Maternal hematopoietic TNF, via milk chemokines, programs hippocampal development and memory

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Tumor necrosis factor α (TNF) is a proinflammatory cytokine with established roles in host defense and immune system organogenesis. We studied TNF function and found a previously unidentified physiological function that extends its effect beyond the host into the developing offspring. A partial or complete maternal TNF deficit, specifically in hematopoietic cells, resulted in reduced milk levels of the chemokines IP-10, MCP-1, MCP-3, MCP-5 and MIP-1β, which in turn augmented offspring postnatal hippocampal proliferation, leading to improved adult spatial memory in mice. These effects were reproduced by the postpartum administration of a clinically used anti-TNF agent. Chemokines, fed to suckling pups of TNF-deficient mothers, restored both postnatal proliferation and spatial memory to normal levels. Our results identify a TNF-dependent ‘lactocrine’ pathway that programs offspring hippocampal development and memory. The level of ambient TNF is known to be downregulated by physical activity, exercise and adaptive stress. We propose that the maternal TNF–milk chemokine pathway evolved to promote offspring adaptation to post-weaning environmental challenges and competition.

RESULTS
Parental TNF deficit increases spatial reference memory
To evaluate the effect of maternal or parental TNF on offspring behavior, we generated wild-type (WT) male offspring by crossing either wild-type or TNF+/− (heterozygote) parents1, all on the C57BL/6NTac (B6Tac) genetic background (Supplementary Fig. 1a). Although the wild-type offspring of wild-type parents (referred to as WToffspring(WTparents/mother), that is, WT(WT) mice) developed in a wild-type parental environment, the wild-type offspring of heterozygote parents (WT(H) mice) were exposed to a receptor-deficient maternal environment both pre- and postnatally. These two groups of offspring were complemented with TNF−/− offspring obtained by crossing either TNF+/− or TNF−/− parents, referred to as KO(H) and KO(KO) mice.

We assessed the spatial learning and memory capabilities of these offspring in the Morris water maze (MWM). Although five consecutive training sessions in the MWM (Supplementary Fig. 1b) resulted in no change in spatial learning6. The use of non-littermate5 and littermate5 controls could explain these discrepancies, as the parental genotype can substantially influence the offspring genotype effect in a non-littermate comparison7. Indeed, we found that a deficit in maternal TNF had a more marked effect on offspring episodic memory than a deficit in the offspring’s own TNF. We observed reduced levels of a group of chemokines in the milk of TNF−/− and TNF+/− mothers, which, when given in supplemental doses, normalized cognitive functions. Further data suggested that the maternal TNF-regulated lactocrine pathway is evolutionarily adaptive and that TNF suppressive drugs taken during the postpartum period may alter the functionality of this pathway and, consequently, long-term spatial memory.
in the gradual learning of the location of the invisible platform in all groups (Fig. 1a), the probe trial on day 6 (in which the platform was removed) revealed a difference between the groups in their ability to recall the location of the platform (Fig. 1b). Specifically, the WT(WT) offspring failed to recall the location, whereas the WT(H) offspring spent significantly more time in the northwest quadrant that previously contained the platform (P < 0.05). KO(H) and KO(KO) mice were also able to recall the platform location (Fig. 1b). An additional 5 d of training resulted in the recall of the platform location in probe trial 2 in all groups, indicating that the WT(WT) group was not incapable of learning, but rather required more training under our experimental conditions to recall memories; regardless of maternal genotype, the effect on recall was still present (Supplementary Fig. 1b,c). No group difference was seen in the visible platform task and in the total distance traveled in the MWM (data not shown).

Associative memory of wild-type mice of mutant parents was assessed in contextual fear conditioning (Supplementary Fig. 1d), which, similar to MWM, requires hippocampal activity, although the two tests differ in sensory and motor demands, as well as motivational aspects. The parental TNF genotype had a significant effect in the contextual (P = 0.006), but not cued, fear test (Fig. 1c–f), suggesting a hippocampal-related, but not amygdala-related, change in the offspring. However, the effect was seen only when maternal TNF was completely eliminated. Although the KO(KO) offspring also exhibited increased freezing during fear conditioning (Fig. 1d), this effect disappeared by the end of the training and before the contextual fear testing began. We conclude that the partial and/or complete genetic inactivation of TNF in the parents, independently of the presence or absence of TNF in the offspring, facilitates the acquisition and recall of hippocampal-dependent memory beyond the normal physiological level in the adult offspring.

