

Rapid linkage of innate immunological signals to adaptive immunity by the brain-fat axis

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Innate immunological signals induced by pathogen- and/or damage-associated molecular patterns are essential for adaptive immune responses, but it is unclear if the brain has a role in this process. Here we found that while the abundance of tumor-necrosis factor (TNF) quickly increased in the brain of mice following bacterial infection, intra-brain delivery of TNF mimicked bacterial infection to rapidly increase the number of peripheral lymphocytes, especially in the spleen and fat. Studies of various mouse models revealed that hypothalamic responses to TNF were accountable for this increase in peripheral lymphocytes in response to bacterial infection. Finally, we found that hypothalamic induction of lipolysis mediated the brain's action in promoting this increase in the peripheral adaptive immune response. Thus, the brain-fat axis is important for rapid linkage of innate immunity to adaptive immunity.

The immune response is a vital mechanism by which an organism copes with harm and damage and is a process that includes innate immunity and adaptive immunity in vertebrates. The innate immune response represents a primary and immediate but rather non-selective defense achieved through the actions of various molecules of the innate immune system (for example, tumor-necrosis factor (TNF) and other cytokines) that are typically produced from cells of the innate immune system, such as macrophages¹⁻⁵. In response to infection or tissue damage, these cells are quickly activated by a group of molecules known as 'pathogen-associated molecular patterns', which include lipopolysaccharide, peptidoglycan and lipoteichoic acids, or by damage-associated molecular patterns, which comprise a broader range of molecules released from stressed or damaged cells¹⁻⁵. Unlike innate immunity, adaptive immunity is a specialized immune response that is mediated by T lymphocytes and B lymphocytes from organs of the immune system, and a prolonged period (4–7 d) is often required for these cells to become effector cells. Innate immunological signals are crucial for the initiation of adaptive immune responses¹⁻⁵; for example, TNF and interleukins have a critical role in stimulating T cells and B cells⁶. While this connection between innate immunity and adaptive immunity has been appreciated locally in peripheral organs of the immune system, an unanswered question is whether this immunological crosstalk requires systemic regulation, such as by the central nervous system (CNS). Here, in the context of research focused on studying hypothalamic pathways of the innate immune system⁷⁻¹¹, we explored whether the brain is important for rapidly conveying signals from the innate immune system to initiate adaptive immunity.

RESULTS

Initiation of adaptive immunity by central TNF action

We used an infection model in which we injected wild-type C57BL/6 mice with *Listeria monocytogenes*, a Gram-positive bacterium that

is a well-established pathogen for the induction of innate and adaptive immune responses^{12,13}. We found that in addition to its rise in the plasma, the TNF concentration in the cerebrospinal fluid (CSF) quickly increased in mice at day 1 after they received an intravenous injection of *L. monocytogenes* (**Supplementary Fig. 1a**). Our immunostaining demonstrated abundant expression of TNF receptor 1 (TNFR1) in the mediobasal hypothalamus (MBH) (**Supplementary Fig. 1b**). We then sought to determine if delivering TNF into the brain of mice had an effect on systemic immunity. To do so, we injected a low dose (10 pg) of TNF into the hypothalamic third ventricle of the brain of wild-type C57BL/6 mice; following this injection, TNF concentrations in the CSF increased similarly to the increase seen in mice infected with *L. monocytogenes* (**Supplementary Fig. 1c**). However, this injection dose did not affect the blood concentration of TNF (37.92 ± 3.94 (TNF injection) versus 38.23 ± 2.74 pg/ml (vehicle injection) (mean \pm s.e.m.); $P = 0.48$ (two-tailed Student's *t*-test)). Because we were interested in determining whether brain responses have a rapid effect on immunity, we used a 3-day experimental course, which reflects an early time point in this immune response and also allows the time window necessary for the induction of cell proliferation. We confirmed that 3-day injections of a low dose of TNF did not lead to sickness in mice (data not shown) and that therefore this pharmacological model was physiologically suitable for this study.

By flow cytometry, we found that such hypothalamic injection of a low dose of TNF did not change the number of macrophages in the peripheral tissues or blood (**Supplementary Fig. 2a–c**). However, such hypothalamic injection of TNF led to a greater abundance of T cells and B cells in the spleen than did injection of vehicle, and these effects were associated with a proportionally greater abundance of splenic CD4⁺ T cells and CD8⁺ T cells in the mice given injection of TNF (**Fig. 1a–e**). Notably, the greater number of CD4⁺ T cells and

