Inactivation of JNK1 enhances innate IL-10 production and dampens autoimmune inflammation in the brain

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Environmental insults such as microbial pathogens can contribute to the activation of autoreactive T cells, leading to inflammation of target organs and, ultimately, autoimmune disease. Various infections have been linked to multiple sclerosis and its animal counterpart, autoimmune encephalomyelitis. The molecular process by which innate immunity triggers autoreactivity is not currently understood. By using a mouse model of multiple sclerosis, we found that the genetic loss of the MAPK, c-Jun N-terminal kinase 1 (JNK1), enhances IL-10 production, rendering innate myeloid cells unresponsive to certain microbes and less capable of generating IL-17–producing, encephalitogenic T cells. Moreover, JNK1-deficient central nervous system myeloid cells are unable to respond to effector T cell inflammatory cytokines, preventing further progression to neuroinflammation. Thus, we have identified the JNK1 signal transduction pathway in myeloid cells to be a critical component of a regulatory circuit mediating inflammatory responses in autoimmune disease. Our findings provide further insights into the pivotal MAPK-regulated network of innate and adaptive cytokines in the progression to autoimmunity.

c-Jun N-terminal kinase | experimental autoimmune encephelomyelitis | innate immunity | macrophages

Although the precise etiology of many autoimmune diseases is still unclear, both environmental and genetic factors have been implicated (1). Experimental autoimmune encephalomyelitis (EAE) serves as a useful prototype to dissect the cellular and molecular mechanisms underlying autoimmunity in multiple sclerosis. In mice, microbial components from mycobacteria or *Bordetella pertussis* can substitute for environmental conditions in the development of EAE. For example, the administration of *B. pertussis* toxin or adjuvant that contains heat-killed *Mycobacterium tuberculosis* is sufficient to trigger EAE in anti-myelin T cell receptor transgenic mice (2, 3). Similarly, housing these transgenic mice in an environment where they are regularly exposed to microbes increases the incidence of spontaneous EAE (3). In nontransgenic mice, immunization with a myelin peptide in the presence of an adjuvant containing mycobacterial components is a common strategy to activate a pathogenic, T cell-mediated, anti-myelin autoimmune inflammation. The triggering of myeloid cell activation by the microbial products initiates the activation of myelin-specific T cells in the peripheral lymphoid organs, resulting in effector T cell invasion of the CNS, neuroinflammation, and limb paralysis, a clinical outcome in EAE.

The c-Jun N-terminal kinases (JNKs) are part of the evolutionarily conserved MAPK pathways that can be activated in a plethora of cell types by mitogenic or proinflammatory signals (4). Once activated, JNKs phosphorylate multiple transcription factors, such as c-Jun, ATF-2, Elk-1, and nuclear factor of activated T cells (NFAT), to impart genomic changes in a cell responding to the environmental stress. There are three different *JNK* genes, *JNK1*, *-2*, and *-3*; each member has multiple isoforms through alternative splicing. JNK1 and -2 are ubiquitously expressed, whereas JNK3 is restricted to the brain, heart, and testis. Studies using gene-targeting disruption of specific JNKs have revealed both overlapping and distinct biological roles in cellular processes such as regulation of adipose cell metabolism, formation of osteoclasts, proliferation, and apoptosis of fibroblasts or neurons (5–8). The functional specification of JNK members is thought to depend on the responding cell types and the nature of the stimuli.

When naïve T cells are stimulated through their T cell receptors and costimulatory molecule CD28, both JNK1 and -2 are activated and have divergent roles in T cell activation, differentiation, and apoptosis *in vitro* (9–12). An *in vivo* consequence of such defects points to the importance of JNK2 in $CD8^+$ T cells to mount antiviral immunity (13) and in the development of autoimmune diabetes in nonobese diabetic (NOD) mice (14). JNK1, however, is implicated in controlling Th2 responses after *Leishmania major* infection (15).

In myeloid macrophages, recognition of pathogen-associated molecular patterns by Toll-like receptors (TLRs) activates NF-κB and MAPKs, which are key molecules in the induction of proinflammatory cytokines, including TNF-α, IFN-γ, IL-6, IL-12, and reactive oxygen/nitrogen intermediates (16). These proinflammatory mediators are critical for killing the pathogens as well as controlling infection by promoting macrophage phagocytosis. Such innate immune response of the myeloid cells also determines the nature of adaptive immune response of $CD4^+$ T cells and the outcome of an inflammation. In addition to JNKs, two other major MAPK pathways (p38 kinases and ERKs) are activated after TLR stimulation. The relative contribution of individual MAPK pathway in TLR signaling remains controversial and appears to depend on the nature of the ligands. For example, p38 and ERKs, but not JNKs, have been shown to be required for macrophage response to mycotoxin (17), bacterial CpG, or endotoxin from Gram-negative bacteria (18), whereas p38 and JNK activation mediate macrophage immunity to Shiga toxin (19). It remains unclear how JNKs may influence macrophage innate response to pathogenic stimuli in the development of T cell-mediated autoimmune inflammation in the brain. Here we show that JNK1 is a reciprocal regulator of myeloid pro- and anti-inflammatory cytokine balance and the encephalitogenicity of brain-reactive T cells.

Results and Discussion

To explore the role of JNK1 in myeloid cell activation and EAE, we used C57BL/6 mice with a disrupted *JNK1* gene. These

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Abbreviations: CFA, complete Freund's adjuvant; DLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; Th, T helper; TLR, Toll-like receptor; Treg, T regulatory cell.

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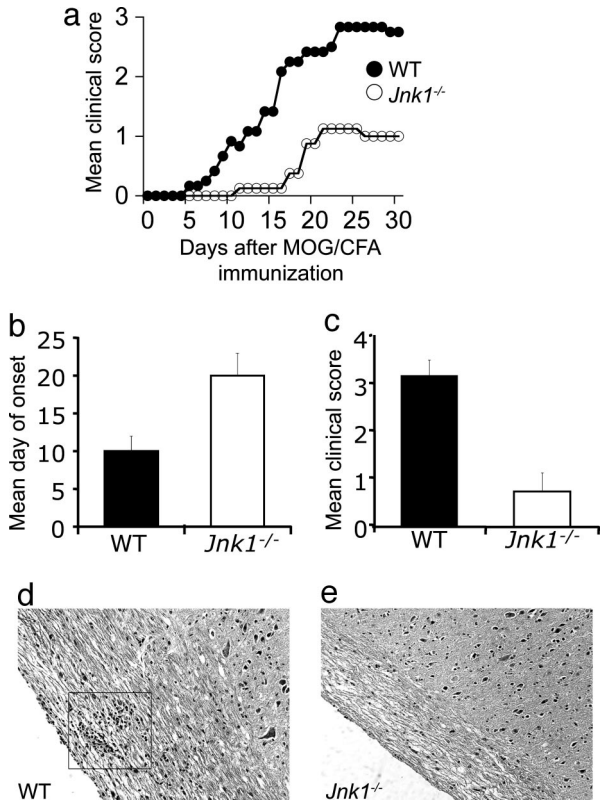

Fig. 1. Reduced induction of EAE in JNK1$^{-/-}$ mice. After immunization with MOG/CFA, mice were monitored daily for the development of clinical signs of EAE. Shown are results in female mice ($n = 8$ per group) (*a*) and male mice ($n = 6$ per group) (*b* and *c*). Data are presented as mean day of onset and mean maximum clinical score. (*d* and *e*) Hematoxylin and eosin staining of spinal cord sections from a WT female mouse with grade 3 EAE (*d*) and an asymptomatic JNK1$^{-/-}$ mouse (*e*). Leukocyte infiltrates are boxed. Data are representative of five experiments.

knockout mice showed no major perturbation in hematopoiesis and T cell development (11). However, after immunization with the encephalitogenic myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide in complete Freund's adjuvant (CFA), JNK1$^{-/-}$ mice showed a substantially reduced disease progression than that seen for wild-type (WT) control mice (Fig. 1). Hormonal influence on the immune system is suspected to contribute to gender difference in EAE susceptibility in various strains of mice (20, 21). For the C57BL/6 strain, no gender difference was noted in our study and others (20, 21). Thus, female (Fig. 1*a*) or male (Fig. 1 *b* and *c*) JNK1$^{-/-}$ mice were equivalently more resistant to EAE than WT C57BL/6 mice. This clinical suppression of EAE correlated with a reduced inflammatory infiltration in spinal cords (Fig. 1 *d* and *e*) and brains (data not shown). Although both JNK1 and -2 are expressed in lymphoid and myeloid cells, JNK2$^{-/-}$ mice are as susceptible as WT mice to EAE (22). Thus, JNK1, but not JNK2, plays a necessary and nonredundant role in the development of EAE. As in other cellular processes, the functional difference between JNK1 and -2 may reside in their differential action on transcription factors (23).

Because EAE requires the generation of myelin-specific CD4 T helper type 1 (Th1) cells (24), we examined whether T cell activation or differentiation in response to MOG peptide immunization was defective in lymphoid organs of JNK1$^{-/-}$ mice. The lack of JNK1 was found to repress the recall proliferative response to MOG (Fig. 2*a*), but not to ConA (Fig. 2*b*), indicating a defect in the generation of MOG-specific T cells. Further, when compared with WT mice, draining lymph node (DLN) cells and splenocytes of the JNK1$^{-/-}$ mice secreted lower amounts of Th1 cell-associated cytokines (IL-2, IFN-γ, TNF-α) and chemokines [macrophage inflammatory protein 1α (MIP-1α/CCL3), regulated upon activation normal T cells expressed and secreted (RANTES/CCL5)] in response to MOG stimulation (Fig. 2*c*). By contrast, JNK1$^{-/-}$ splenocytes produced equivalent levels of IL-4, the signature Th2 cell cytokine (Fig. 2*c*). Because JNK1$^{-/-}$ T cells can preferentially differentiate to Th2 cells (9), we think that such enhanced ability may contribute to the overall normal level of IL-4, despite a reduced proliferation or generation in response to MOG in JNK1$^{-/-}$ mice. In addition, antigen-presenting cells of the innate immune system were reported to be important sources of IL-4 in response to MOG immunization (25). We speculate that JNK1 might negatively regulate IL-4 production in these innate cells. All together, our data support an impairment of MOG-specific Th1 responses in the JNK1$^{-/-}$ mice.

Recent data suggest that further differentiation of effector/memory T cells into a subset that produces the cytokine IL-17 is crucial for the development of EAE (26). We found that JNK1$^{-/-}$ lymphoid cells were profoundly defective in IL-17 production relative to WT cells (Fig. 2*c*). Because IL-17 is produced by memory and effector T cells (27), we investigated whether there was an impairment in the generation of these T cells in JNK1$^{-/-}$ mice in response to MOG/CFA immunization. By using flow cytometric analysis, we detected a much lower frequency of CD44^{+}CD62L^{-} memory CD4^{+} T cells and activated CD134^{+}CD4^{+} T cells in immunized JNK1$^{-/-}$ mice (Fig. 2 *d* and *e*). The appearance of CD134^{+}CD4^{+} T cells normally increases during EAE, and their significance is evidenced by prevention of disease in mice that were either deficient in CD134 or treated with a blocking anti-CD134 antibody (Ab) (28). Thus, the absence of JNK1 blocks the generation of memory/effector CD4^{+} T cells in response to MOG, resulting in fewer IL-17–producing encephalitogenic T cells in the circulation. Interestingly, the development of virus-specific CD4 T cells was also thwarted in JNK1$^{-/-}$ mice as compared with WT or JNK2$^{-/-}$ mice after lymphochoriomeningitis virus infection (13).

Cytokines, such as granulocyte/macrophage colony-stimulating factor (GM-CSF) and IL-3, promote the activation and expansion of the myeloid cell population in response to immunization (29). Activated macrophages produce various inflammatory mediators, including IL-6 and the chemokine KC/CXCLI (30). We found that these myeloid cytokines were made in much lower amounts in MOG/CFA-immunized JNK1$^{-/-}$ mice (Fig. 2*c*), suggesting a perturbation in the myeloid cell response when JNK1 activity is absent. To further test this hypothesis, we generated JNK1$^{-/-}$ and WT bone marrow-derived monocytes/macrophages and stimulated them with heat-killed *M. tuberculosis* (which is present in CFA). *B. pertussis* toxin was not tested here because it stimulates primarily the ERK and p38, but not the JNK, pathways (2). Fig. 3*a* shows that the lack of JNK1 in macrophages selectively repressed their production of the inflammatory cytokine TNF-α but enhanced their production of the anti-inflammatory cytokine IL-10. Further, IFN-γ and the chemokine KC were reduced in JNK1$^{-/-}$ macrophages, but neither IL-1α nor IL-1β was affected (Fig. 3 *b* and *c*). When exposed to other microbial products, such as LPS (Fig. 3*e*) or CpG (data not shown), JNK1$^{-/-}$ macrophages secreted WT levels of TNF-α and IL-10. The production of other cytokines, such as IL-12, was not altered in the absence of JNK1, regardless of the microbial sources used in the stimulation (Fig. 3 *d* and *e*). Thus, the myeloid JNK1 signaling pathway regulates the pro- and anti-inflammatory balance in response to certain microbial components, such as *M. tuberculosis*, but not others. That JNK1 was dispensable for the macrophage response to LPS was consistent with our previous report (15). Because the T cell

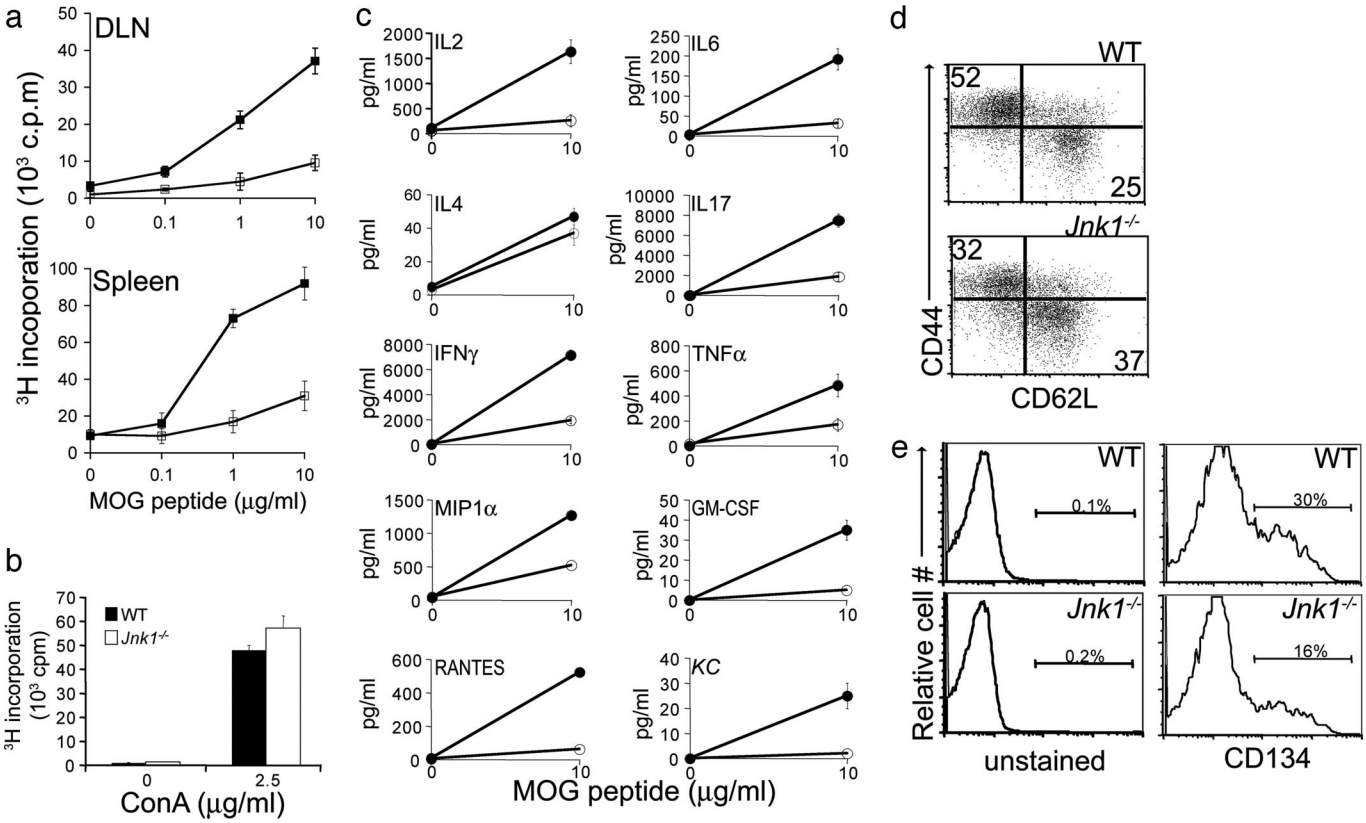

Fig. 2. Impaired generation of MOG-reactive T cells in JNK1$^{-/-}$ mice. Seven days after immunization with MOG/CFA, DLNs and splenocytes were isolated from WT (filled squares) or JNK1$^{-/-}$ (open squares) mice to measure their proliferative responses to MOG (*a*) or ConA (*b*). (*c*) The same number of splenocytes from MOG-immunized WT (filled circles) or JNK1$^{-/-}$ (open circles) mice were restimulated with MOG, and the production of cytokines and chemokines was measured. (*d* and *e*) Flow cytometric analysis of a gated splenic CD4$^+$ T cell population for a memory CD44$^+$CD62L^{lo} phenotype (*d*) or for the expression of CD134 (*e*) 12 days after immunization with MOG/CFA. Results are representative of three experiments.

cytokine IFN-γ is a strong activator of macrophages, we investigated whether the addition of IFN-γ would restore the production of TNF-α in JNK1$^{-/-}$ myeloid cells. Although exogenous IFN-γ increased the levels of TNF-α (from a mean of 0.42 to 3.9 ng/ml), JNK1$^{-/-}$ macrophages still produce less TNF-α and more IL-10 than WT cells (Fig. 3*f*). Hence, the altered development of encephalitogenic Th1 cells and IL-17–producing effector T cells in JNK1$^{-/-}$ mice is likely to be associated with the requirement of JNK1 activity in the myeloid compartment. The cytokine IL-10 is known to inhibit myeloid cell antigen-presenting and effector functions as well as counteract the activity of IL-12 (31). Thus, in the absence of JNK1 function in myeloid cells, immune suppression can result at several levels, including pathogenic autoreactive T cell generation, effector cytokine imbalance, and resistance to EAE induction.

To verify that lymphoid JNK1 activity is dispensable for the generation of pathogenic CD4 T cells and inflammation in the brain, we examined the ability of naïve JNK1$^{-/-}$ lymphoid cells to become encephalitogenic in a host with normal JNK1 function. We isolated naïve CD4 T cells from JNK1$^{-/-}$ or WT mice and transferred them into *Rag1*-deficient recipients, which otherwise lack T cells. All host cells, including myeloid, therefore had an intact JNK1 gene, whereas only CD4 T cells were either defective or normal for their JNK1 function. After immunization with MOG/CFA, all recipients exhibited a comparable incidence, severity, and clinical course of EAE (Fig. 4*a*), indicating that both JNK1$^{-/-}$ and WT T cells had comparable encephalitogenic capacity. Histological examination of the CNS also revealed a comparable degree of leukocyte infiltration and cellular composition (data not shown). Thus, the absence of JNK1 in T cells does not alter their ability to mount a pathogenic autoimmune response to myelin. This finding is surprising in light of previous studies, in which purified JNK1$^{-/-}$ CD4 T cells showed defects in T cell activation (9) or differentiation *in vitro* (11). Although the exact reasons for such differences are unclear, possible explanations include the mouse genetic background, the stimulation method, and the complexity of an *in vivo* environment. In addition, IL-17 is key to EAE and was not measured in those studies.

To further support the importance of nonlymphoid JNK1 activity in neuroinflammation, we performed adoptive transfer of MOG-reactive, effector WT T cells into either WT or JNK1-deficient hosts. According to the prevailing concept, the clinical signs of EAE are manifested in this model after the transferred encephalitogenic effector T cells infiltrate the CNS and stimulate local myeloid cells (e.g., microglia, macrophages, or dendritic cells), which present myelin to them for their reactivation *in situ* (32, 33). As expected, WT mice receiving MOG-reactive effector WT T cells developed clinical signs of EAE (Fig. 4*b*). In sharp contrast, JNK1$^{-/-}$ mice that received WT effector T cells failed to develop disease (Fig. 4*b*). Furthermore, these pathogenic WT T cells failed to stimulate JNK1$^{-/-}$ macrophage or microglial activation as judged by minimal CD11b/Mac-1 immunoreactivity on JNK1$^{-/-}$ spinal cord sections (Fig. 4*c*). Because CD25$^+$CD4$^+$ T regulatory cells (Tregs) have been implicated in suppressing autoimmune T cell activation and EAE (34), we investigated whether the resistance to the adoptive EAE in JNK1$^{-/-}$ mice could also be attributed to a higher number of Tregs or an enhanced suppressive function. Treg number in naïve JNK1$^{-/-}$ mice was comparable with that

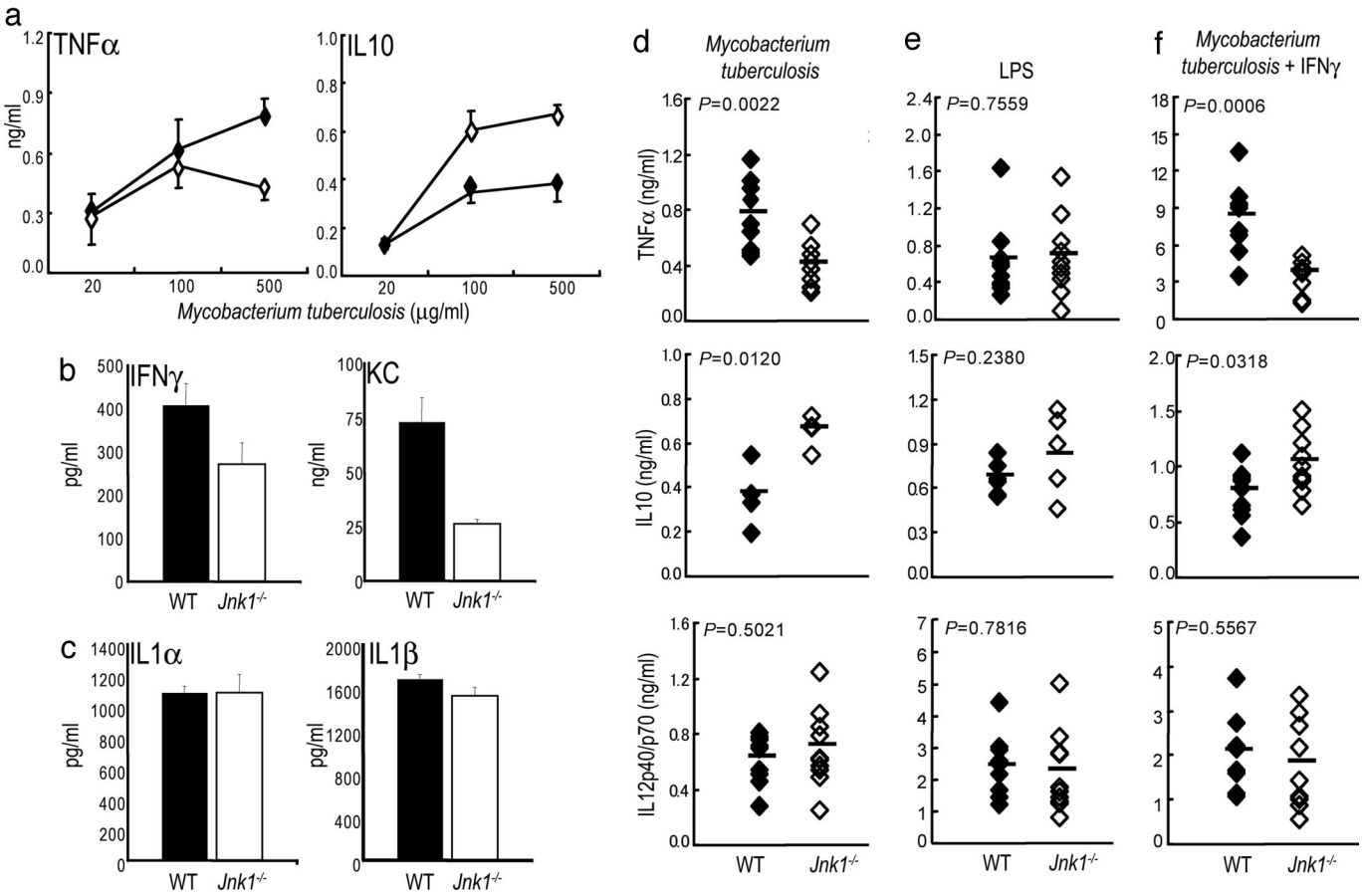

Fig. 3. JNK1$^{-/-}$ myeloid cells produce less TNF-α and KC but more IL-10. (*a*) Production of TNF-α and IL-10 by WT (filled diamonds) and JNK1$^{-/-}$ (open diamonds) bone marrow-derived macrophages after stimulation with titrated amounts of heat-killed *M. tuberculosis*. Data represent the mean of 12 and 7 mice per group for TNF-α and IL-10, respectively. Error bars indicate SE. (*b* and *c*) Secretion of IL-1α, IL-1β, IFN-γ, and the chemokine KC by macrophages after stimulation with 500 μg/ml *M. tuberculosis*. Each bar represents the mean of three mice per group. (*d–f*) Production of the cytokine TNF-α, IL-10, and IL-12 by macrophages when cultured with LPS (10 ng/ml) or *M. tuberculosis* (500 μg/ml) in the presence or absence of IFN-γ (20 units/ml) for 24 h. Symbols represent individual mice, and the mean is indicated by a solid line. *P* values were calculated with Student's *t* test.

in WT mice (7% vs. 8%, respectively). In addition, we found no difference between JNK1$^{-/-}$ and WT Treg in their ability to suppress the activation of CD25^{-}CD4^{+} T cells by using the standard *in vitro* coculture assay (Fig. 4*d*). Hence, EAE resistance in JNK1$^{-/-}$ recipients is not due to enhanced Treg function on the transferred encephalitogenic effector T cells. Rather, one possible scenario is that microglial activation, which is crucial for EAE progression (35), requires JNK1 activity to mediate signals from effector T cell proinflammatory cytokines, such as IL-17. Of interest, inhibitors of the JNK pathways were shown to block IL-17 responsiveness in human macrophages (36). Also, JNK1$^{-/-}$ macrophages did not seem to respond well to the effector T cell cytokine IFN-γ (Fig. 3*f*). Because CNS microglia are bone-marrow derived, our data suggest that JNK1 may positively regulate T cell inflammatory signaling in microglia and macrophages for EAE progression in mice. Alternatively, JNK1 may modulate TLR and myeloid differentiation factor 88 (MyD88) signaling in microglia and macrophages within the CNS for the progression of EAE. In support of this hypothesis, stimulation of TLR on microglia has been shown to enable them to efficiently present myelin antigens to CD4^{+} T cells (37). Moreover, mice deficient in either MyD88 or TLR9 did not develop EAE after the adoptive transfer of WT encephalitogenic T cells (38). Our observation that WT encephalitogenic T cells failed to induce EAE in JNK1$^{-/-}$ recipient mice upon transfer points to a potential role for JNK1 in the regulation of TLR signaling in CNS myeloid cells. The use of an adoptive transfer system is particularly useful to examine microglial activation and EAE progression without the complication of the adjuvant effect, which persists in the immunization model. In fact, in addition to its activation of the innate immunity in lymphoid organs, *M. tuberculosis* in CFA can cause other systemic changes (e.g., myelopoeisis, blood–brain barrier damage, and activation of CNS resident cells) that result in lowering the threshold for the induction of neuroinflammation (39–41). As illustrated in Fig. 3, the absence of JNK1 selectively affects the production of cytokines in response to heat-killed *M. tuberculosis*. The production of IL-1 was normal, suggesting that certain systemic changes induced by CFA likely remain intact in JNK1$^{-/-}$ mice. Hence, these systemic factors may contribute to the mild EAE in MOG/CFA-immunized JNK1$^{-/-}$ mice (Fig. 1), in which the initial immunization failed to produce a critical quantity of encephalitogenic T cells (Fig. 2). Because CFA was not given to recipient mice in the adoptive transfer model, the CFA influence that facilitates neuroinflammation is absent, thereby increasing the threshold for the activation of CNS microglia and progression to EAE. Therefore, the CFA effect likely contributes to the mild disease in the immunization model and its absence to the lack of disease in the adoptive transfer model.

Collectively, our studies point to a critical and nonredundant role for JNK1 activity in nonlymphoid cells as being an important regulator of inflammation in the brain and possibly other organs.

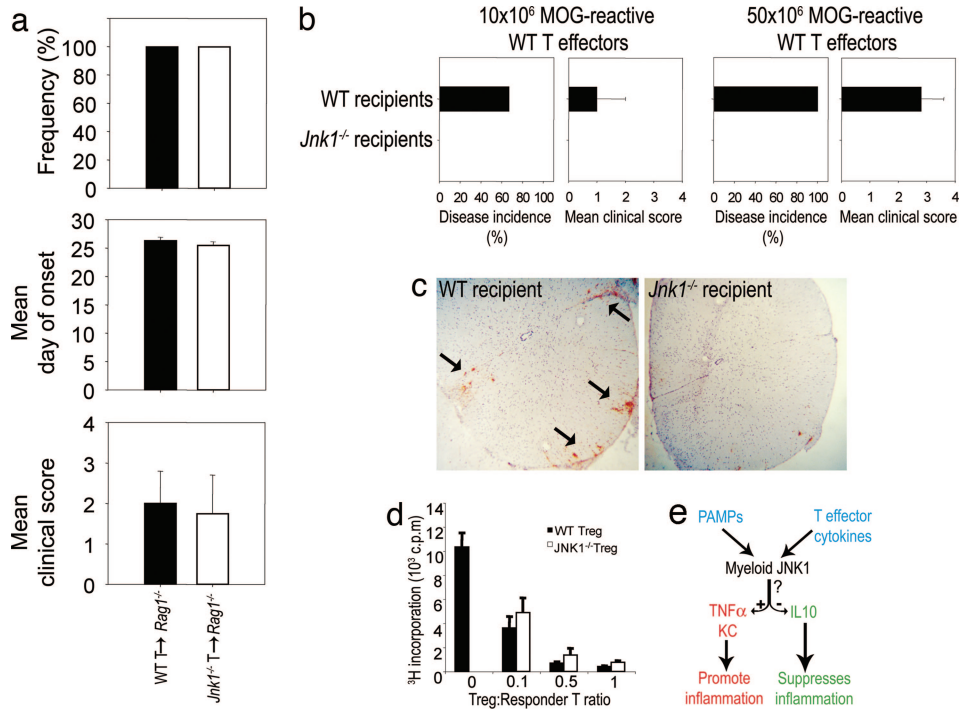

Fig. 4. Adoptive transfers of naïve or effector T cells. (*a*) WT or JNK1$^{-/-}$ naïve CD4$^+$ T cells equivalently provoked EAE in *Rag1*$^{-/-}$ hosts ($n = 4$ per group) in response to immunization with MOG/CFA. (*b*) MOG-reactive WT effector T cells failed to induce EAE in JNK1$^{-/-}$ recipient mice ($n = 4$). This procedure was repeated in a separate experiment with another four recipients per group. (*c*) Spinal cord sections from a WT recipient with EAE and an asymptomatic JNK1$^{-/-}$ recipient were immunostained for CD11b/Mac-1$^+$ (arrows) activated microglia and macrophages. (*d*) WT or JNK1$^{-/-}$ Tregs (CD25$^+$CD4$^+$) had the same potency in suppressing the activation of WT responder effector T cells. (*e*) A proposed model implicating myeloid JNK1-mediated regulation of counteracting immune cytokines and responses.

We propose that JNK1 activity in myeloid cells is induced in response to microbial pathogens and functions as a reciprocal regulator of counteracting cytokines that shape the outcome of the innate and acquired immune response (Fig. 4*e*). Invading microbial pathogens are detected by TLRs on myeloid cells. Although TLR signaling depends heavily on myeloid differentiation factor 88 (MyD88) (16), JNK1 activity may further fine-tune the innate myeloid cell response. Of note, it may do so selectively depending on the types of pathogens, TLRs, or inflammatory milieu. Our findings that JNK1 contributes to both positive and negative regulation of counteracting myeloid-derived cytokines suggest that this pathway is a viable target for therapeutic intervention in autoimmune and infectious diseases.

Methods

Mice and Immunization. JNK1$^{-/-}$ mice (11) were backcrossed onto the C57BL/6 genetic background for at least nine generations, and WT C57BL/6 control mice were purchased from The Jackson Laboratory (Bar Harbor, ME). RAG1$^{-/-}$ mice were bred and maintained in our animal facilities. EAE was elicited in female or male mice at 8–12 weeks of age by s.c. tail-base injection of 50 μg of MOG$_{35-55}$ peptide (35-MEVGWYRSPF-SRVVHLYRNGK-55) in CFA containing 500 μg of heat-inactivated *M. tuberculosis* (H37RA; DIFCO Labs, Sparks, MD), and 200 ng of *B. pertussis* toxin (LIST Biological Labs, Inc., San Jose, CA) was injected i.p. on days 0 and 2. Mice were monitored daily for clinical signs of EAE and scored as follows: 1, flaccid tail; 2, inability to right and one hind limb paralysis; 3, paralysis of both hind limbs with or without incontinence; 4, paralysis of all limbs; and 5, moribund. Supplementary food and water were provided on the cage floor for disabled animals. Animal maintenance and all experimental protocols were in accordance with Yale Animal Research Committee.

Adoptive Transfer of MOG-Reactive Effector T Cells. C57BL/6 WT mice were immunized as described above. Ten days later, a single cell suspension was prepared from DLNs, and cells (3×10^6 per ml) were cultured in the presence of 10 μg of MOG and 5 units/ml recombinant human IL-2 in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FCS, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 4 days of culture, nonadherent T cells were collected by centrifugation on Ficoll, and 10–50 $\times$ 10^6 T cells were injected i.v. into naïve WT or JNK1$^{-/-}$ recipient mice. Each mouse also received 200 ng of pertussis toxin on days 0 and 2 after transfer.

Adoptive Transfer of Naïve T Cells. Naïve T cells (CD4$^+$CD62L^{hi}CD44^{lo}) from WT or JNK1$^{-/-}$ mice were isolated on a FACS Vantage sorter, and 10 $\times$ 10^6 were injected in the tail vein of RAG1$^{-/-}$ recipient mice. After 24 h, all recipients were immunized with MOG as described above.

***In Vitro* Recall Response Assay.** Cells from spleens or DLNs were isolated 6 days after immunization and were cultured for 4 days in the presence of indicated amounts of MOG peptide or Con A (Sigma, St. Louis, MO). To assess proliferation, cultures were pulsed with 1 uCi (1 Ci = 37 GBq) of [^{3}H]thymidine during the last 18 h of incubation. The mean [^{3}H]thymidine uptake in triplicate wells was measured as cpm. To measure production of cytokines and chemokines, supernatants were collected after 2–4 days of culture with MOG and subjected to analysis by specific enzyme-linked immunosorbent assays ELISA (for IL-2, IL-4, and IFN-γ), FlowCytomix (for IL-6 and TNF-α), and Bio-Plex [for MIP-1α, RANTES, KC, granulocyte/macrophage colony-stimulating factor (GM-CSF), and IL-17] according to the manufacturer's instructions. Paired Abs against cytokines in ELISA were purchased from PharMingen (San Diego, CA).

FlowCytomix multiplex bead kits were obtained from BenderMedSystems (Burlingame, CA), and Bio-Plex multiplex bead kits from Bio-Rad Laboratories (Hercules, CA).

Flow Cytometry. Cells were stained with fluorochrome-conjugated primary Abs for 30 min at 4°C in PBS containing 1% FCS and 0.01% sodium azide, washed twice, acquired on FACSCalibur flowcytometer (BD Biosciences, San Diego, CA), and analyzed with FlowJo software (Tree Star, Ashland, OR). All Abs against CD4, CD25, CD44, CD62L, and CD134 were purchased from PharMingen.

Histology. Spinal cords were removed after intracardial perfusion with ice-cold PBS and were fixed in 10% formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. Immunohistochemistry on 10-μm frozen sections was performed as described (42). The primary rat Ab against mouse CD11b/Mac-1 was obtained from PharMingen, and the biotinylated secondary Ab was from Vector Laboratories (Burlingame, CA).

Cytokine Production by Bone Marrow-Derived Macrophages. Bone marrow cells were cultured in DMEM (Invitrogen) with 20% FBS and 30% L929 supernatant containing macrophage-stimulating factor for 6 days. Macrophages (1×10^6 cells per ml) were then cultured in DMEM supplemented with 5% FBS in the presence of heat-killed *M. tuberculosis*, LPS, CpG, or medium alone for 24 h. The concentration of cytokines and chemokines was determined in culture supernatants by ELISA (PharMingen and eBioscience, San Diego, CA) and Bio-Plex analysis (Bio-Rad), according to the manufacturer's guidelines.

Treg Suppression Assay. $CD25^-CD4^+$ responder T cells (5×10^4 cells per well) and $CD25^+CD4^+$ Tregs were separated on a MoFlo cytometer and cultured at the indicated ratio in 96-well round-bottom plates with T cell-depleted spleen cells as accessory cells (3,000 R irradiated) and 0.5 μg/ml anti-CD3 Ab (clone 2C11) for 72 h. Cultures were pulsed with [^{3}H]thymidine for the last 6 h of culture.

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