**Maternal hematopoietic TNF is linked to offspring memory**

TNF is primarily expressed in the immune system (macrophages, neutrophils and lymphocytes), but is also expressed in the CNS (in glia and neurons). We inactivated both maternal TNf alleles in the hematopoietic system and brain using a loxP-flanked Tnf allele in combination with polyinosinic-polyctydidylic acid (polyIC)-inducible Mx1-cre and the nestin-cre transgenes, respectively (Supplementary Fig. 2a). Real-time PCR–based quantification of the Tnf allele revealed its extensive deletion in the spleens of 9-week-old Mx1-cre females.
Figure 2  Hematopoietic system specific inactivation of the Tnf gene in the mother results in enhanced memory. (a) Inducible Mx1-cre expression resulted in recombination at the TnfloxP/loxP allele, which led to a reduction in the overall expression of this allele in the spleen, but not in brain. In contrast, nestin-cre expression resulted in a substantial reduction of the TnfloxP/loxP allele in brain (for example, cortex and hippocampus), neurons (for example, CA1 and dentate gyrus (DG) of the hippocampus) and glia (for example, corpus callosum, CC) but not in spleen. (b) Reduction of Tnf mRNA was limited to the spleen in TnfloxP/loxP; Mx1-cre mice, whereas this reduction was brain specific in TnfloxP/loxP; nestin-cre mice. (c) Probe trial 1 of the MWM with the offspring of TnfloxP/loxP; Mx1-cre and TnfloxP/loxP; nestin-cre mothers. There was an effect of the platform location in both groups (ANOVA: TnfloxP/loxP; Mx1-cre, F3,56 = 35.69, P < 10−5; N = 7 and 9 mice per group; TnfloxP/loxP; nestin-cre, F3,80 = 42.43, P < 10−5; N = 9 and 13 mice per group), but a group × location interaction was seen only with the offspring of TnfloxP/loxP; Mx1-cre mothers (F3,56 = 3.09, P = 0.03) and LSD test revealed that offspring of TnfloxP/loxP; Mx1-cre mice, but not TnfloxP/loxP; nestin-cre mothers, had an increased memory of the platform location (∗P < 0.05, ***P < 0.0005). Data are from three independent experiments. (d) Increased freezing of the offspring of TnfloxP/loxP; Mx1-cre (N = 13 and 13 mice per group), but not TnfloxP/loxP; nestin-cre mothers (N = 7 and 10 mice per group), during contextual fear testing (t test: TnfloxP/loxP; Mx1-cre, *P = 0.014; TnfloxP/loxP; nestin-cre, *P = 0.42). Box-whisker plots represent the first three quartiles (25%, median and 75%) and values 1.5× the interquartile range below (lower horizontal line) and above the third quartile (upper horizontal line). All data are presented as mean ± s.e.m.

(ΔΦloxP/loxP; Mx1-cre), injected with polyIC 3 weeks earlier, although the allele was largely intact in the cortex and hippocampus (Fig. 2a). In nestin-cre (ΔΦloxP/loxP; nestin-cre) females, Tnf was deleted in the cortex, hippocampus, isolated CA1 and dentate gyrus neurons, and glia rich corpus callosum, whereas its level in spleen remained close to that in the TnfloxP/loxP mice (Fig. 2a). PCR quantification of Tnf mRNA levels confirmed the spleen- and brain-specific gene deletions in the TnfloxP/loxP; Mx1-cre and TnfloxP/loxP; nestin-cre mice, respectively (Fig. 2b). Consistent with these results, control (ΔΦloxP/loxP) spleens contained 378 ± 50 pg Tnf per g of protein, as measured by ELISA, whereas the level in TnfloxP/loxP; Mx1-cre spleen was undetectable (<70 pg per g of protein), indicating that at least 80% of the Tnf protein was eliminated. The hematopoietic specificity of Mx1-cre-mediated recombination was further verified by the cre-reporter Gt(Rosa26)Sor<tm1Sor/J> strain 5 (Supplementary Fig. 2b). Taken together, expression of the Mx1-cre and nestin-cre transgenes resulted in the tissue-specific deletion of the Tnf allele and reduction of TNF mRNA and protein, beyond 50%, which is the level sufficient to produce the spatial memory phenotype.

Male offspring of polyIC-inducible TnfloxP/loxP; Mx1-cre mothers, as compared with those of TnfloxP/loxP mothers, showed a better than normal performance in the probe trial (Fig. 2c) and training sessions of the MWM test (Supplementary Fig. 3a), whereas the performance of the offspring of TnfloxP/loxP; nestin-cre and TnfloxP/loxP mothers was comparable (of note, mice of the B6 strain achieved learning with five training sessions that was suboptimal in B6Tac mice; Fig. 1b). Improved memory was seen in all groups after an additional 5 days of training, resulting in no difference between the offspring of TnfloxP/loxP; Mx1-cre and TnfloxP/loxP mothers (Supplementary Fig. 3b). Offspring of TnfloxP/loxP; Mx1-cre mothers, injected with polyIC, had no enhanced learning or memory, indicating that polyIC and the Mx1-cre transgene had no confounding effects by themselves (Supplementary Fig. 3c). Offspring of TnfloxP/loxP; Mx1-cre, but not TnfloxP/loxP; nestin-cre, mothers also showed increased freezing during contextual fear testing (Fig. 2d) and tone-shock pairings (Supplementary Fig. 3d), but not during cued fear testing (data not shown). In summary, a TNF deficit that was limited to the maternal hematopoietic system was sufficient to augment both spatial and associative memory beyond their normal physiological levels in the offspring.

Maternal TNF regulates hippocampal proliferation

Both hippocampal spatial and associative memory are positively correlated with neuronal proliferation in the adult dentate gyrus. We tested whether this correlation applies to the offspring of Tnf−/− mothers exhibiting higher than normal hippocampal-dependent memory. Using BrdU labeling, we found increased proliferation in postnatal day 14 (P14) in WT(H), KO(H) and KO(KO) offspring, but not at P5, P35 and adult (Fig. 1b and Supplementary Fig. 3a). This trend was more pronounced in the KO(H) offspring (P = 0.074 and 0.098, respectively); Supplementary Fig. 3a), proportionately with the increase in the number of BrdU+ cells (Supplementary Fig. 4b); thus, the fraction of BrdU+ cells was comparable across the groups and represented 6.7–7.8% of all neurons in the granule cell layer (GCL; Supplementary Fig. 4c). However, the total cell number in the GCL was normalized by the time mice reached adulthood, similar to that of the BrdU+ cells. Increased proliferation at P14 was specific for the dorsal dentate gyrus, as other neurogenic areas, such as the hilus, the subventricular zone and rostral migratory stream, showed no maternal genotype–dependent change in the offspring of mutant mothers (Supplementary Fig. 4d,e). The number of ‘surviving’ BrdU+ cells in the dentate gyrus 3 weeks after labeling at P14 (>95% NeuN+) was different among the
Figure 3 Increased proliferation in the developing dentate gyrus is linked to enhanced adult spatial memory in the offspring of TNF mutant mothers. (a–e) Proliferation in the dorsal (d) subgranular zone (SGZ) at P5, P14 and adult as measured 2 h after BrdU labeling. At P14, ANOVA revealed a group difference in the number of BrdU+ cells (F3,16 = 7.38, P = 0.003, N = 5 mice per group; LSD post hoc, *P < 0.05, **P < 0.005, ***P < 0.0005). (d) Maternal TNF genotype had no effect on QNP proliferation (F3,16 = 0.69, P = 0.57), but did increase ANP proliferation (F3,16 = 7.9, P < 0.001, N = 5 mice per group; LSD post hoc, *P < 0.05, **P < 0.005, ***P < 0.0005) at P14. (e) Confocal micrographs of WT(WT) and WT(H) SGZs with arrows showing QNPs characterized by BrdU and Sox-2 expression and GFAP+ apical extensions toward the molecular layer of the dentate gyrus. Scale bars represent 20 μm. (f) Representative micrographs showing an increased number of BrdU+ Tbr2+ ANPs in the SGZ of WT(H), KO(H) and KO(KO) offspring as compared with the WT(WT) offspring at P14. Scale bars represent 50 μm. All data are presented as mean ± s.e.m.

Maternal TNF deficit causes adult hippocampal changes

Next we tested whether there are persistent functional and/or structural changes in dentate gyrus granule cells as a result of the transient maternal effect that can potentially explain the enhanced memory of adult WT(WT) mice. RNA-Seq–based expression profiling of WT(H) versus WT(WT) adult granule cells identified 121 upregulated and 121 downregulated genes (>2.5 fold, Q < 0.01). Gene ontology analysis revealed that the functional category most significantly enriched in differentially expressed genes was ‘neurotransmission at synapses’ (P = 6.48 × 10⁻⁶, Ingenuity pathway analysis), consisting of six genes that were all substantially upregulated in WT(H) granule cells (Supplementary Table 1). We were surprised to find that five of these genes are linked to acetylcholine (ACh), an excitatory neurotransmitter that is involved in encoding new episodic memories in the hippocampus12. Specifically, the genes encode the nicotinic cholinergic receptor α3, β3 and β4 subunits (Chrna3, 7.2-fold increase; Chrnb3, 14.8-fold; Chrnbd4, 16.4-fold) that can combine and form pentameric ACh receptors13, a solute carrier family 5 protein that transports choline from outside to inside of the cell (Slc5a7, 7.7-fold), and choline O-acetyltransferase (Chat, 14.6-fold), the ACh synthetic enzyme. An additional gene encodes α-synuclein (Sncg, 2.9-fold), a presynaptic vesicular membrane protein with a physiological role
in neurotransmitter release. Although the cholinergic genes were either lowly expressed or not present in adult granule cells, in situ hybridization revealed their robust expression in postnatal dentate gyrus at P4 and P14 and, at reduced levels, at P28, with no or very low expression in P56 adult dentate gyrus (Allen Developing Mouse Brain Atlas, http://developingmouse.brain-map.org/). Similarly, α-synuclein is expressed in young, but not mature, granule cells in the dentate gyrus14. It has been proposed that ACh receptors in young granule neurons respond to cholinergic inputs from the forebrain that densely innervate the subgranule zone and could contribute to their high excitability15. Retention of ACh receptor expression in adult WT(H) neurons suggests that their extended excitability could contribute to enhanced spatial memory12. The increased expression of Slc5a7 and Chat in WT(H) granule cells could also contribute to the increased hippocampal memory by producing ACh. Notably, acetylcholinesterase inhibitors, by preventing the degradation of ACh, are used in the management of Alzheimer’s disease. Overall, these data indicate that the developmentally programmed transcriptional silencing of some genes (although less significantly, F1,148 = 1.9, P = 0.04; N = 6, 7, 8, 10, 11 mice per group) and values 1.5× the interquartile range below the first three quartiles (25%, median and 75%) in general, with inhibition on glutamate release and excitation16, their downregulation in WT(H) granule cells could add to the excitatory effect of the upregulated cholinergic genes.

A similar expression profiling of CA1 pyramidal neurons from adult WT(H) versus WT(WT) mice yielded 27 upregulated and 121 downregulated genes. Functional analysis returned no highly significant clustering of genes, and the overlap in differentially expressed genes between the dentate gyrus granule and CA1 pyramidal neuron was only 17%. Finally, only the cholinergic Chrm4 gene, first identified as upregulated in WT(H) granule cells, showed differential expression in WT(H) CA1 pyramidal cells, but the direction of change was opposite. These data suggest that CA1 pyramidal neurons are not affected the way that the dentate gyrus granule cells are by the maternal TNF deficit.

Another neuronal characteristic that is modulated by early life environment and that can also be correlated with cognitive performance is dendritic morphology and complexity17. Golgi-Cox staining and Sholl analysis of the dendritic length of dentate gyrus granule cells of adult WT(H) and WT(WT) offspring revealed a maternal genotype effect (Fig. 5a). Although the proximal quarter of the arbor (between 0 and 60 µm from the neuron) was similar, WT(H) neurons exhibited longer dendritic length in more distal areas (60–240 µm) (Fig. 5b). Although spine density was not altered in either the proximal or more distal areas in the arbor (Fig. 5c), the longer dendritic length indicates an increased number of spines and, presumably, synaptic contacts per neuron. These data suggest that the TNF deficient maternal environment leads to permanent morphological changes in dentate gyrus granule cells that could contribute to the enhanced cognitive performance of WT(H) mice (see discussion).

The maternal TNF genotype effect is postnatal

Crossfostering B6Tac WT(WT) pups to Thy1+/− or Thy1−/− mothers (WToffspring(WTpre/Hpostnatal) and WT(WT/KO)) within 24 h of...
Birth resulted in the recall of the platform location in probe trial 1 in MWM, whereas offspring crossfostered to wild-type mothers (WT(WT/WT)) failed to identify the platform location, as expected (Fig. 6a). Wild-type mice exposed to the prenatal Tnf−/− maternal environment (WT(H/WT)) also failed to recall the platform location. Although Tnf−/− pups crossfostered to wild-type mothers (KO/KO/WT) recalled the platform location, the level of memory was significantly lower than that of wild-type pups crossfostered to Tnf−/− mothers (WT(WT/KO)) (P = 0.012). Overall, these data indicate that the postnatal Tnf−/− maternal environment is required for the high level of adult spatial memory observed in WT(H) mice when limited training is used (in probe trial 1).

A postnatal exposure was also sufficient to enhance fear contextual memory, but only when the offspring were crossfostered to Tnf−/− mothers (Fig. 6b); an effect that is consistent with previous data (Fig. 1a). Finally, proliferation in dentate gyrus was increased at P14 when exposure to the Tnf−/− maternal environment was limited to the postnatal period (Fig. 6c), consistent with a causative link between postnatal proliferation and adult memory (Fig. 4b). These postnatal effects could be a result of altered maternal care and behavior18, but Tnf−/− mothers exhibited no apparent changes in any of the established maternal care behaviors, including arched-back nursing, licking and grooming of the pups during the light and dark periods, latency to retrieve pups, and nest-building behavior (Supplementary Fig. 8a–f), suggesting a non-behavioral transmission.

Postpartum antibody to TNF elicits the offspring phenotypes

We then tested whether infliximab, a chimeric mouse-human antibody to TNF19 that is widely used in the treatment of autoimmune and chronic inflammatory diseases, could reproduce the offspring effects seen with the genetic deletion of maternal TNF when administered to lactating mothers. In contrast with receptor-based anti-TNF agents, infliximab is selective to TNF, as it does not neutralize the similar cytokine lympho-toxin alpha20. Infliximab neutralizes mouse TNF21 and it has been used to prevent TNF-induced inflammatory responses in numerous acute and chronic rodent models22,23. As a result of its long half life, infliximab is typically administered once a week at 10 µg per kg concentration for chronic effect22,23.

Administration of the drug at postpartum days 1 and 7 to B6Tac mothers increased dentate gyrus proliferation in the P14 offspring (Fig. 6d). Furthermore, adult offspring of mothers treated at postpartum days 1, 7 and 14 with infliximab or antibody to mouse TNF exhibited a higher level of spatial memory in the MWM than the control offspring (Fig. 6e). Controls were pooled from offspring born to mothers injected postnatally with either BSA or IgG1 isotype control antibodies were pooled, as their behavior did not differ. All data are presented as mean ± s.e.m.

TNF deficit alters the chemokine composition of milk

A conceivable postnatal non-behavioral mechanism of the maternal TNF genotype effect may involve milk. As reported24, TNF was below
the detectable levels in postpartum day 2 mouse milk of wild-type mothers (<30 pg ml⁻¹, ELISA). However, we detected significantly reduced levels of IP-10, MIP-1β and MCP-1 (P < 0.05, see Fig. 7a) for individual chemokines; MCP-3 was significantly reduced only when uncorrected for multiple testing, P = 0.04) in both Tnf⁻/⁻ and Tnf⁻/- milk, a maternal genotype–dependent pattern similar to that seen with increased proliferation and enhanced MWM cognitive performance in the offspring (Fig. 7a). In contrast, the levels of MCP-5, lymphotactin and eotaxin were reduced only in Tnf⁻/- milk. Levels of KC/GRO, MIP-1α, MIP-3β and MDC were unchanged in Tnf⁻/- and Tnf⁻/- milk, whereas another 24 cytokines and growth factors were undetectable (multiplex immunoassay Luminox, Myriad RBM, Mouse Cytokine MAP A,B,C). These data suggest that TNF, produced by milk macrophages or other immune cells, acts locally on immune and epithelial cells in the mammary gland to modulate the expression of milk chemokines. These data are consistent with reports showing that IP-10 and MCP-1, cytokines that are expressed in the gut30 (which normally has more immune cells than the rest of the body), given the lower than normal levels of IP-10 (a chemokine that directs lymphocytes), and MIP-1β (attracts monocytes as well as lymphocytes), and MIP-1β (attracts monocytes) in Tnf⁻/- milk. Alternatively, the production and differentiation of lymphocytes and monocytes could be altered because chemokines are also involved in the maturation of immune cells31, especially during the neonatal period characterized by a substantial expansion of the immune system. In turn, these immunological changes could elicit increased proliferation in the hippocampus, as immune cells can communicate, via cytokines, with the brain22 and have been shown to modulate neuronal progenitor proliferation28,29.

Figure 7 Reduced milk chemokine levels in TNF-deficient mothers are responsible for the WT(H) phenotypes. (a) Chemokine levels in postpartum day2 milk of Tnf⁻/⁻ and Tnf⁻/- mothers (two-way ANOVA: genotype effect, F₂,110 = 24.40, P < 0.0001; group × chemokine, F₅,110 = 4.31, P < 0.0001; Tukey HSD post hoc, *P < 0.05, N = 5, 3, 5 mice per group). (b) A cocktail of five recombinant cytokines at 3× and 10x doses given by gavage daily (1×: 4 pg per g IP-10, 25 pg per g MIP-1β, 10 pg per g MCP-1, 4 pg per g MCP-3 and 0.7 pg per g MCP-5) between P1 and P14 reduced proliferation in P14 dentate gyrus (ANOVA; F₃,118 = 5, 6, 6, 5 mice per group; LSD post hoc, *P < 0.05, **P < 0.01, ***P < 0.0005). (c) The cytokine cocktails given between P1 and P21 reduced adult MWM memory in probe trial 1 at the 3x and 10x doses (two-way ANOVA: platform location, F₃,75 = 24.98, P < 10⁻⁵; group × location, F₅,118 = 0.024; N = 8, 5, 7, 9 mice per group; LSD post hoc, *P < 0.05, **P < 0.01). The results were consistent with reports showing that IP-10 and MCP-1 respectively, brain development and behavior28,29.

To determine whether the reduced levels of milk chemokines can be directly linked to the hippocampal and behavioral changes in the WT(H) offspring, we administered a cocktail of five chemokines that were substantially downregulated in Tnf⁻/- milk (Fig. 7a) to wild-type pups by daily gavage from P1 to P21 while they were nursed by Tnf⁻/- mothers. Both a 3× and a 10x cocktail (3× or 10x times the amount present in wild-type milk consumed daily; ~0.1 ml milk per g of pup weight) reduced proliferation in P14 dentate gyrus (Fig. 7b). In addition, both the 3x and 10x cocktails in WT(H) offspring reduced reference memory in the MWM compared with control WT(H) mice (Fig. 7c).

Next, we measured cytokine levels in the blood of P10 pups of wild-type and mutant mothers, but found no substantial differences in any of the four chemokines that showed reduced levels in Tnf⁻/- milk, or in another 12 cytokines that were also detectable (Supplementary Table 2). Levels of another 19 of the total of 35 tested cytokines were undetectable. However, we found a significant increase in the number of circulating white blood cells (WBCs, P = 0.019) in P10 pups of Tnf⁻/-, as compared with wild type, mothers (Fig. 7d). Both flow cytometry–based and manual counting of WBCs revealed a significant increase in the number of lymphocytes and monocytes of pups from Tnf⁻/- mothers (P = 0.02 and 0.004, respectively; Fig. 7d). This increase in circulating lymphocytes and monocytes could be a result of their reduced retention in the gut30 (which normally has more immune cells than the rest of the body), given the lower than normal levels of IP-10 (a chemokine that attracts lymphocytes), MCP-1 and MCP-5 (attract monocytes as well as lymphocytes), and MIP-1β (attracts lymphocytes) in Tnf⁻/- milk.
DISCUSSION

Here we identified a previously unknown function of the pro-inflammatory cytokine TNF. We found that the partial or complete inactivation of the Tnf gene in the maternal hematopoietic system results in a life-long enhancement of episodic memory in the offspring; specifically, of spatial reference memory in the MWM and contextual fear response. Notably, performance in these behavioral tasks was not influenced by the offspring’s own TNF level. Although a partial inactivation of maternal TNF (in Tnf<sup>+/−</sup> mothers) was sufficient to elicit the improved MWM performance in the offspring, its complete (in Tnf<sup>−/−</sup>) and near complete (in TnfloxP/loxP, Mx1-cre) elimination was required to increase fear memories, indicating a difference in TNF dosage dependence between the two hippocampal processes that are both spatial learning and memory related, but differ in sensory and motor demands and in motivational aspects. Finally, crossfostering studies revealed that the maternal effect was a result of the postnatal environment. Given that newborn mice are ‘underdeveloped’ relative to human infants, it is possible that a similar maternal programming in human would begin earlier, during fetal life.

The identified function of TNF differs from its known immunological functions in several ways. First, the effect of TNF is manifested across a generation, whereas other known functions of TNF have been described in the host itself. Second, the identified function is related to behavior, whereas other known functions are associated with either host defense or organ development. Third, this function is related to physiological levels of TNF, whereas most other known functions have been associated with excessive or pathological levels of TNF (for example, in host defense and inflammation).

The maternal TNF deficit–related increase in memory recall was not only strongly correlated with, but also directly linked to, the rate of postnatal ANP proliferation in the offspring dentate gyrus. Specifically, the increased performance of WT(H) offspring could be brought back to the level characteristic of the WT(WT) offspring by normalizing the otherwise enhanced dentate gyrus proliferation. A correlation between increased granule cell precursor proliferation and enhanced spatial memory has been described in adult rodents as a result of voluntary running and exposure to enriched environment<sup>10,33</sup>. These adult effects are temporal, and both the proliferation rate and memory eventually return to their normal levels. In contrast, increased proliferation during the late postnatal period in the offspring of TNF mutant mothers, albeit temporal in its own, resulted in a permanent increase in spatial memory.

Given that the maternal effect is concluded at weaning (at P21) and that the increase in granule cell proliferation was normalized soon after weaning, persistent functional and/or structural changes must be present in the WT(H) offspring that underlie their adult cognitive phenotype. We found evidence for the dysregulation of the developmental program in WT(H) neurons; namely, cholinergic genes that are active in young neurons, but silenced during maturation, were upregulated in adult WT(H) granule cells, suggesting that they were still active in mature neurons. The opposite changes occurred in a number of neuropeptide-related genes. Cholinergic neurotransmission promotes, whereas neuropeptides generally suppress, neuronal activity<sup>12,18</sup>, suggesting that the net effect in WT(H) neurons is increased neuronal activity. We also found structural changes, specifically increased dendritic length in WT(H) neurons, as a result of the maternal TNF–deficient environment, that may be the direct consequence of increased neuronal activity, as branching and dendritic complexity are readily increased by neuronal activity<sup>34</sup>. Finally, both increased neuronal activity and dendritic complexity are consistent with the enhanced hippocampal memory of WT(H) mice, as numerous groups have reported that enriched environment increases neuronal activity, dendritic complexity and spatial learning<sup>25</sup>.

Maternal TNF does not interact directly with neuronal precursors, but rather acts via the regulation of a group of milk chemokines. Because of the delayed production of gastric acid and pancreatic proteases in neonates<sup>27</sup>, these chemokines can reach the offspring gut intact and modulate the enteric immune system. Indeed, we found evidence for substantial changes in the number of circulating WBCs, specifically lymphocytes and monocytes, in the pups of Tnf<sup>+/−</sup> mothers. Immune cells, located in the meningeal space or perivascular areas in the brain, can have substantial effects on brain function and behavior<sup>21,22</sup>. Indeed, depletion of lymphocytes reduces spatial memory and replenishment with T cells normalizes cognitive performance<sup>36</sup>. However, further studies will be necessary to determine whether the increased number of circulating lymphocytes and monocytes in the pups of Tnf<sup>+/−</sup> mothers are indeed linked to the increased hippocampal proliferation and enhanced spatial memory of these mice.

Although most TNF-related studies during pregnancy/postpartum are focused on diseases with pathologically high TNF levels<sup>37,38</sup>, there are conditions associated with reduced ambient TNF. The level of ambient TNF is known to be downregulated by physical activity/exercise and adaptive stress<sup>39–41</sup> via glucocorticoids<sup>42</sup> and low TNF levels reflect low inflammatory physiology<sup>39</sup>. Drugs can also reduce TNF levels. The level of TNF is reduced by TNF antibody products<sup>19</sup>, such as infliximab and adalimumab, to achieve therapeutic benefit in rheumatoid arthritis and other inflammatory diseases<sup>43</sup>. Given that rheumatoid arthritis often has its onset at reproductive age, these medications are also used during pregnancy and the postpartum period. Other drugs that have potent anti-TNF activity and that are used during pregnancy and postpartum include the antidepressant compound buproprion<sup>44</sup> and some 5-HT<sub>2A</sub> receptor antagonists<sup>45</sup>.

What is the evolutionary benefit for the offspring to respond to maternal TNF and consequently adjust their spatial reference memory capacity? Given that TNF production is suppressed by glucocorticoids in the range induced by adaptive stress, physical activity and exercise<sup>39–41</sup>, TNF levels could be a measure of challenges and competition in a natural environment that is reported, via a lactocrine pathway, to the developing offspring. It is important to point out that only moderate and acute increases in maternal glucocorticoids lead to adaptive adjustments in the offspring<sup>46</sup>, whereas large and sustained increases have detrimental effects<sup>47</sup>. Consistent with the idea of the maternal environment in offspring adaptation, voluntary wheel running of female mice during pregnancy and lactation increased postnatal hippocampal proliferation transiently in their offspring<sup>48</sup>, a pattern reminiscent of that seen in the offspring of TNF-deficient mothers. In addition, low-dose glucocorticoids, administered through the drinking water provided during lactation, has been shown to permanently increase offspring spatial memory<sup>49</sup>. We hypothesize that a more challenging and competitive environment programs a higher level of spatial reference memory during the postnatal period in the offspring because of its benefit to the survival of the individual. Because of the cost associated with maintaining a higher level of cognitive performance, a less challenging or competitive environment would program a lower level of cognitive performance that is still appropriate for survival under these conditions. Alternatively, the negative effect of maternal chemokines on offspring cognition could be a trade-off of their beneficial effect on the maturation of the neonatal immune system<sup>50</sup>. However, this scenario does not explain why a mechanism has not been developed that would protect the brain against this undesirable peripheral effect. Thus, we propose that the maternal TNF-dependent mechanism of programming adult spatial reference memory is adaptive in nature.
METHODS
Methods and any associated references are available in the online version of the paper.

Accession codes. RNA expression data have been deposited to the GEO database under the accession code GSE52069.

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AUTHOR CONTRIBUTIONS
B.L., B.Z. and M.T. conceived, designed and analyzed the experiments. B.L., E.L., B.Z., G.G., M.B., S.K. and J.G.T. performed the experiments. M.T. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Animal experiments were carried out in accordance with the Weill Cornell Medical College Institutional Animal Care and Use Committee guidelines. All mice were group housed up to five per cage with 12-h light/dark cycle with lights on at 6 a.m. Food and water were available ad libitum. Tnfcre/cre (B6;129S-Tnftm1Gkl/J) mice and their appropriate wild-type controls (B6;129S2F2/J) were obtained from Jackson Laboratory. The mice were backcrossed onto the B6Tac background four times. nestin-cre mice52 (B6.Cg-Tg(Nes-cre)1Kln/J) and Mx1-cre mice53 (B6.Cg-Tg(Mx1-cre)1Cgn/J) were purchased from Jackson Laboratory. The generation of Tnflox/lox;Tnfcre/cre mice on the B6 background was previously described51. Cre recombinase expression was induced in females by the intraperitoneal injection of polyIC (250 µg, Sigma-Aldrich) at 6 weeks of age as described previously52. 3 weeks later, females were bred to generate conditional Tnf mutants. R26R-LacZ and Gfp-Tk1 mice54 (B6.Cg-Tg(Gfp-Tk1)1.7Mvs/J) were obtained from Jackson Laboratory. Crossfostering was performed within 24 h of birth. Male Tk1+ pups received 12.5 mg per kg GCV by intraperitoneal injection (Cytovene-IV, Roche Lab, NDC 0004-6940), 1–6 times between P5 and P10. Lactating wild-type B6/Tac mice received 10 Ìg/g Remicade (mfliximmab, Janssen Biologics BV) in 0.1 ml intraperitoneal at postpartum day 1, 7 and 14. Another group of mice received purified NA/LE hamster antibody to mouse/rat Tnf (BD Phprarmining 557570, 300 µg per mouse) at postpartum day 1, 7 and 14. Control groups received either 10 µg per g BSA or purified NA/LE hamster IgG1 isotype control (BD Phprarmining 554709, 300 µg per mouse) at postpartum day 1, 7 and 14. Milk was collected at postpartum day 2–3 from mammary glands by a vacuum operated system (10–50 µl per mouse), 1 min after the administration of 2 IU oxytocin in 0.1 ml (ref. 55). Dams and her litter were separated for 2 h before milking. The cocktail of chemokines (dissolved in 1 mg ml−1 BSA solution) was administered by daily oral gavage to pups with animal feeding needles (24 gauge, Harvard Apparatus) from birth until P14 or P21. Based on similar prior behavioral experiments, no less than 5–6 mice per group were used, but when difficult to obtain mother/offspring genotypes were not a limiting factor, the number of mice used was at least 8, but typically more than 10. At least three litters per group were used. Given the low frequency of appropriate genotypes in the conditional knockout experiments, 5–6 litters were used. The same or similar numbers of mice were selected from individual litters randomly and assigned to the groups. During all behavioral tests, the investigators who performed the tests were blind to the genotype and treatment of the mice. Moreover, all behavioral tests were fully automated with no human input on data collection.

Immunohistochemistry. At P5, P14 and at adult age (4–6 months), mice were given a single 100 mg per kg subcutaneous injection of BrdU (Sigma, B5002) followed 2 h later by transcardial perfusion with 4% paraformaldehyde (wt/vol). Immunohistochemistry was performed as previously described56 by using mouse monoclonal antibody to BrdU (1:500, Novocastra, MCA2060) and biotinylated horse antibody to mouse antibody (1:200, Vector Laboratories, BA2000). For BrdU detection, sections were exposed to 2 µg ml−1 pepsin in 0.01 M hydrochloric acid for 10 min at room temperature. Sections were then subjected to antigen retrieval in 10 mM EDTA, 5 mM Tris, pH 8.0 at 95–100 °C for 15–20 min for antigen recovery, blocked with donkey antibody to rabbit IgG–Alexa Fluor 488 (1:200, A-21206), antibody to goat IgG–Alexa Fluor 555 (A-21432) and/or antibody to mouse Alexa Fluor 647 (A-31571) secondary antibodies (Jackson ImmunoResearch). The dorsal CA1 pyramidal layer of 5–6 WT(WT) and WT(H) male mice of 12–14 weeks of age from three or more litters. RNA-seq libraries were prepared using the Illumina HiSeq2000 sequencing platform, generating approximately 45 million 51 nucleotide reads per pool. Reads were processed and aligned to the mm9 reference genome (NCBI v37 build) using TopHat v1.2.0, which incorporates the Bowtie algorithm to perform splice junction mapping of RNA-seq transcripts. The aligned reads were then processed by Cufflinks v1.3.0. Reads were assembled into transcripts, their abundance estimated, and tests for differential expression and regulation between the tissue samples were performed. We used the “−G” argument, which calculates the expression levels for all known/annotated transcripts using the Cuffcompare library to identify the abundance of transcripts from the reference transcriptome using the Tophat alignments and concurrently test for differential expression. The unit of measurement for the relative abundances of transcripts is fragments per kilobase of exon per million fragments mapped (FPKM), and the measure for differential expression is the log2-fold change of the treatment FPKM divided by the control FPKM.

Milk cytokine levels. Milk samples from individual mice were diluted 1:1 in ice-cold protease buffer (0.15 mM spermine, 0.5 mM spermidine, 1 mM PMSE, 1× complete protease inhibitor in phosphate-buffered saline) and were centrifuged for 10 min (2,300g, 4 °C). Supernatant was analyzed for cytokines and chemokines by multiplex Luminox immunoassay by Myriad RBM (A, B and C panels).

Behavioral procedures. The cognitive tests were conducted using male offspring aged 8–16 weeks. Mice were first tested in open field for overall activity, followed 2 d later by the MWM as described previously58. 1 week after the completion of this test, mice were tested for fear conditioning according to a previously described protocol59. All the water maze and fear condition tests run between 1–5 p.m. (i.e., during the light cycle). Maternal care behavior was assessed both during the light and dark phases60.

Statistical analysis. Data are shown as mean ± s.e.m. No data or mice were excluded. One- or two-way ANOVAs or t tests were used to compare groups. Homogeneity of variance was assessed by Levene’s test. LSD or Tukey HSD post hoc analyses were used to assess statistical significance. If the assumption for equal variances and normality was not met, the Dunnett’s T3 post hoc test algorithm was used. Differences between groups were considered to be significant when P < 0.05.

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