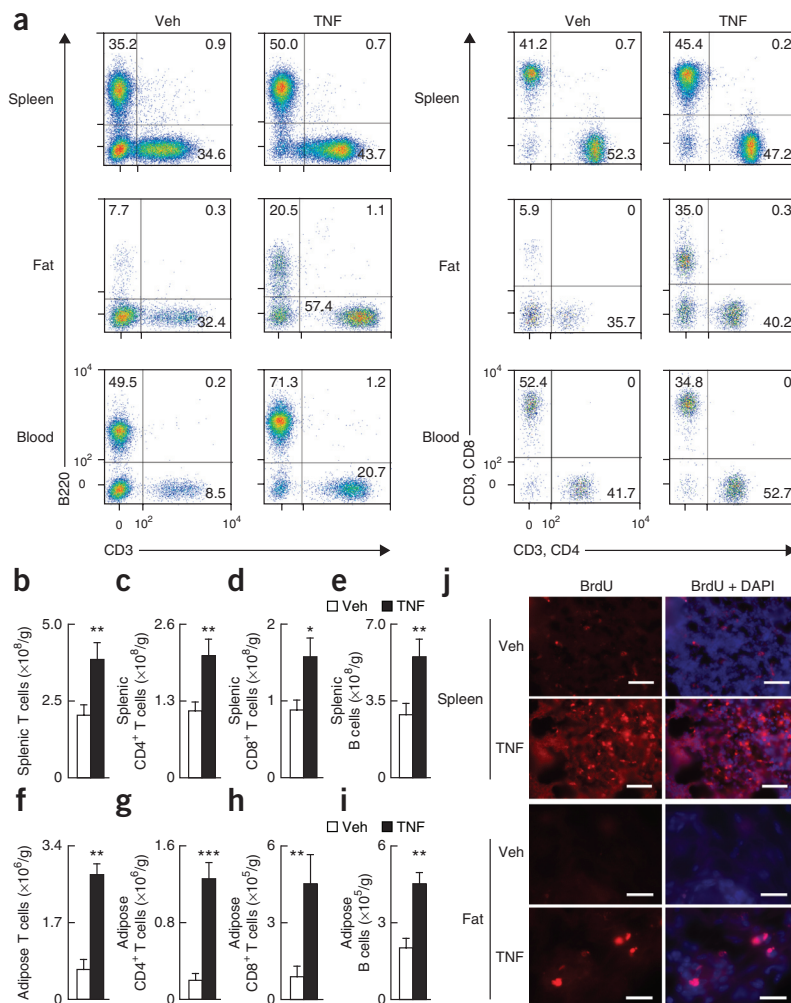
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Figure 1 Central injection of TNF rapidly increases the abundance of splenic and adipose lymphocytes. **(a)** Flow cytometry of T cells (CD3⁺), B cells (B220⁺), CD4⁺ cells (CD3⁺CD4⁺) and CD8⁺ cells (CD3⁺CD8⁺) in the spleen (top), epididymal fat (middle) and blood (bottom) of C57BL/6 mice ($n = 8-11$ per group) given daily injection of TNF (10 pg) or vehicle (Veh) into hypothalamic third ventricle for 3 d. Numbers in quadrants indicate percent cells in each throughout. **(b–i)** Quantification of T cells **(b,f)**, CD4⁺ T cells **(c,g)**, CD8⁺ T cells **(d,h)** and B cells **(e,i)** (all identified as in **a**) in the spleen **(b–e)** and epididymal fat **(f–i)** of the mice in **a** ($n = 8-11$ per group). **(j)** BrdU staining (red) of spleen and epididymal fat sections of C57BL/6 mice ($n = 3-4$ per group) given injection of TNF or vehicle as in **a**, followed by intraperitoneal injection of BrdU at 2 h before collection of tissues; staining with the DNA-binding dye DAPI (blue) shows all cells. Images represent 3–4 mice per group. Scale bars, 50 μ m. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (two-tailed Student's *t*-test). Data are representative of at least three independent experiments with similar results (mean and s.e.m. in **b–i**).

CD8⁺ T cells was more notable in the epididymal fat of mice given injection of TNF than in the spleen of these mice (**Fig. 1a,f–h**), and the number of B cells was also greater in the epididymal fat of these mice given injection of TNF than in that of mice given injection of vehicle (**Fig. 1a,i**). The increase in the abundance of lymphocytes in these mice given injection of TNF was comparable to the increase in these cells observed in mice after 3 d of bacterial infection (**Supplementary Fig. 2d–k**). We predicted that increased number of splenic and adipose lymphocytes in mice given injection of TNF might lead to an increased number of lymphocytes in the circulation. Flow cytometry of the blood of these mice supported this prediction (**Fig. 1a** and **Supplementary Fig. 3a–d**). We also obtained other tissues (such as liver, skeletal muscle, heart and kidney) from mice given injection of TNF and subjected them to flow cytometry but found few lymphocytes in these tissues and found that their abundance did not change upon injection of TNF (data not shown).

To further demonstrate the immunological relevance of the findings reported above, we profiled subsets of T cells and B cells after hypothalamic injection of TNF. First, we used flow cytometry to analyze ICOS⁺PD-1⁺ and PD-L1⁺ cells, as these populations represent activated T lymphocytes and B lymphocytes, respectively. We observed a greater number of ICOS⁺PD-1⁺ cells in the epididymal fat and spleen of mice given injection of TNF than in that of mice given injection of the vehicle control (**Supplementary Fig. 3e–h**), which suggested that T cells and B cells in these tissues were activated in response to hypothalamic injection of TNF. Staining with the thymidine analog BrdU revealed that mice given injection of TNF showed greater cell proliferation in the spleen and epididymal fat than did those given injection of vehicle (**Fig. 1j**). Subsequently, we used flow cytometry to profile various CD4⁺ helper T cell subsets, including T_H1 cells (CXCR3⁺CD4⁺), T_H2 cells (CXCR3[−]CD4⁺) and T_H17 cells (CCR6⁺CD4⁺). Injection of TNF into the hypothalamic ventricle increased or tended to increase the abundance of each of these



helper T cell subsets in the spleen and epididymal fat (**Supplementary Fig. 3i–n**). We analyzed regulatory CD4⁺ T cells on the basis of the transcription factor Foxp3 and the T cell-activation marker CD25; however, this hypothalamic injection of TNF did not increase the number of CD4⁺Foxp3⁺CD25⁺ cells in the spleen or fat relative to the injection of vehicle (data not shown). In summary, the action of TNF in the brain had the effect of increasing and activating peripheral lymphocytes.

Brain TNF action initiates adaptive immunity in infection

To evaluate if the observed effects of TNF were brain specific, we subjected C57BL/6 mice to peripheral (intraperitoneal) injection of TNF with the same low dose (10 pg) used in our hypothalamic injection experiments. Flow cytometry confirmed that peripheral injection of this low dose of TNF did not change the number of CD4⁺ T cells, CD8⁺ T cells or B cells in the spleen, epididymal fat or blood (data not shown). For comparison, we administered TNF into C57BL/6 mice at a pathological, high dose (5 ng) via intraperitoneal injection, which mimicked the increase in serum TNF that developed after bacterial infection. The TNF concentrations in the CSF of these mice given injection of TNF increased about threefold above the normal baseline concentration in control mice given injection of vehicle (data not shown). These mice given injection of a high dose of TNF over 3 d had a greater abundance of CD4⁺ T cells, CD8⁺ T cells and B cells in the spleen and epididymal fat than did control mice given injection of vehicle (data not shown). We thus

hypothesized that an infection-induced increase in circulating TNF led to the observed effects of central TNF, given that the MBH vasculature is permeable¹⁴ and this permeability is enhanced under infection¹⁵. To test this idea, we investigated whether bacterial infection-induced adaptive immune responses could be altered by the suppression of TNF signaling in the brain. Therefore, we 'pre-injected' the TNF antagonist WP9QY into the hypothalamic ventricle of C57BL/6 mice 1 d before infection with *L. monocytogenes* and then maintained daily hypothalamic injections of WP9QY for 3 d before analyzing cells from the mice by flow cytometry. This treatment with WP9QY substantially dampened the induction of adipose CD4⁺ T cells and CD8⁺ T cells, as well as B cells, by infection (Fig. 2a–e). Also, these mice treated with WP9QY showed impairment in the increase in the abundance of these lymphocytes in the spleen after injection of bacteria (Fig. 2a,f–i). Hence, all these data supported the hypothesis that TNF signals in the brain contributed to the initiation of adaptive immune responses.

Hypothalamic TNF action initiates adaptive immunity

Because the MBH, especially its arcuate nucleus, had high expression of TNFR1, we reasoned that the MBH would be critical for the action of central TNF in increasing the abundance of peripheral lymphocytes. To investigate this, we assessed whether blocking TNFRs in the MBH was sufficient to affect the adaptive immune response during infection with *L. monocytogenes*. Using an established method for site-specific delivery of lentiviral short hairpin RNA (shRNA)¹⁶, we generated mice with MBH-directed knockdown of TNFRs. By this approach, we knocked down TNFR1 as well as TNFR2 to eliminate possible redundancy between these two isoforms. Expression of TNFR1 and TNFR2 protein in the MBH was reduced by knockdown via shRNA (Fig. 3a). We treated those mice, as well as mice given injection of control lentivirus, with intravenous injection of *L. monocytogenes*; at 3 d after infection, we collected various tissues and subjected them to flow cytometry. We found that infection-induced increases in the abundance of adipose CD4⁺ T cells, CD8⁺ T cells and B cells were severely impaired in mice in which TNFRs were knocked down compared with those in mice given injection of nontargeting control shRNA (Fig. 3b–f). Knockdown of TNFRs also significantly impaired the induction of splenic CD4⁺ T cells, CD8⁺ T cells

and B cells by infection (Fig. 3b,g–j). In addition, we generated mice in which TNFRs were inhibited in pro-opiomelanocortin neurons in the MBH and found that this manipulation partially diminished the effects of central TNF in increasing the abundance of adipose CD4⁺ T cells, CD8⁺ T cells and B cells (Supplementary Fig. 4a–d). Thus, on the basis of these collective results, we propose that the mediobasal region of the hypothalamus is important in linking brain TNF signals to adaptive immunity.

To further study the role of hypothalamic TNF in initiating adaptive immune responses, we used a genetic mouse model that is deficient in TNFR1 and TNFR2 (refs. 17,18). We crossed TNFR1-deficient (*Tnfrsf1a*^{−/−}) mice with TNFR2-deficient (*Tnfrsf1b*^{−/−}) mice to generate mice with homozygous doubly deficiency in TNFR1 and TNFR2 (called 'TNFR-null' here). TNFR-null mice are extremely vulnerable to bacterial infection^{17,18}. We confirmed that adaptive immune responses to infection were severely impaired in these mice (data not shown). In this context, we investigated whether the impairment in adaptive immunity in the TNFR-null mice could be reversed, perhaps partially, by restoring TNFRs in the MBH. To do so, we injected lentivirus expressing TNFR1 bilaterally into the MBH of these TNFR-null mice by MBH-directed injection^{8,9,11,16}. TNFR-null mice given injection of lentivirus lacking TNFR1 served as a viral control group. By immunostaining, we verified that lentiviral expression of TNFR1 led to restoration of TNFR1 protein in the MBH, and especially in the subregion of the arcuate nucleus, of the TNFR-null mice (Fig. 4a). Thus, we generated a mouse model in which TNFR was absent from the whole body except for the MBH; this provided a unique tool with which to selectively delineate the MBH-specific role of TNFRs in the immune response.

We challenged the mice described above (TNFR-null mice given lentiviral delivery of TNFR1 and those given injection of the control lentivirus) by injection of *L. monocytogenes* to induce adaptive immune responses. The TNFR-null mice that received the control lentiviral vector had fewer splenic and adipose CD4⁺ T cells, CD8⁺ T cells and B cells than did their counterparts given lentiviral delivery of TNFR1 (Fig. 4b–j), with the abundance in the former being similar to

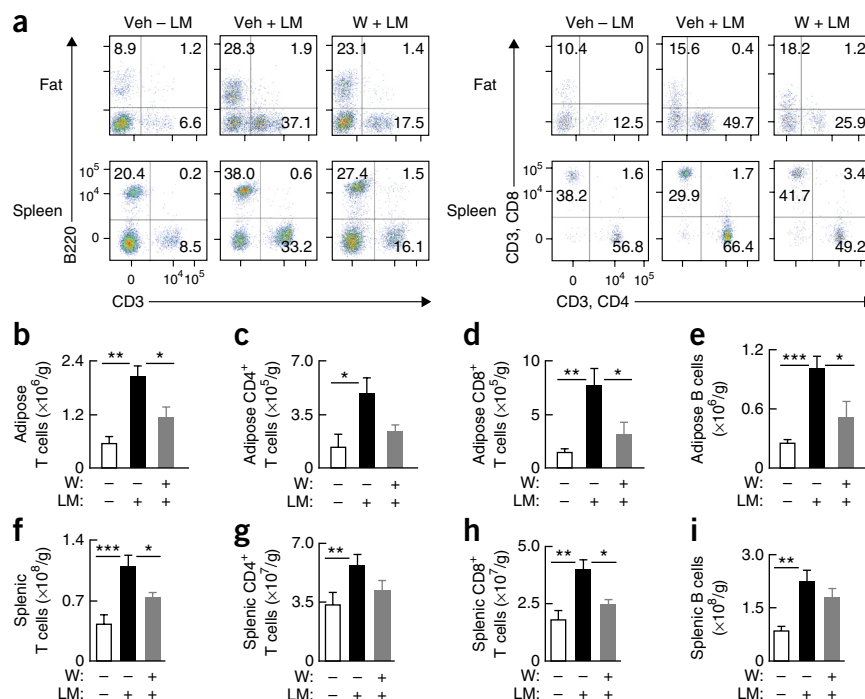


Figure 2 The bacterial infection-induced adaptive immune response requires brain TNF. (a) Flow cytometry of T cells, CD4⁺ T cells, CD8⁺ T cells and B cells (all as in Fig. 1a) in the epididymal fat and spleen of C57BL/6 mice ($n = 6-8$ per group) given pre-injection of the TNF antagonist WP9QY (W) or the vehicle artificial cerebrospinal fluid (Veh) into the hypothalamic ventricle 1 d before infection with *L. monocytogenes* (+LM) or vehicle (−LM) and then maintained with daily hypothalamic injection of WP9QY or vehicle for 3 d. (b–i) Quantification of T cells (b,f), CD4⁺ T cells (c,g), CD8⁺ T cells (d,h) and B cells (e,i) (all as in a) in the epididymal fat (b–e) and spleen (f–i) of mice as in a ($n = 6-8$ per group). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (analysis of variance (ANOVA) and Tukey's *post-hoc* test). Data are representative of two independent experiments with similar results (mean and s.e.m. in b–i).

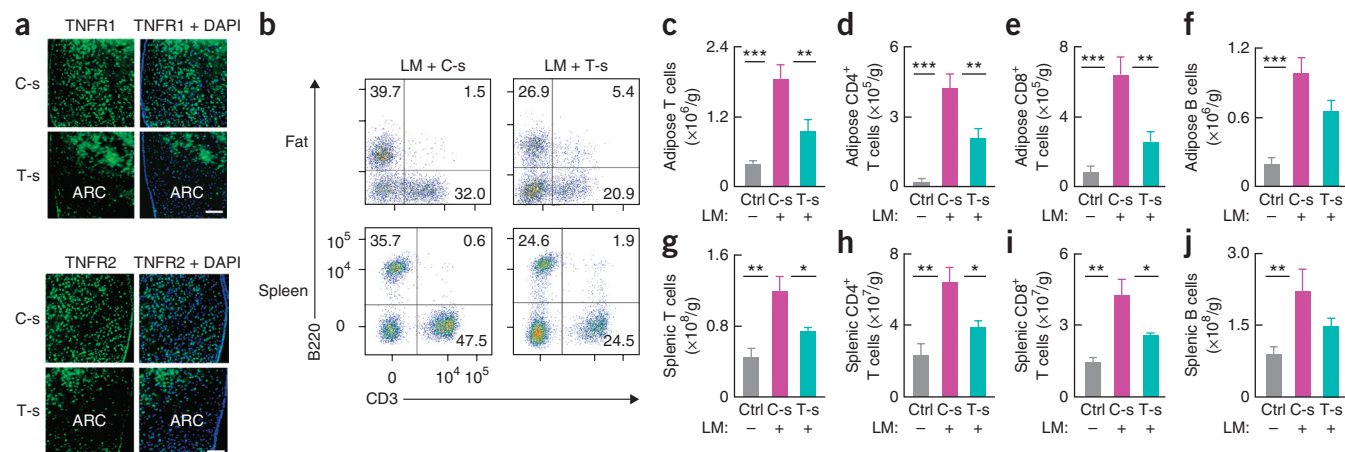


Figure 3 Hypothalamic TNFRs are required for the adaptive immune response to infection. (a) Immunostaining of TNFR1 or TNFR2 (green) in the MBH of C57BL/6 mice ($n = 3-4$ per group) given bilateral injection of lentiviral shRNA specific for mRNA encoding TNFR1 and TNFR2 (T-s) or nontargeting control shRNA (C-s) in the arcuate nucleus (ARC) (DAPI staining as in Fig. 1j). Scale bars, 200 μ m. (b) Flow cytometry of T cells (CD3⁺) and B cells (B220⁺) in the epididymal fat and spleen of mice as in a, given intravenous injection of *L. monocytogenes* or vehicle or no such injection (basal control (Ctrl)) and then assessed 3 d later ($n = 5-7$ mice per group). (c-j) Quantification of T cells (c,g), CD4⁺ T cells (d,h), CD8⁺ T cells (e,i) and B cells (f,j) (all identified as in Fig. 1a) in the epididymal fat (c-f) or spleen (g-j) of mice as in b ($n = 5-7$ per group). * $P < 0.05$ and ** $P < 0.01$ (ANOVA and Tukey's *post-hoc* test). Data are representative of two independent experiments with similar results (mean and s.e.m. in c-j).

that seen in TNFR-null mice that did not receive injection into the MBH (data not shown). In contrast, the delivery of TNFR1 into the MBH completely or partially restored the increase in the number of adipose lymphocytes in TNFR-null mice in response to infection (Fig. 4b-f). Also, the number of splenic T cells and B cells in these mice was greater than that in TNFR-null mice infected with control lentivirus, although to a lesser extent than in fat tissues (Fig. 4g-j). As a measurement of immunological function in combating infection, we found fewer viable bacteria in TNFR-null mice given lentiviral injection of TNFR1 into the MBH than in mice given the lentiviral vector control (Supplementary Fig. 4e-g), which indicated that restoration of TNFR1 in the MBH was able to decrease the severity of infection in various tissues of the TNFR-null mice. Thus, the TNF pathway in the hypothalamus was required for the induction of adaptive immunity to bacterial infection.

Lipolysis links the CNS to the adaptive immune response

We subsequently sought to determine which adipose factors might be responsible for the brain TNF's action in mobilizing cells of the adaptive immune system. To address this question, we measured the abundance of mRNA encoding innate immunity cytokines such as interleukins 1 β and 6 and observed that hypothalamic injection of TNF did not change the abundance of mRNA encoding these cytokines in the fat (data not shown); this challenged the proposal that adipose innate immunity is a key mediator for the effect of central TNF on adaptive immunity. In contrast, such delivery of TNF increased the expression of mRNA encoding lipolytic molecules in the fat, including hormone-sensitive lipase (*Lipe*), lipoprotein lipase (*Lpl*) and a member of the acyl-CoA synthetase long-chain family (*Acs11*) (Fig. 5a). Lipolysis is a characteristic of infection, and some fatty acids have been linked to the activation of Toll-like receptors

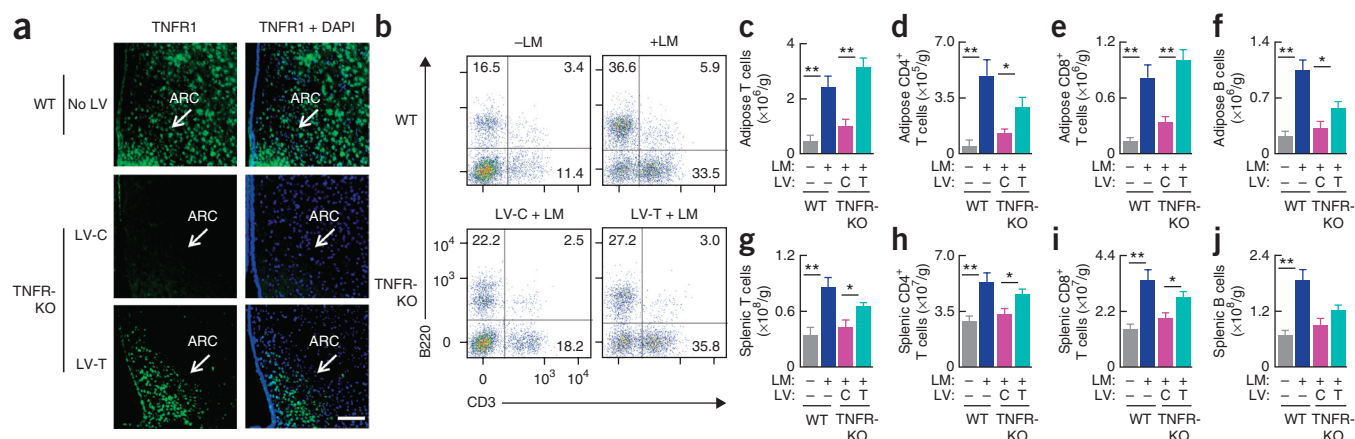


Figure 4 Hypothalamic restoration of TNFRs improves adaptive immunity in TNFR-null mice. (a) Immunostaining of TNFR1 (green) in the MBH of TNFR-null mice (TNFR-KO) given bilateral injection of a lentiviral construct encoding TNFR1 (LV-T) or empty lentiviral construct (LV-C) into the arcuate nucleus, and of wild-type mice (WT) given no lentivirus ($n = 3-4$ mice per group) (DAPI staining as in Fig. 1j). Scale bars, 200 μ m. (b) Flow cytometry of T cells (CD3⁺) and B cells (B220⁺) in the epididymal fat of mice as in a, given intravenous injection of *L. monocytogenes* or vehicle and then assessed 3 d later ($n = 5-7$ mice per group). (c-j) Quantification of T cells (c,g), CD4⁺ T cells (d,h), CD8⁺ T cells (e,i) and B cells (f,j) (all identified as in Fig. 1a) in the epididymal fat (c-f) and spleen (g-j) of mice as in b ($n = 5-7$ per group). * $P < 0.05$ and ** $P < 0.01$ (ANOVA and Tukey's *post-hoc* test). Data are representative of two independent experiments with similar results (mean and s.e.m. in c-j).

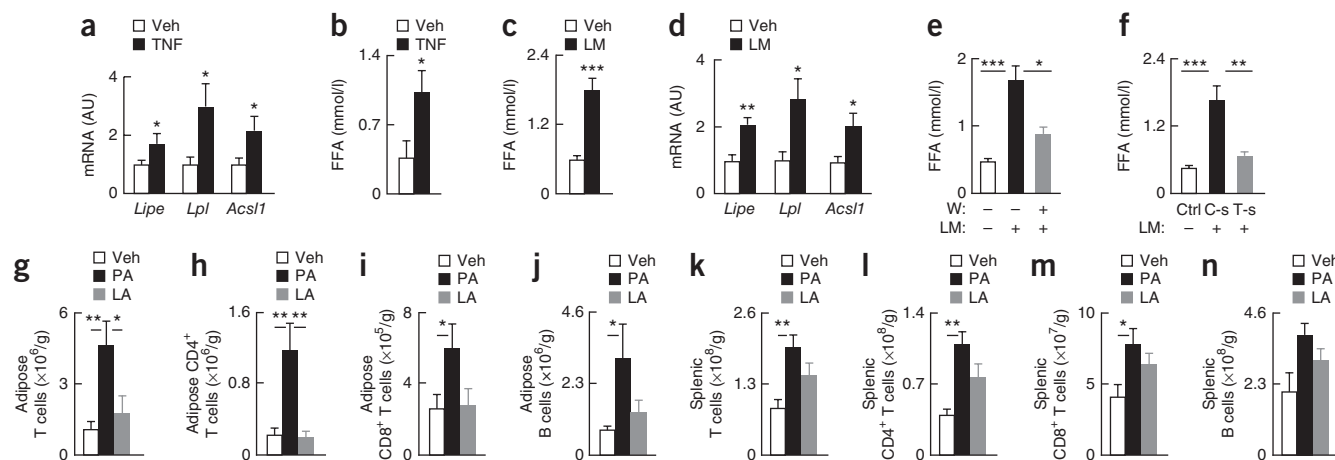


Figure 5 Central induction of lipolysis mediates initiation of the adaptive immune response. (a–f) Expression of *Lipe*, *Lpl* and *Acs1* in adipose (a,d) and concentration of free fatty acids (FFA) in serum (b,c,e,f) of C57BL/6 mice ($n = 4$ –6 per group) given injection of TNF (10 μ g) or vehicle into the hypothalamic third ventricle (as in Fig. 1) (a,b) or injection of *L. monocytogenes* or vehicle (c,d) or treated with WP9QY and injection of *L. monocytogenes* (as in Fig. 2) (e) or with shRNA targeting TNFR1 and TNFR2 or control shRNA (as in Fig. 3) (f) (time points as in Figs. 1–3, respectively). AU, arbitrary units. (g–n) Quantification of T cells (g,k), CD4⁺ T cells (h,l), CD8⁺ T cells (i,m) and B cells (j,n) (all identified as in Fig. 1a) in the epididymal fat (g–j) and spleen (k–n) of C57BL/6 mice ($n = 5$ –6 per group) given daily intraperitoneal injection of palmitic acid (PA), linoleic acid (LA) or vehicle (Veh) for 3 d, assessed by flow cytometry. * $P < 0.05$ and ** $P < 0.01$ (ANOVA and Tukey's *post-hoc* test). Data are representative of at least three independent experiments with similar results (mean and s.e.m.).

in adipose macrophages^{19–23}, but whether lipolysis is important for the initiation of adaptive immune responses by the brain-fat axis has remained unexplored, despite published studies showing a role for lipids in T cell development²⁴. We directed our research attention to the brain-fat axis by investigating whether the brain might regulate lipolysis to aid adaptive immunity. Indeed, hypothalamic injection of TNF substantially increased fatty acids in the blood by eliciting concentrations that were ~2.6 times higher than the basal levels in mice given injection of vehicle (Fig. 5b). In agreement with that, the blood concentrations of fatty acids in mice infected with *L. monocytogenes* were about three times higher than those in uninfected control mice (Fig. 5c). That effect was consistent with higher expression of genes encoding molecules involved in lipolysis in the fat of bacteria-infected mice than in uninfected control mice (Fig. 5d). By analyzing fatty acid species, we found that central injection of TNF or bacterial infection substantially increased the serum concentration of several long-chain fatty acids (Supplementary Fig. 5a). In agreement with these results, endotoxin-treated mice are reported to increase lipolysis and the concentration of fatty acids in the blood²⁵. Thus, we hypothesized that brain-induced release of lipids might work as a link for the relationship among the brain, fat and cells of the adaptive immune system.

To directly evaluate the potential role of fatty acids in adaptive immunity, we analyzed *L. monocytogenes*-infected mice in which the function of brain TNF or hypothalamic TNFRs was inhibited. We found that bacteria-induced increases in blood fatty acids were attenuated in these mice when brain or hypothalamic TNF was inhibited pharmacologically (Fig. 5e) or genetically (Fig. 5f). We then investigated whether the administration of a long-chain fatty acid species could lead to an increase in the abundance of lymphocytes. Using an experimental duration of 3 d, we injected either palmitic or linoleic acid at a suitable dose (5 mg per kg body weight per day) intraperitoneally into C57BL/6 mice. We found that mice treated with palmitate had a greater abundance of adipose CD4⁺ T cells, CD8⁺ T cells and B cells than did vehicle-treated mice (Fig. 5g–j). Palmitate also increased the number of splenic lymphocytes (Fig. 5k–n); however, treatment with palmitate at this dose and duration did not clearly

increase the number of macrophages in the fat or spleen (data not shown). In contrast to palmitate, linoleic acid at this dose did not increase the number of adipose lymphocytes (Fig. 5g–j) but had an effect in increasing the abundance of splenic lymphocytes (Fig. 5k–n). We also tested oleic acid but found that its injection at the same dose did not increase the number of lymphocytes in the fat or spleen (data not shown). Thus, despite the finding that the effects of individual fatty acid species on tissue lymphocytes were dynamic, these observations supported the general idea that brain-induced lipolysis has a role in linking the brain-fat axis to adaptive immune response.

Central TNF-induced lipolysis initiates adaptive immunity

We simultaneously designed loss-of-function experiments to investigate whether fatty acids could be inhibited to cause an impaired effect of brain TNF in stimulating adaptive immunity. In these experiments, we gave C57BL/6 mice intraperitoneal injection of the fatty-acid-synthase inhibitor cerulenin at a dose of 10 mg per kg body weight per day, for 3 d, then gave these mice daily injections of TNF (10 μ g) into the hypothalamic third ventricle. While the abundance of lymphocytes was greater in the fat of mice given injection of TNF than in that of mice given injection of vehicle, these changes in the fat were completely prevented by pre-treatment with cerulenin (Fig. 6a–d). Notably the effects of central TNF in increasing the number of splenic T cells and B cells were also abolished by pre-treatment with cerulenin (Fig. 6e–h), which suggested that release of fatty acids from the fat had an effect on initiating the increase in the abundance of splenic lymphocytes.

We sought to determine whether the lipid-based effect noted above also applied to bacterial infection. To investigate this possibility, we administered that same dose of cerulenin (10 mg per kg body weight per day) to mice on the day before other treatments, followed by a single intravenous injection of *L. monocytogenes* or vehicle while daily intraperitoneal injections of cerulenin continued for 3 d. Flow cytometry revealed that treatment with cerulenin reduced the magnitude of bacterial infection-induced abundance of lymphocytes in the fat as well as the spleen (Fig. 6i–p). For comparison, we simultaneously analyzed macrophages in these tissues and found that treatment with cerulenin

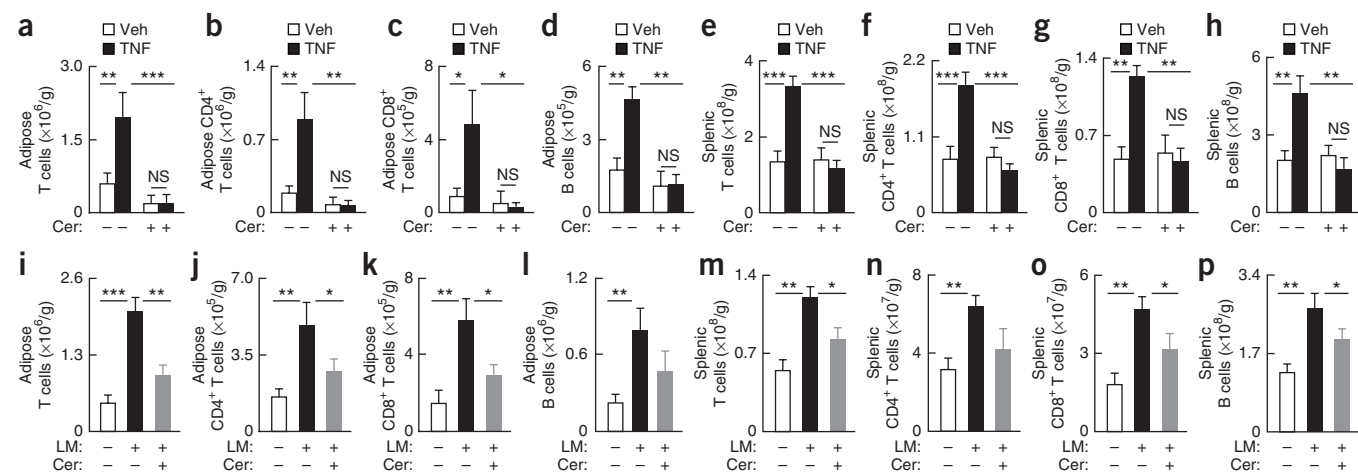


Figure 6 Inhibition of fatty acids attenuates brain TNF- or infection-induced adaptive immunity. (a–h) Quantification (by flow cytometry) of T cells (a,e), CD4⁺ T cells (b,f), CD8⁺ T cells (c,g) and B cells (d,h) (all identified as in Fig. 1a) in the epididymal fat (a–d) and spleen (e–h) of C57BL/6 mice ($n = 5–7$ per group) given injection of TNF plus intraperitoneal injection of cerulenin (Cer +) or vehicle (saline; Cer –) on the day before other treatments, followed by daily injection of TNF (10 pg) or vehicle (artificial cerebrospinal fluid) (key) into the hypothalamic third ventricle, together with daily intraperitoneal injection of cerulenin, for 3 d. (i–p) Quantification of T cells (i,m), CD4⁺ T cells (j,n), CD8⁺ T cells (k,o) and B cells (l,p) in the epididymal fat (i–l) and spleen (m–p) of C57BL/6 mice ($n = 5–7$ per group) given injection of *L. monocytogenes* or vehicle and intraperitoneal injection of cerulenin or vehicle on the day before other treatments, followed by a single intravenous injection of *L. monocytogenes* or vehicle while daily intraperitoneal injections of cerulenin continued for 3 d. NS, not significant; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (ANOVA and Tukey's *post-hoc* test). Data are representative of at least three independent experiments with similar results (mean and s.e.m.).

did not prevent bacteria from increasing the abundance of these cells of the innate immune system (data not shown). To gain further insight into the brain-fat connection in the adaptive immune response, we performed denervation of the epididymal fat to break the brain-fat axis. Indeed, sympathetic denervation of white fat has been established as an approach with which to inhibit lipolysis^{26–28}. We found that denervation of fat reduced the effects of brain TNF in increasing the number of adipose lymphocytes (Fig. 7a–e). The effects of brain TNF in increasing the abundance of splenic T cells and B cells were also reduced by fat denervation (Fig. 7f–i), which suggested that the epididymal fat was important in conveying the brain signal into the spleen. In summary,

the induction of lipid release by the brain was important for adaptive immunity in a manner that was independent of innate immunity.

Lipolysis requires leptin release to increase lymphocytes

The data presented above supported the proposal that lipolysis is a vital mediator of the effects of the CNS in regulating cells of the adaptive immune system. On this background, we reasoned that lipolysis might require other secretory factors from the fat in this regulation, which would be likely, as lipolysis itself can be induced in metabolic conditions unrelated to immunity, such as starvation. In addition to the difference between these conditions in the magnitude and patterns

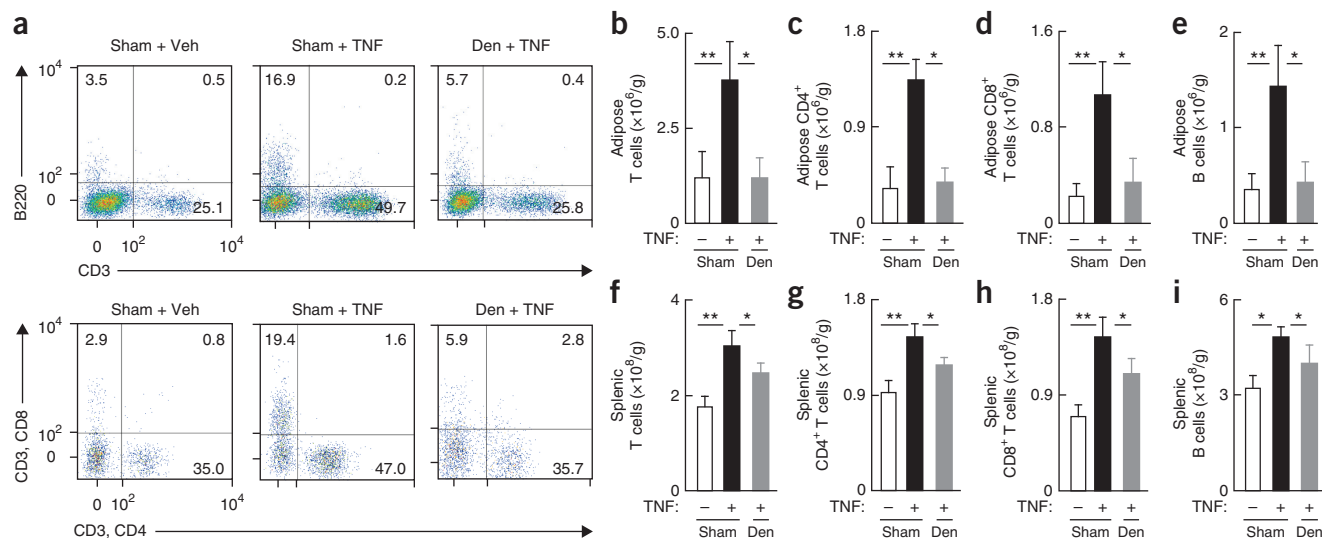
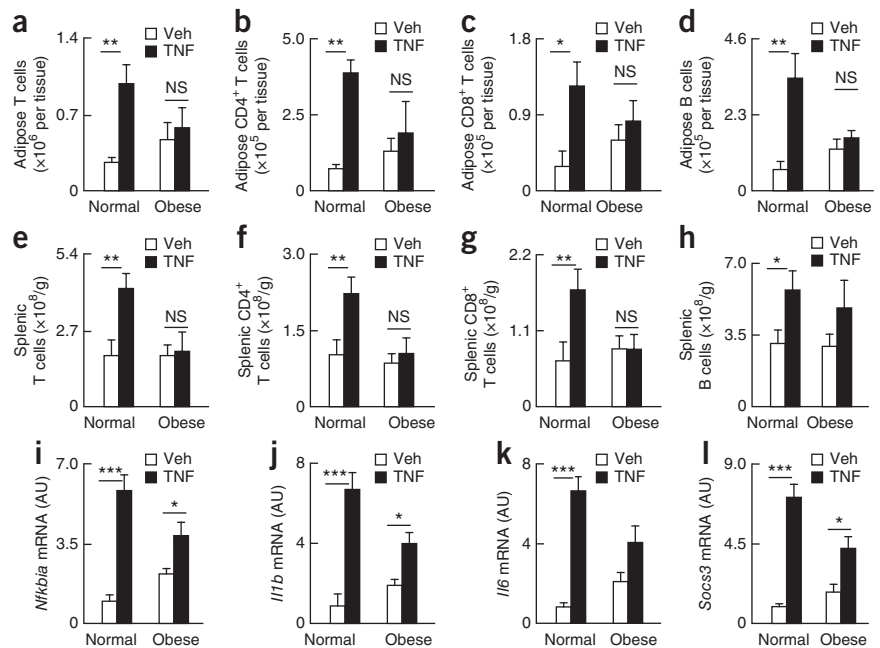


Figure 7 Acute effect of the brain-fat axis on adaptive immunity is mediated by adipose nerves. (a) Flow cytometry of T cells, B cells, CD4⁺ T cells and CD8⁺ T cells (all identified as in Fig. 1a) in the epididymal fat of C57BL/6 mice ($n = 7–8$ per group) that underwent denervation of the epididymal fat (Den) or sham surgery (Sham) and simultaneous implantation of a cannula into the hypothalamic third ventricle and, following 1 week of recovery, were given daily injection, for 3 d, of TNF (10 pg) or vehicle via the cannula. (b–i) Quantification of T cells (b,f), CD4⁺ T cells (c,g), CD8⁺ T cells (d,h) and B cells (e,i) (as in a) in the epididymal fat (b–e) and spleen (f–i) of mice as in a ($n = 7–8$ per group). * $P < 0.05$ and ** $P < 0.01$ (ANOVA and Tukey's *post-hoc* test). Data are representative of three independent experiments with similar results (mean and s.e.m. in b–i).

Figure 8 Chronic neuroinflammation desensitizes the central regulation of adaptive immunity. (a–h) Quantification of T cells (a,e), CD4⁺ T cells (b,f), CD8⁺ T cells (c,g) and B cells (d,h) (all identified as in Fig. 1a) in the epididymal fat (a–d) and spleen (e–h) of adult C57BL/6 mice fed a high-fat diet to develop obesity (Obese) and age- and sex-matched control mice fed normal chow (Normal), all given daily injections, for 3 d, of TNF or vehicle into the hypothalamic third ventricle. (i–l) Expression of genes encoding inflammatory molecules in the hypothalamus of mice as in a–h. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (ANOVA and Tukey's *post-hoc* test). Data are representative of two independent experiments with similar results (mean and s.e.m.).



of lipolysis, we further appreciated that infection-induced lipolysis was profoundly accompanied by enhanced release of adipokines such as leptin. This characteristic differs from the features of starvation during which leptin release from the fat is in fact suppressed. Of note, systemic leptin has been shown to enhance the function of organs of the immune system²⁹; moreover, an acute increase in leptin has a considerable metabolic effect on increasing lipolysis. We analyzed blood samples from several mouse models in this study and found that serum leptin concentrations increased in mice in response to central injection of TNF or bacterial infection and that TNFRs in the MBH were required for this effect (Supplementary Fig. 5b–e). We then studied the potential relationship between the release of fatty acids and that of leptin in affecting the adaptive immune response. Through the use of pre-treatment with cerulenin, we found that inhibition of lipolysis impaired the injection intraperitoneal of leptin in increasing the abundance of lymphocytes (Supplementary Fig. 6a–h), which indicated that lipolysis mediated the leptin-induced adaptive immune response. Notably, we also found that blocking systemic leptin through the neutralization of leptin led to compromised effects of palmitate in increasing the abundance of T cells and B cells (Supplementary Fig. 7a–h), which suggested that the increase in the release of leptin from fat was required for the effect of lipolysis in increasing the abundance of lymphocytes. Thus, fatty acids and leptin released from adipose tissue acted together with each other in inducing adaptive immune responses, although the underlying crosstalk mechanism remains to be explored.

Chronic neuroinflammation impairs the adaptive immune response

Published studies have shown that the baseline levels of innate immunity signals are high in the hypothalamus under conditions of obesity and associated metabolic disorders^{7–11}. