neuropathic *NOD.Aire*$^{GW/+}$ mice (n = 3). The data shown are representative of three independent flow cytometry experiments. (*F*) Visualization of Hallmark "IFNg response" pathway expression determined by VISION analysis using a UMAP projection. Blue indicates low pathway expression while yellow indicates relatively high pathway expression. (*G*) Visualization of Hallmark "TNF signaling via NF-κB pathway" expression determined by VISION analysis using a UMAP projection. (*H*) Heatmap showing average expression per cluster of genes included in the Hallmark TNF signaling via NF-κB pathway.

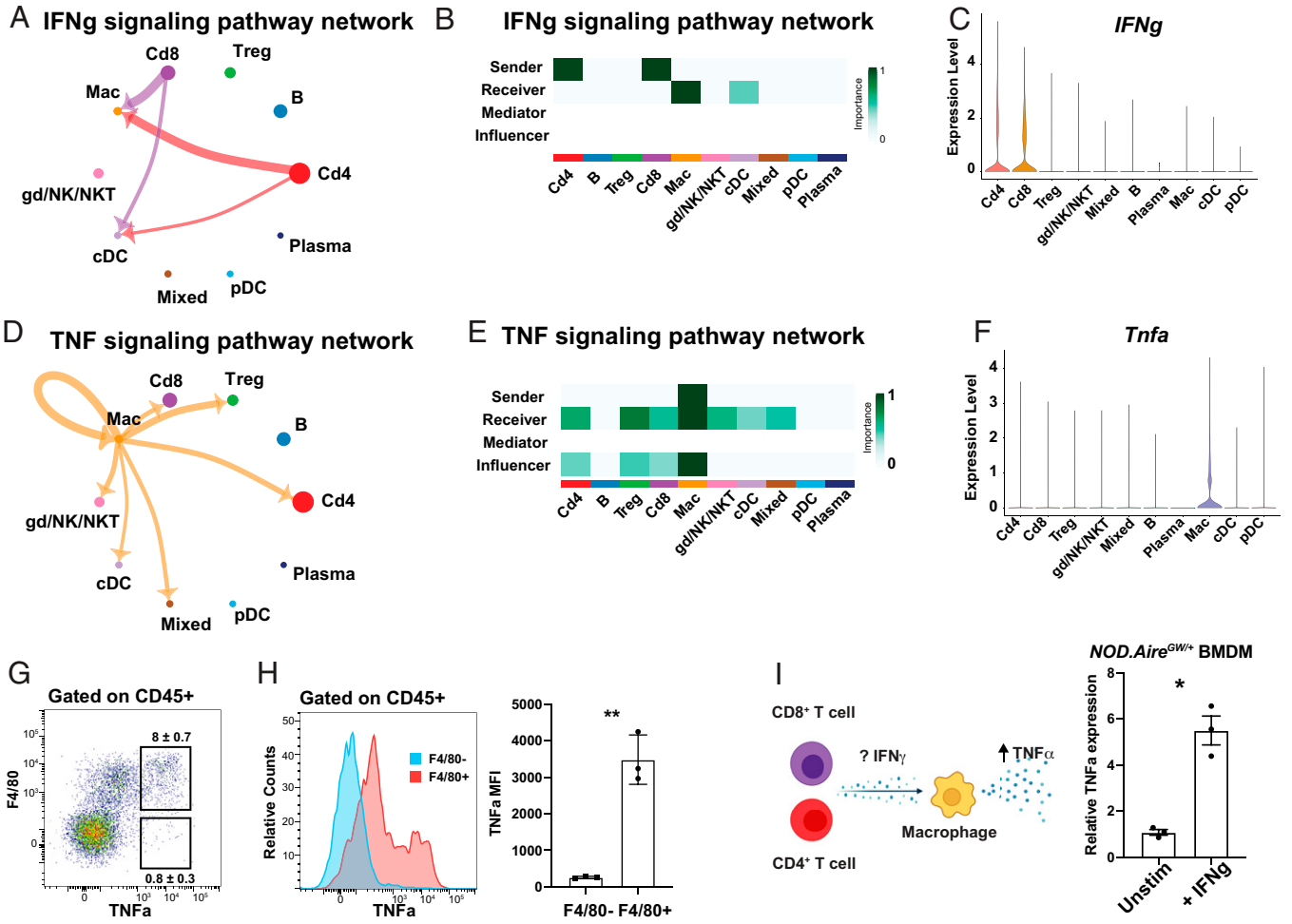

Fig. 2. T cell-derived IFN-γ drives macrophage TNF production. (*A* and *D*) Circle plot visualization of NOD.Aire$^{GW/+}$ sciatic nerve immune cell types significantly sending and receiving IFN-γ (*A*) or TNF-α (*D*) signals determined by CellChat analysis. (*B* and *E*) Heatmap of CellChat analysis depicting dominant cell types involved in IFN-γ (*B*) and TNF-α (*E*) signaling. (*C* and *F*) Violin plot showing expression of *IFNg* (*C*) and *Tnf* (*F*) by cell type from the scRNA-seq data. (*G*) Flow cytometry quantification of TNF-α production by distinct immune cell populations (CD45$^+$ F4/80$^+$ vs. F4/80$^-$) in the sciatic nerves of neuropathic *NOD.Aire*$^{GW/+}$ mice. Data shown are representative of three independent experiments. (*H*) Representative histograms and mean fluorescence intensity (MFI) of TNF-α expression among F4/80$^+$ vs. F4/80$^-$ cells. Data shown are representative of three independent experiments. $^{**}P < 0.01$ by Student's *t* test. (*I*, *Left*) Schematic of hypothesis that IFN-γ from CD4 and CD8$^+$ T cells stimulates macrophage production of TNFα in *NOD.Aire*$^{GW/+}$ nerves. (*Right*) *Tnf* expression relative to the housekeeping gene *GAPDH* by RT-qPCR in BMDM from neuropathic NOD.AireGW/$^+$ mice without (Unstim) or with (+IFN-γ) IFN-γ stimulation. $^*P < 0.05$ by Student's *t* test.

In addition, CellChat analysis identified a distinct cell–cell communication pattern mediated by TNF-α. Macrophages were the primary TNF-α source, and multiple immune cell types, including macrophages themselves, were TNF-α responders (Fig. 2 *D* and *E*). Visualization of TNF-α signaling by circle plot revealed autocrine signaling by macrophages had the highest communication probability, with macrophage TNF-α signaling to Tregs, CD4$^+$ T effectors, and NK/NKT cells having the next highest communication strength (Fig. 2*D*). Heatmap visualization of network centrality revealed macrophages, CD4$^+$ T cells, Treg, and CD8$^+$ T cells as both "influencers" that control cell–cell communication (26) via TNF-α signaling, as well as "receivers" of TNF-α signaling. This heatmap depicted macrophages as the most important source, influencer, and recipient of TNF-α signaling (Fig. 2*E*). Consistent with the notion that macrophages are the predominant TNF-α source, comparison of gene expression across cell clusters showed that TNF-α expression was highest in macrophages (Fig. 2*F*). Flow cytometric analysis of sciatic nerves from neuropathic *NOD.Aire*$^{GW/+}$ mice verified that TNF-α protein was expressed predominantly by F4/80$^+$ macrophages (Fig. 2*G*). Mean fluorescence intensity of TNF-α expression among CD45$^+$ immune cells was significantly higher in F4/80$^+$ macrophages compared with nonmacrophage populations (Fig. 2*H*).

We next focused on delineating signals that stimulate macrophage TNF-α expression during active autoimmunity. Because IFN-γ has been reported to induce macrophage TNF-α expression (27), we reasoned that IFN-γ, which is made by T cells in nerves from neuropathic *NOD.Aire*$^{GW/+}$ mice, may be the signal that stimulates macrophage TNF-α expression (Fig. 2 *I*, *Left*). To test this model, we ideally would test the effects of IFN-γ on macrophages from preneuropathic *NOD.Aire*$^{GW/+}$ mice. However, generating sufficient numbers of same-aged *NOD.Aire*$^{GW/+}$ mice from which to pool sciatic nerve macrophages was prohibitive. We therefore isolated bone marrow-derived macrophages (BMDM) from neuropathic *NOD.Aire*$^{GW/+}$ mice and incubated them in the presence or absence of IFN-γ (Fig. 2 *I*, *Right*). BMDM exposed to IFN-γ significantly up-regulated TNF-α expression compared with unstimulated BMDM. In contrast, HMGB1, which is expressed widely within peripheral nerve immune cells of neuropathic *NOD.Aire*$^{GW/+}$ mice (*SI Appendix*, Fig. 2*A*), failed to up-regulate TNF-α expression in *NOD.Aire*$^{GW/+}$ BMDM (*SI Appendix*, Fig. 2*B*). HMGB1 has previously been reported to induce macrophage

TNF-α expression through its function as an endogenous Toll-like receptor 4 (TLR4) ligand (28). Taken together, our findings suggest a model in which $CD4^+$ and $CD8^+$ T cells produce IFN-γ, which acts upon macrophages to stimulate TNF-α production.

TNF-α–Mediated Signaling Is Up-Regulated in Sciatic Nerves Targeted by Autoimmunity. Immune cell infiltration is a hallmark of peripheral nerve autoimmunity in mice and humans (29). By flow cytometry, $CD45^+$ immune cell numbers are exponentially increased in sciatic nerves of neuropathic *NOD.Aire*$^{GW/+}$ mice compared with steady-state *NOD.WT* (Fig. 3*A*). Nevertheless, resident immune cells are present in nondiseased nerves under homeostatic conditions and have recently been analyzed by scRNA-seq in wild-type C57BL/6 (*B6.WT*) and NOD (*NOD.WT*) mice (data from Gene Expression Omnibus [GEO] accession no. GSE142541) (15). By performing combined analysis of sciatic nerve immune cell scRNA-seq data from steady-state (*B6.WT* and *NOD.WT*) vs. autoimmune (*NOD.Aire*$^{GW/+}$) mice, we aimed to delineate changes in cell composition and phenotype that may underlie the development of autoimmunity. Cell type-clustering identified 12 major immune populations consisting of $Cd4^+$ T cells (Cd4: *Cd3g*, *Cd4*), $Cd8^+$ T cells (Cd8: *Cd3g*, *Cd8a*); proliferating T cells (Pro_T: *Cd3g*, *Stmn1*, *Mki67*, *Top2a*); a mixed population of γδ T and innate lymphoid cells (mixed: *Cd3g*, *Gata3*, *Rora*, *Rorc*); γδ T, NK, NKT cells (gd/NK/NKT: *Trdc*, *Nkg7*, *Ncr1*, *Klrb1a*); B cells (B: *Cd79a*, *Cd79b*, *Ighm*, *Ms4a1*); plasma cells (plasma: *Prdm1*, *Tnfrsf17*); macrophages (MC: *Fcgr1*, *Cd14*); cDC (*Ciita*, *H2-Oa*, *Btla*); and pDCs (*Btla*, *Siglech*) (Fig. 3*B*, *SI Appendix*, Fig. 3*A*, and Dataset S2). Additionally, two T cells clusters were identified that expressed coinhibitory receptors Tigit (Tigit_T: *Tigit*, *Cd3g*) and CTLA-4 (Ctla4_T: *Ctla4*, *Cd3g*). Cells derived from *B6.WT*, *NOD.WT*, and *NOD.Aire*$^{GW/+}$ were present in all clusters except for $Ctla4^+$ T cell, which derived solely from autoimmune *NOD.Aire*$^{GW/+}$ (Fig. 3*C*). Although the NOD strain is known to be more prone to autoimmunity than the C57BL/6 strain due to predisposing genetic polymorphisms, *B6.WT* and *NOD.WT* immune cells were similarly distributed across clusters (Fig. 3*C*). This suggests that, in healthy sciatic nerves, each identified cell type was associated with a common cell lineage rather than derivation from the C57BL/6 or NOD strain. Nevertheless, it is important to keep in mind that multiple immune changes have been described in *NOD.WT* mice, which predispose these mice to multiorgan autoimmune diseases including type 1 diabetes.

Compared with immune cells present in steady-state sciatic nerves, T cells were overrepresented in autoimmune infiltrates of *NOD.Aire*$^{GW/+}$ nerves. (*SI Appendix*, Fig. 3*B*). This may reflect defective negative selection of autoreactive T cell clones in this Aire-deficient mouse model (30). We next used CellChat to compare cell–cell signaling patterns between immune cells from autoimmune vs. steady-state sciatic nerves. Increased information flow, a measure of communication probability between cell cluster pairs (20), was evident in *NOD.Aire*$^{GW/+}$ nerves compared with *NOD.WT*. Of the 47 pathways that are significantly expressed in *NOD.Aire*$^{GW/+}$ sciatic nerves, 37 pathways were unique to *NOD.Aire*$^{GW/+}$, while 10 pathways were also expressed in *NOD.WT* (*SI Appendix*, Fig. 3*C*). Pathways with information flow found only in autoimmune *NOD.Aire*$^{GW/+}$ sciatic nerves included "IFNII" (IFN-γ) and "TNF" signaling pathways (*SI Appendix*, Fig. 3*C*). Corroborating our previous analysis (Fig. 2 *D*–*F*), macrophages were the main source of TNF-α, and TNF-α signals were received by multiple innate and adaptive cell types in *NOD.Aire*$^{GW/+}$ sciatic nerve (Fig. 3 *D*, *Right*). The lack of connecting lines/arrows on the *NOD.WT* circle plot, on the other hand, indicated the absence of significant TNF-α signaling (Fig. 3 *D*, *Left*).

TNF-α signaling occurs through two receptors (TNFR1 and TNFR2, encoded by *Tnfrsf1a* and *Tnfrsf1b*, respectively), and both *Tnfrsf1a* and *Tnfrsf1b* expression were increased in *NOD.Aire*$^{GW/+}$ immune cell groups from *NOD.Aire*$^{GW/+}$ sciatic nerves compared with *NOD.WT* (Fig. 3*E*). Expression of *Tnfrsf1a* was significantly increased in macrophage and cDC groups, while *Tnfrsf1b* was significantly increased in T cell, gd/NK/NKT, mixed, and macrophage populations. Additionally, *Tnf* expression was increased in macrophage populations of neuropathic *NOD.Aire*$^{GW/+}$ sciatic nerve immune cells compared with *NOD.WT* (Fig. 3*F*), and this increase was verified by quantitative real-time PCR (RT-qPCR) (Fig. 3*G*) and immunohistochemical staining. (Fig. 3*H*). Together, these analyses suggest that autoimmune vs. steady-state nerves are distinguished by up-regulation of autocrine/paracrine TNF-α signaling among immune cells.

Macrophage scRNA-seq Profiles Are Distinct in Autoimmune vs. Injury Macrophages. Accumulating data suggest an important, proinflammatory role for macrophages in autoimmune peripheral neuropathy. Similar to the sciatic nerves of *NOD.Aire*$^{GW/+}$ mice, macrophage infiltration is seen in nerve biopsies of patients with inflammatory neuropathies (31, 32). Moreover, we have previously reported that macrophages play a proinflammatory role in SAPP, since depletion of phagocytes with clodronate liposomes ameliorates neuropathy (14). Like autoimmune disease, crush injury also induces macrophage infiltration, but these injury-response macrophages play a very different role (33, 34). Rather than promoting autoimmune demyelination, injury macrophages repair tissue damage through secretion of growth factors, phagocytosis of myelin debris, and activation of Schwann cells to promote peripheral nerve remodeling (35–39). Recent scRNA-seq analysis of sciatic nerve macrophages at day 1 and day 5 postinjury by Ydens et al. (40) has provided high resolution characterization of macrophage populations involved in nerve injury repair.

We first sought to compare the transcriptional profiles of steady-state and disease-associated (injury and autoimmune) macrophages by integrating scRNA-seq datasets from each of these conditions (*SI Appendix*, Fig. 4*A*). Steady-state data were obtained from previously published datasets [GSE142541 (15) and GSE144708 (40)], which consisted of two datasets from C57BL/6 ("steady-state B6-1" and "steady-state B6-2") and one dataset from NOD ("steady-state NOD") wild-type mice. Additionally, data from sciatic nerve macrophages 1 d postnerve injury ("D1 injury") and 5 d postnerve injury ("D5 injury") (GSE144708) (40) were utilized. These data were integrated with data from "autoimmune" macrophages from neuropathic *NOD.Aire*$^{GW/+}$ mice.

Macrophages clustered into seven groups (0 to 6) suggesting macrophage heterogeneity (*SI Appendix*, Fig. 4*A*). Interestingly, groups 3 and 5 derived largely from steady-state *B6.WT* and *NOD.WT* mice, groups 0 and 4 derived largely from D1 injury mice, group 1 derived from D5 injury mice, and groups 1 and 2 derived from D5 injury and autoimmune mice (*SI Appendix*, Fig. 4*B*). The nonoverlapping distribution of steady-state vs. disease-associated macrophages suggests that the gene-expression profiles of healthy, steady-state macrophages are distinct from disease-associated macrophages.

To query further the relationship between injury and autoimmune macrophages, we separately compared the published scRNA-seq data (available from GEO accession no. GSE144708) (40) from injury macrophages with our data from autoimmune macrophages. Next, 556 *NOD.Aire*$^{GW/+}$ macrophages were integrated and compared against 2,763 macrophages 1 d postnerve injury ("D1 injury") and 1,949 macrophages 5 d postnerve injury ("D5 injury") from a nerve crush injury model. Clustering analysis revealed seven distinct groups (Fig. 4*A* and Dataset S3). Macrophages from groups 1 and 2 derived largely from D1 injury macrophages (Fig. 4 *A* and *B*) and expressed genes associated with alternatively activated M2 macrophage (*Arg1*, *S100a4*, *Chil3*)

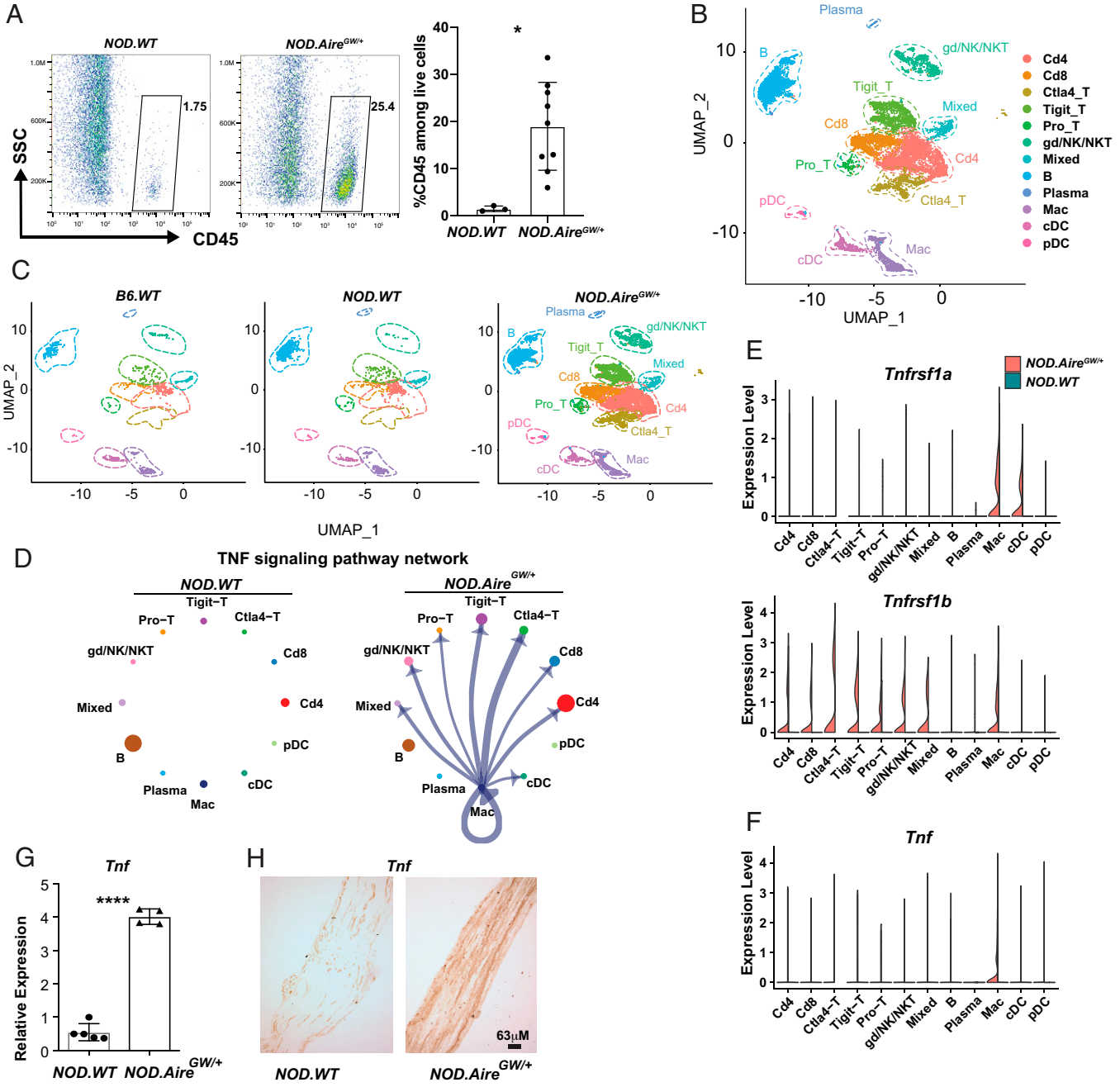

Fig. 3. Autoimmune sciatic nerves up-regulate autocrine/paracrine TNF-α signaling that is mediated by macrophages. (*A*) Example flow cytometry plots and quantification of sciatic nerve leukocyte infiltration in female *NOD.WT* and neuropathic *NOD.Aire*$^{GW/+}$ mice (>23 wk of age). *$P = 0.01$ by Student's *t* test. (*B*) UMAP plot of 12 different immune cell clusters after integration of *NOD.Aire*$^{GW/+}$ immune cells with *B6.WT* and *NOD.WT* datasets. Cd4, CD4$^+$ T cells; Cd8, CD8$^+$ T cell; CTLA-4 -T, CTLA$^+$ T cell; Pro-T, proliferating T cell; Tigit-T, Tigit$^+$ T cell. (*C*) UMAP plots from *B* split by condition. Positions of each cluster from *B* are delineated by their respective colored dotted lines. (*D*) Comparison of CellChat analyses between CD45$^+$ immune cells of *NOD.Aire*$^{GW/+}$ and *NOD.WT* mice visualized using a circle plot. The lack of lines connecting cell clusters for the *NOD.WT* condition indicates the lack of significant TNF-α signaling. (*E* and *F*) Split violin plots comparing expression of *Tnfrsf1a, Tnfrsf1b* (*E*) and *Tnf* (*F*) in *NOD.WT* vs. *NOD.Aire*$^{GW/+}$ immune cell groups. (*G*) *Tnf* expression relative to cyclophilin, measured by RT-qPCR. RNA was isolated from whole sciatic nerves of *NOD.WT* and neuropathic *NOD.Aire*$^{GW/+}$ mice. ****$P < 0.0001$ by Student's *t* test. (*H*) Immunohistochemical staining for TNF-α in sciatic nerves from *NOD.WT* and neuropathic *NOD.Aire*$^{GW/+}$ mice.

and suppression of T cell activation (*Arg1*, *Pkm*) (*SI Appendix*, Fig. 5 *A*–*C*) (41–45). Macrophages from groups 3 and 4 derived largely from D5 injury macrophages (Fig. 4 *A* and *B*) and up-regulated the MHCII molecule H2-Aa (4) (*SI Appendix*, Fig. 5 *B* and *C*). At the same time, macrophages from groups 3 and 4 up-regulated genes associated with an antiinflammatory phenotype (*Sepp1*, *Retnla*, *Trem2*) (*SI Appendix*, Fig. 5 *A* and *C*) (46–48), suggesting a heterogeneous population with pro- and antiinflammatory functions. Day 5 injury-derived macrophages in group 5 expressed the highest levels of *Retnla*, consistent with an antiinflammatory phenotype (*SI Appendix*, Fig. 5).

Additionally, we examined the markers for group 7, where nearly all (98.5%) of the autoimmune macrophages from neuropathic *NOD.Aire*$^{GW/+}$ mice were localized. The cluster up-regulated multiple chemokines (*Cxcl10*, *Cxcl9*, *Ccl5*), and a proinflammatory factor important in assembly of NLRP3 inflammasome (*Rack1*) (*SI Appendix*, Fig. 5 *B* and *C*) (49–51). The MHCII molecule H2-Aa was also highly expressed by group

7 macrophages (*SI Appendix*, Fig. 5 *B* and *C*). Notably, *Tnf* expression was highest in cluster 7 macrophages (Fig. 4*C*), and VISION analysis revealed "TNF-α signaling via NF-κB" to be among the pathways with high autocorrelation scores and with highest signaling in cluster 7 (*SI Appendix*, Fig. 5*D*). Subsequent gene set enrichment analysis (GSEA) also revealed that "IFN-γ response" and "TNF-α signaling via NF-kB" were the top two most highly up-regulated pathways in group 7 macrophages (Fig. 4*D*). In support of a proinflammatory phenotype, oxidative phosphorylation was down-regulated in cluster 7 macrophages (Fig. 4*D*). Because alternatively activated, immunosuppressive macrophages rely on anabolic metabolism processes, such as oxidative phosphorylation, a skew away from oxidative phosphorylation in autoimmune macrophages suggests a shift away from an immunosuppressive state (41, 52).

Despite their seemingly disparate functions and distinct transcriptional profiles, injury and autoimmune macrophages may be linked. Macrophages are highly plastic, and peripheral nerve injury can lead to autoimmunity. For example, unilateral partial sciatic nerve ligation predisposes to the development of autoimmunity in the alternate sciatic nerve (53). This temporal connection suggests the possibility that reparative macrophages recruited by injury may be polarized to become pathogenic, resulting in sciatic nerve degeneration and acute or chronic neuropathy. To test this possibility, we performed pseudotime trajectory analysis using Monocle3 and SeuratWrapper packages to determine the transcriptional fate of immunoregulatory macrophages in groups 1/2 in silico (Fig. 4*E*) (54). Single-cell trajectory analysis revealed changes that place group 1/2 macrophages and group 3/4 macrophages on the same path, as suggested by their derivation from D1 injury and D5 injury, respectively. Interestingly, group 7 macrophages (derived from autoimmune *NOD.Aire*$^{GW/+}$ nerves) were shown as an extension of the D5 injury trajectory. This placement suggests a possible phenotypic change from a reparative state at the end of nerve injury repair to a proinflammatory state with autoimmunity.

Autoimmune Peripheral Neuropathy Is Ameliorated with Anti–TNF-α Antibody Treatment and Genetic TNFR Ablation. Given the prominent TNF-α signaling in autoimmune peripheral nerves, we sought to determine whether TNF-α is required for autoimmune demyelination of peripheral nerves. We first tested the effects of antibody-mediated TNF-α blockade in an adoptive transfer model of PNS autoimmunity in which activated splenocytes from neuropathic *NOD.Aire*$^{GW/+}$ donors are transferred to immunodeficient *NOD.SCID* recipients (10). Immune-mediated neuropathy develops uniformly in recipient mice by 10 wk after transfer, allowing for a highly reproducible and robust system to test the effects of anti– TNF-α antibody. While all 7 recipient mice developed neuropathy without treatment, anti–TNF-α antibody treatment prevented neuropathy in 13 of 14 recipients (Fig. 5*A*). Histological analysis and flow cytometry of sciatic nerves revealed significantly decreased immune infiltration in anti–TNF-α treated *NOD.Aire*$^{GW/+}$ mice (Fig. 5 *B* and *C*). We have previously reported that neuropathic *NOD.Aire*$^{GW/+}$ mice show multiple changes consistent with demyelinating neuropathy, including increased latency, decreased conduction velocity, and decreased amplitude of nerve conduction (23). Similarly, isotype control-treated recipients of neuropathic *NOD.Aire*$^{GW/+}$ splenocytes also showed these abnormalities (Fig. 5*D*). Nerve conduction studies of recipients treated with anti–TNF-α antibody, however, showed normalization of these parameters, with decreased latency, increased conduction velocity, and increased amplitude (Fig. 5*D*). Together, these findings demonstrate that antibody-based TNF-α blockade protects against immune infiltration and demyelination of peripheral nerves.

We next set out to verify the effects of TNF-α blockade on the development of PNS autoimmunity using a genetic approach. Because TNF-α signals through two receptors (TNFR1 and TNFR2, encoded by *Tnfrsf1a* and *Tnfrsf1b*, respectively), we generated *NOD.Aire*$^{GW/+}$ mice deficient in both TNFR1 and -2 (*NOD.Aire*$^{GW/+}$ *Tnfrsf1a*$^{-/-}$ *Tnfrsf1b*$^{-/-}$). In line with our data that anti–TNF-α antibody protects from neuropathy, TNFR1 and TNFR2 double-deficiency protected *NOD.Aire*$^{GW/+}$ mice from SAPP development. While all *NOD.Aire*$^{GW/+}$ mice ($n = 16$) developed neuropathy by 30 wk of age, *NOD.Aire*$^{GW/+}$ *Tnfrsf1a*$^{-/-}$ *Tnfrsf1b*$^{-/-}$ mice ($n = 19$) were completely protected (Fig. 5*E*). Thus, blocking TNF-α activity, either through treatment with anti–TNF-α antibody or through genetic deficiency in TNFR1 and -2, prevents the development of autoimmune peripheral neuropathy.

Because our data show that macrophages are the prime target of TNF-α (Figs. 1*G*, 2 *D* and *E*, and 3*D*), we sought to delineate TNF-α's effects on the transcriptional profile of macrophages. The BD Rhapsody platform, a microwell capture system coupled to a highly multiplexed PCR amplification of a defined set of 500 macrophage immune response genes, was used to collect single cell data. BMDMs isolated from *NOD.Aire*$^{GW/+}$ mice were incubated for 8 h, with or without TNF-α (55). Comparison of differential gene expression identified multiple changes in response to in vitro TNF-α stimulation (Fig. 5*F*). Genes that were significantly increased in their transcription included multiple autoimmunity-associated genes (e.g., *Clec4e*, *Cd40*) and proinflammatory genes (e.g., *Marcksl1*, *Irf1*, *Irf7*) (56–63). Several up-regulated genes were also involved in leukocyte activation, recruitment, and adhesion as well as immune infiltration (e.g., *Cxcl1*, *Cxcl2*, *Cxcl10*, *Ccl5*, *Icosl*, *Vcam1*) (64–80). Of special interest was *Clec4e*, a necrotic debris sensor that induces the production of inflammatory cytokines after recognizing cell death (56). *Clec4e* is essential for the maintenance of an inflammatory macrophage phenotype in acute renal inflammation and activates and recruits T cells in central nervous system autoimmunity (57, 81). It is possible, therefore, that *Clec4e* may play a similarly pathogenic role in PNS autoimmunity following macrophage stimulation by TNF-α. Finally, *Tnf* itself is up-regulated in response to TNF-α stimulation. These data confirm our CellChat analysis (Fig. 2*D*) that TNF-α functions in an autocrine, positive feedback loop to drive autoimmune disease progression.

TNFR1 Promotes PNS Autoimmunity and Induces Expression of Multiple Autoimmunity-associated Genes in *NOD.Aire*$^{GW/+}$ Macrophages. Our data indicate that *NOD.Aire*$^{GW/+}$ mice are protected from SAPP by double-deficiency of TNFR1 and TNFR2 (Fig. 5*E*). Signaling through TNFR1 and -2 have different functional outcomes in immune cells, and loss of TNFR1 alone has been shown to protect against a number of TNF-α–mediated autoimmune conditions (82–84). Thus, it is possible that single deficiency in TNFR1, which is involved in proinflammatory signaling, is sufficient to protect. To test this, we determined the effects of genetic TNFR1 deficiency on neuropathy development. Indeed, TNFR1 deficiency was sufficient to protect *NOD.Aire*$^{GW/+}$ mice from SAPP ($n = 8$; *NOD.Aire*$^{GW/+}$ *Tnfrsf1a*$^{-/-}$ *Tnfrsf1b*$^{+/-}$) (Fig. 6*A*). By flow cytometry and on histological examination, sciatic nerves from TNFR1-deficient (*Tnfrsf1a*$^{-/-}$) *NOD.Aire*$^{GW/+}$ mice were significantly less infiltrated than TNFR1-sufficient littermates (*Tnfsrsf1a*$^{+/-}$) (Fig. 6 *B* and *C*). Additionally, nerve conduction studies on sciatic nerves showed that TNFR1 deficiency protected against demyelination. While nerve conduction studies of TNFR1-sufficient *NOD.Aire*$^{GW/+}$ mice revealed significant abnormalities consistent with demyelination, studies of TNFR1-deficient *NOD.Aire*$^{GW/+}$ mice demonstrated shorter latencies, increased amplitudes, and increased conduction velocities (Fig. 6*D*). In contrast, TNFR2 deficiency failed to protect *NOD.Aire*$^{GW/+}$ mice from SAPP ($n = 8$; *NOD.Aire*$^{GW/+}$ *Tnfrsf1a*$^{+/-}$ *Tnfrsf1b*$^{-/-}$) (Fig. 6*A*). Thus, TNFR1 is required for the development of autoimmune

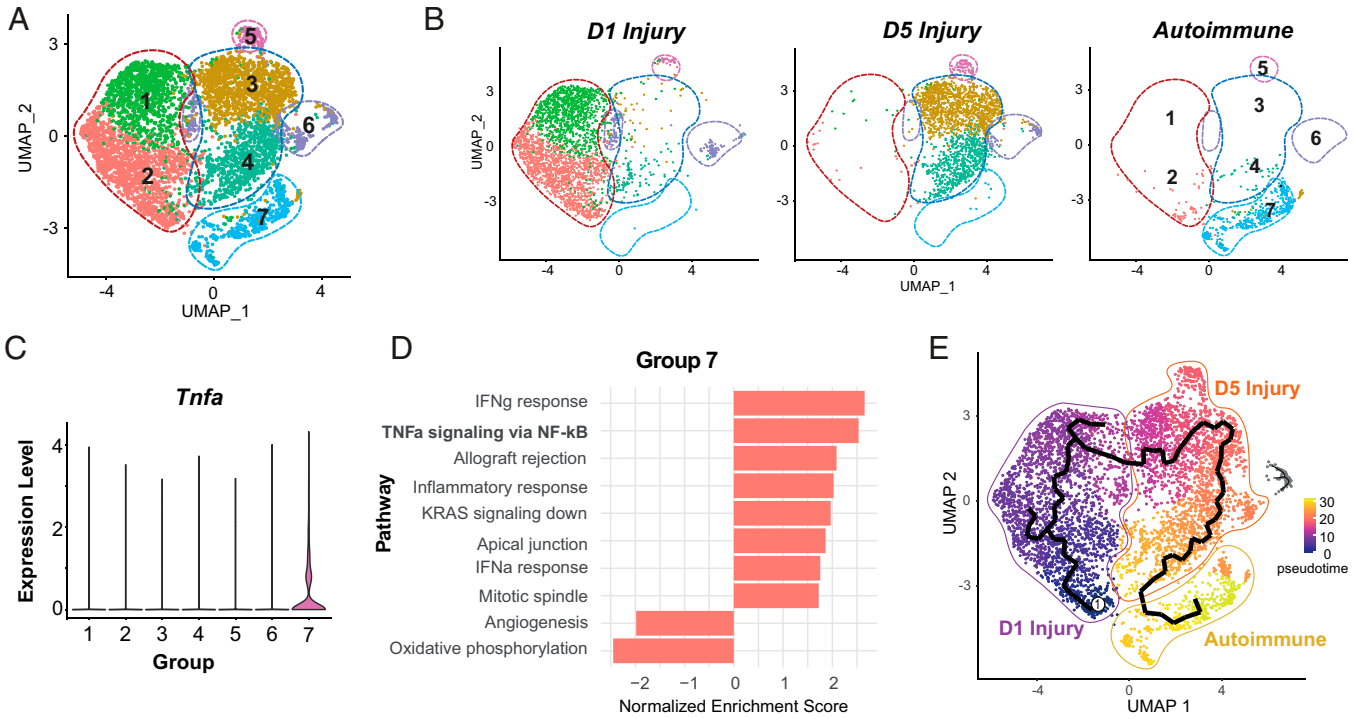

Fig. 4. Autoimmune *NOD.Aire*$^{GW/+}$ macrophages up-regulate *Tnf* and other proinflammatory pathways compared with injury macrophages. (*A*) UMAP plot of seven different macrophage clusters after integration of *NOD.Aire*$^{GW/+}$ macrophages with D1 and D5 injury macrophage datasets. (*B*) UMAP plots from *A* split by condition. Positions of each cluster from *A* are delineated by their respective colored dotted lines. (*C*) Violin plots displaying expression of *Tnf* across different groups. (*D*) GSEA plots with murine hallmark signature datasets from MSigDB (adjusted $P < 0.05$, normalized enrichment score < 7). Plot compares differentially expressed genes (Wilcoxon rank sum test: adjusted $P < 0.05$, average $\log_2$ fold-change > 0.25) from cluster 7 against the rest of the clusters. (*E*) UMAP plot from *A* with pseudotime trajectory overlay. Cells are colored pseudochronologically with purple indicating earliest time points and yellow indicating latest time points.

peripheral neuropathy, and TNFR1 deficiency is sufficient to prevent immune infiltration and autoimmune demyelination.

While our data show that TNFR1 signaling is prominent in multiple immune cell populations (Fig. 2*D*), it is possible that TNFR1 on nonimmune peripheral nerve cells (e.g., Schwann cells, fibroblasts, endothelial cells) is required for autoimmune demyelination. In support of a role for TNF-α signaling on Schwann cells, a previous report has suggested that TNF-α signaling inhibits Schwann cell proliferation in the PNS (85). To determine the importance of TNFR1 on nonimmune cells, we tested neuropathy development in an adoptive transfer model in which immunodeficient recipients were either deficient or sufficient for TNFR1. *NOD.SCID Tnfrsf1a*$^{-/-}$, or *NOD.SCID Tnfrsf1a*$^{+/-}$ mice received 10^6 splenocytes from neuropathic *NOD.Aire*$^{GW/+}$ mice and were followed for neuropathy development. Interestingly, no difference was seen in clinical neuropathy, immune infiltration, or nerve conduction studies between the two groups (*SI Appendix*, Fig. 6), suggesting that TNFR1 expression on nonimmune cells is dispensable. Taken together, these data point to a critical role for TNFR1 signaling on immune cells in the development of autoimmune peripheral neuropathy.

Discussion

Inflammatory neuropathies are characterized by pathologic immune infiltration into peripheral nerves, but little is known about the composition of these cell types and how they cooperate to destroy myelin. Here, we use scRNA-seq analysis to uncover a TNF-α signaling axis that is induced by IFN-γ and required for initiation of autoimmune demyelination. While it is known that IFN-γ is required for SAPP and produced by a significant proportion of autoreactive T cells infiltrating sciatic nerves, the identity of cells targeted by IFN-γ and the phenotypic changes induced were previously not clear. Our data show that IFN-γ acts upon macrophages most prominently, and that an important outcome of IFN-γ signaling is macrophage up-regulation of TNF-α (*SI Appendix*, Fig. S8). Induction of macrophage TNF-α production sets off an autocrine TNF-α$^+$ feedback loop, which signals macrophages to undergo a phenotype switch to a proinflammatory state. In BMDMs, TNF-α signaling leads to up-regulation of multiple proinflammatory genes, including *Clec4e* and multiple chemotactic factors (e.g., *Cxcl1*, *CXCl10*, *Cxcl2*, *Ccl5*). Additionally, TNF-α may provide signals to multiple additional immune cell types, including cDCs, γδ T cells, NK cells, NKT cells, and T cells. Through these signals, TNF-α may promote immune-mediated injury of nerve myelin.

TNF-α cell–cell communication networks in autoimmune sciatic nerves were centered on macrophages, a highly plastic cell type whose phenotypic state is influenced by the immune microenvironment. Macrophages from neuropathic *NOD.Aire*$^{GW/+}$ sciatic nerves differed transcriptionally from those in nerves after crush injury, further demonstrating the diversity of macrophage phenotypes found within the PNS. Differentially regulated pathways included those involved in cytokine signaling as well as metabolism. This alteration in metabolic pathways was interesting to us, given that the activation state of macrophages is dependent on underlying metabolic processes (52). Whereas classically activated, proinflammatory macrophages rely on catabolic processes (such as glycolysis), alternatively activated macrophages utilize anabolic processes, such as oxidative phosphorylation (9, 10). Down-regulation of the oxidative phosphorylation pathway in autoimmune macrophages compared with injury macrophages therefore suggests that autoimmune macrophages are in a

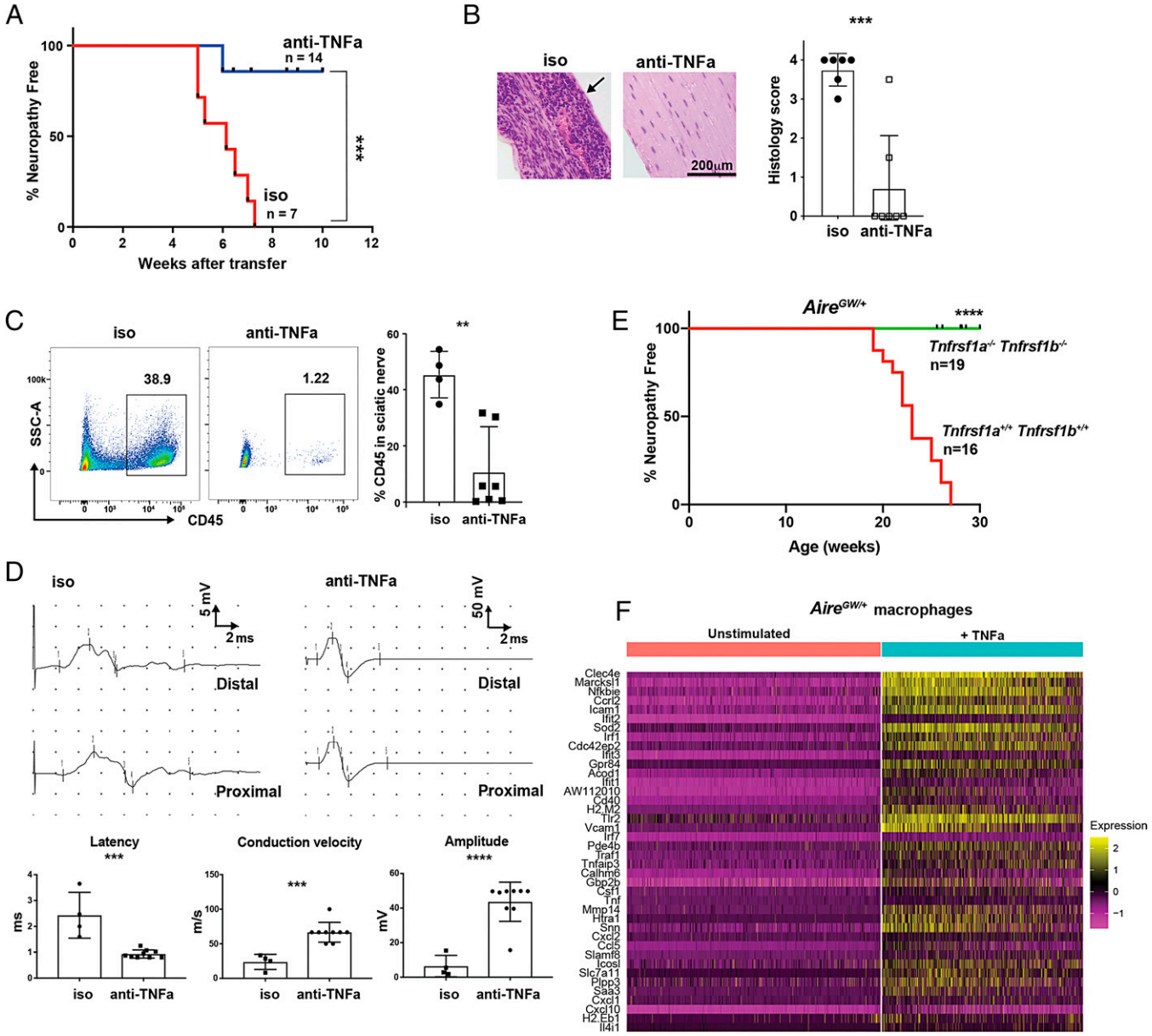

Fig. 5. Autoimmune peripheral neuropathy is ameliorated with anti–TNF-α antibody treatment and genetic TNFR1/2 ablation. (*A–D*) 10^6 splenocytes from neuropathic NOD.Aire$^{GW/+}$ female mice (15 to 25 wk of age) were transferred by retro-orbital injection into 8- to 12-wk-old female immunodeficient NOD.SCID recipients. Twenty-four hours after splenocyte transfer, recipients were treated with intraperitoneal anti–TNF-α (clone XT3.11) or isotype control (iso; HPRN) antibody. Next, 300 μg of antibody was given for the first dose, then 150 μg weekly for 10 wk. Mice were monitored for 10 wk after adoptive transfer. (*A*) Neuropathy incidence curve. ***$P < 0.0005$ by log-rank test. (*B*) Histology scores of H&E-stained sciatic nerves. Arrow highlights area of dense immune infiltration. Each dot represents an individual mouse. ***$P < 0.0005$ by Student's *t* test. (*C*) Flow cytometric analysis of CD45$^+$ immune cells present in the sciatic nerves Each dot represents an individual mouse. **$P < 0.005$ by Student's *t* test. (*D*) Representative EMG traces of sciatic nerves. Peak latency, conduction velocity, and amplitude are shown. Note that the height of one square is 5 mV for isotype-treated and 50 mV for anti–TNF-α treated mice. Each dot represents an individual mouse. ***$P < 0.0005$ and ****$P < 0.00005$ by Student's *t* test. (*E*) Neuropathy incidence curve that shows onset of clinical symptoms in *NOD.Aire*$^{GW/+}$ mice that are genetically deficient or sufficient in TNFR1 and TNFR2. ***$P < 0.0005$ by log-rank test. (*F*) Heatmap of top up-regulated genes using the BD Rhapsody platform in *NOD.Aire*$^{GW/+}$ BMDMs stimulated with TNF-α (50 ng/mL) or vehicle control for 8 h.

proinflammatory state. It is possible that proinflammatory cytokine signaling may precipitate this altered metabolic state in nerves affected by autoimmunity and will be addressed in future studies. It is also worth noting that our analysis only included macrophages from days 1 and 5 after crush injury, so may not reflect macrophage phenotypic states at other timepoints after injury.

The TNF-α signaling axis presents a potentially powerful clinical target for treating patients with PNS autoimmunity. Immunomodulatory therapies that target TNF-α have been efficacious in multiple inflammatory and autoimmune diseases (e.g., rheumatoid arthritis, psoriatic arthritis, inflammatory bowel disease, and psoriasis) but are not currently in use for inflammatory neuropathies (86). Most recently, anti–TNF-α therapy has shown efficacy in preserving pancreatic β-cell function in new-onset type 1 diabetes patients (87, 88) and in immune-related adverse events related to immune checkpoint therapy (89). Past studies have suggested a link between TNF-α and CIDP pathogenesis in human patients. In situ hybridization of proinflammatory cytokine expression in sural nerve biopsies found that TNF-α localized to the inner rim of perineurial, epineurial, and endoneurial blood vessels, as well

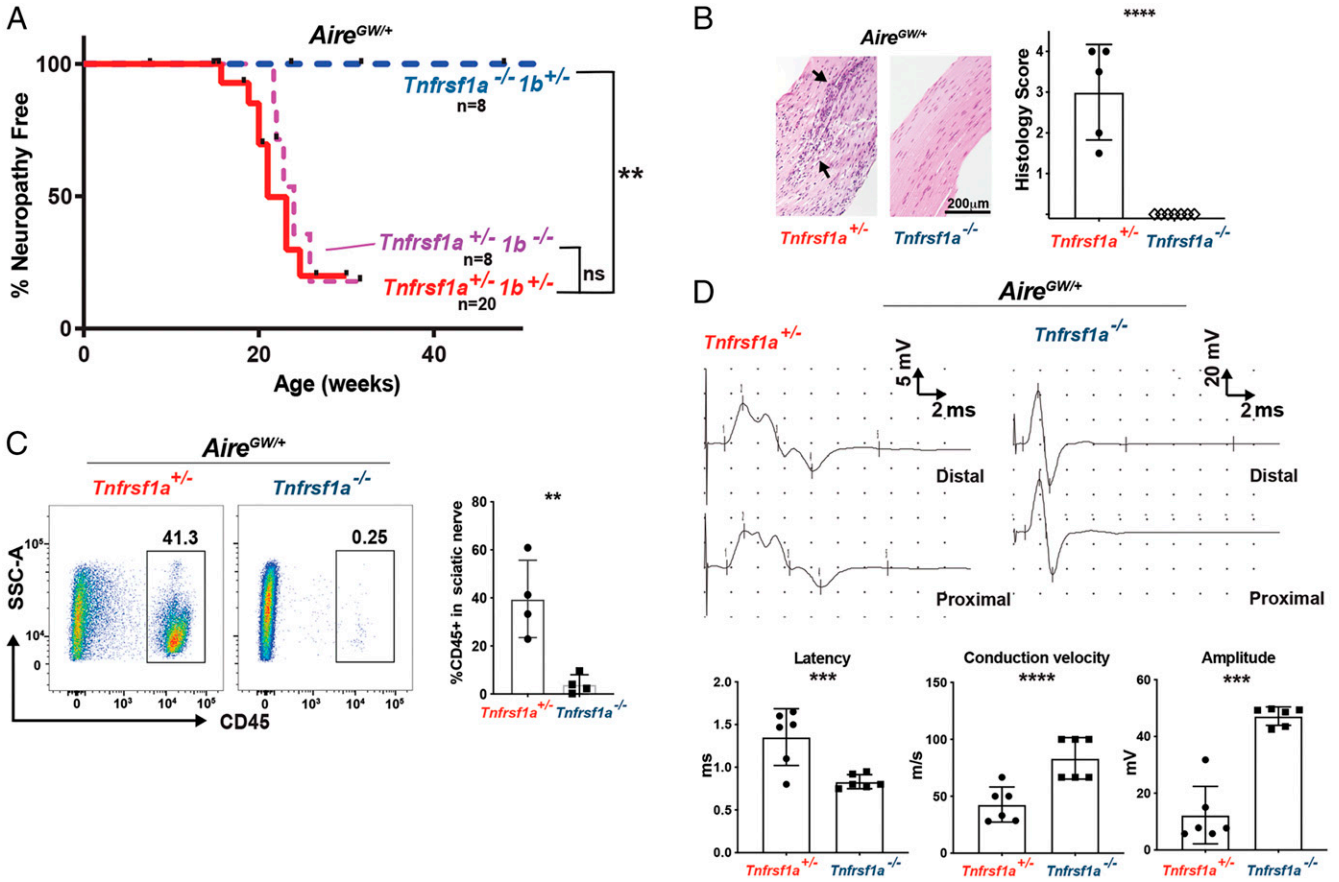

Fig. 6. TNFR1 deficiency protects from SAPP. (*A*) Neuropathy incidence curve for *NOD.Aire*$^{GW/+}$ mice that are either genetically deficient or sufficient in TNFR1 or TNFR2. $^{**}P < 0.005$ by log-rank test. ns = not significant by log-rank test. (*B*) Histology scores of H&E-stained sciatic nerves from TNFR1 deficient and sufficient mice. $^{****}P < 0.00005$ by Student's *t* test. (*C*) Flow cytometric analysis of CD45^{+} immune cells present in the sciatic nerves of TNFR1-deficient and sufficient mice. $^{**}P < 0.005$ by Student's *t* test. (*D*) Representative EMG traces of TNFR1-deficient or sufficient mice at 20- to 23-wk old. Peak latency, conduction velocity, and amplitude are shown. $^{***}P < 0.0005$ and $^{****}P < 0.00005$ by Student's *t* test.

as infiltrating immune cells (90). Additionally, elevated serum levels of TNF-α are associated with increased severity of neurological dysfunction and weakness in proximal and distal muscles (91). These findings preliminarily implicate TNF-α in the development and progression of CIDP in humans, but literature on the possible therapeutic effects of TNF-α signaling inhibition in the context of CIDP is limited. In one retrospective study, CIDP patients receiving the TNF-α blocker etanercept responded positively to standard dose treatment, suggesting possible efficacy (92). Our data that antibody-based TNF-α blockade and genetic ablation of TNF-α signaling protect against the development of PNS autoimmunity thus provides preclinical evidence that TNF blockade with an anti–TNF-α antibody may serve as an effective therapeutic intervention by altering immune events leading to PNS autoimmunity.

An important consideration in using TNF-α blockade for therapeutic purpose is that TNF-α signals through two receptors, TNFR1 and TNFR2. TNFR1 is expressed ubiquitously on most cells throughout the body, while TNFR2 expression is limited to certain cell types, including immune cell subsets, such as Treg cells and macrophages (93, 94). Through binding to each receptor, TNF-α activates distinct intracellular signaling pathways. Soluble TNF-α (sTNF-α) binding to TNFR1 activates proinflammatory and apoptotic pathways, while membrane-bound TNF-α (mTNF-α) binding to TNFR2 promotes cell survival (93, 94). Because of the skewed and greater expression of TNFR2 on Treg cells, TNF-α–TNFR2 binding can promote immune tolerance by expansion of Tregs. Our data indicate that TNFR1 blockade is sufficient to protect against PNS autoimmunity. This protection may be through direct means, namely lack of a proinflammatory signal via TNFR1. Alternatively, neuropathy protection with TNFR1 deficiency may also potentially be due to secondary up-regulation of TNFR2 in the absence of TNFR1 (95). Our data suggest the former, since mice doubly deficient in TNFR1 and 2 (*NOD.Aire*$^{GW/+}$ *Tnfrsf1a*$^{-/-}$ and *Tnfrsf1b*$^{-/-}$) are also protected from neuropathy development. Thus, TNFR1 deficiency protects from neuropathy development independent of TNFR2 function.

Paradoxically, TNF-α blockade has been sporadically linked to the development of autoimmune conditions, including those for which TNF blockade has been effective as a treatment. For example, TNF-α blockade is used to treat inflammatory bowel disease (IBD), but TNF-α blockade also increases the risk of developing IBD in non-IBD patients receiving anti–TNF-α for other indications (96). Anti–TNF-α agents are also highly effective for the treatment of psoriasis, yet have been associated with the development of psoriasis in patients treated with TNF-α blockade (97). Thus, potentially paradoxical outcomes with TNF-α blockade is seen in multiple immune-mediated conditions. What underlies this effect is not clear, but may be related to loss of TNF-α–mediated inhibition of pDC that produce type 1 IFNs (97, 98). Sporadic cases of inflammatory neuropathies have also been associated with TNF-α blockade when given for other indications (99). Whether this may also be due to removal of TNF-α–mediated pDC inhibition or is attributable to other mechanisms remains to be determined.

Finally, our studies revealed that peripheral nerves affected by autoimmunity contain heterogeneous immune cell types,

many of which were identified as receivers of TNF-α signaling. These include cDCs, γδ T cells, NK cells, NKT cells, and multiple T cell subtypes. How TNF-α signaling alters the phenotype of these heterogeneous immune cells, the potential role for these heterogeneous immune cell types in PNS autoimmunity, and whether these immune cells can be manipulated for therapeutic purpose remains to be determined. Of special interest was the identification of NKT and γδ T cells as receivers of TNF-α signaling within autoimmune nerves. NKT and γδ T cells function at the intersection of innate and adaptive immunity and recognize glycolipid antigens. Because self-glycolipids have been implicated as targets of autoimmunity in GBS (100), it is possible that NKT and γδ T cells may also play a pathogenic role in SAPP and that TNF-α signaling may alter their phenotypic state. In addition to immune cells, TNF-α signaling may also act upon Schwann cells, endothelial cells, and other nonimmune peripheral nerve cells during autoimmunity. For example, TNF-α signaling has previously been reported to inhibit the proliferation, but not the viability, of Schwann cells in the PNS (85). Further studies are therefore warranted to understand how these effects may be potential contributors to the pathogenesis of inflammatory neuropathies.

Materials and Methods

Mice. *NOD.Aire*$^{GW/+}$ mice (18), *NOD.SCID* (JAX 001303), *NOD.Tnfrsf1a*$^{-/-}$ *Tnfrsf1b*$^{-/-}$ (JAX 024314) mice were housed in a pathogen-free barrier facility at the University of North Carolina, Chapel Hill (UNC-CH) and the University of California, Los Angeles (UCLA). Experiments were performed on female mice and complied with the Animal Welfare Act and the NIH guidelines for the ethical care and use of animals in biomedical research. Experiments were also in compliance with the guidelines of the Institutional Animal Care and Use Committees at UNC-CH and UCLA and were approved by the UNC-CH and UCLA Animal Research Committee.

Adoptive Transfer and Anti–TNF-α Antibody Treatment. Adoptive transfer was performed as previously described (14). In brief, splenocytes from neuropathic female *NOD.Aire*$^{GW/+}$ mice (15 to 25 wk of age) were activated with anti-CD3 (eBioscience, clone 145-2C11; 1 μg/mL in PBS) and anti-CD28 (BD Pharmingen, clone 37.51; 1 μg/mL in PBS) overnight at 4 °C or for at least 2 h at 37 °C and washed with PBS. Next, 1×10^6 activated splenocytes were transferred in sterile PBS using retro-orbital injection into immunodeficient female *NOD.SCID* or *NOD.SCID Tnfrsf1a*$^{-/-}$ recipients (8 to 12 wk of age) sedated with 2% isoflurane in oxygen. For antibody treated cohorts, anti–TNF-α antibody (clone: XT3.11, Bio X Cell, Cat#: BE0058) or isotype control antibody (clone: HRPN, Bio X Cell, Cat# BE0088) was administered (300 μg for the first dose, then 150 μg per week for 10 wk) starting at 1 d after transfer.

Neuropathy Assessment and Further Analyses. Assessment of neuropathy development, nerve histology, and nerve conduction studies were performed as previously described (14, 23). In brief, mice were evaluated weekly (spontaneous model) or every other day (adoptive transfer model) for clinical signs of neuropathy. For the spontaneous model, mice were monitored for neuropathy starting at 12 wk of age. For the adoptive transfer model, mice were monitored for neuropathy starting 3 wk after transfer. Scoring for clinical signs of neuropathy were performed as follows: 0 = no signs of neuropathy present; 1 = mild hind limb weakness; 2 = pronounced bilateral hind limb weakness; 3 = reduced or absent ability to grip cage grating; and 4 = moribund. Mice were considered neuropathic when they reached a score of 2 in order to minimize false positives. At this time, mice were subjected to electrophysiologic studies and then killed for further studies. Mice in adoptive transfer model were killed at 11 wk after transfer if neuropathy was not seen. For spontaneous models, mice were killed at 30 wk (*NOD.Aire*$^{GW/+}$ *Tnfrsf1a*$^{-/-}$ *Tnfrsf1b*$^{-/-}$) or 50 wk of age (*NOD.Aire*$^{GW/+}$ *Tnfrsf1a*$^{-/-}$ *Tnfrsf1b*$^{+/-}$) if neuropathy was not seen. Nerve conduction studies were performed using a Teca Synergy T2X EMG system, as described previously (14, 23).

Flow cytometric and scRNA-seq analysis of sciatic nerves were performed as described in *SI Appendix, Materials and Methods*. For nerve histology, sciatic nerves were fixed in 10% buffered formalin for at least 96 h then were embedded in paraffin, sectioned, and stained with H&E by the UNC Animal Histopathology Core. Immune infiltration was scored while blinded to genotype, as described in *SI Appendix, Materials and Methods*.

Data Availability. The data reported in this paper have been deposited in the National Center for Biotechnology Information (NCBI) GEO database (accession no. GSE180498) (101). Previously published data by Wolbert et al. (15) (data is accessible at NCBI GEO database accession no. GSE142541) and Ydens et al. (40) (data accessible at NCBI GEO database accession no. GSE144708) were used for this work.

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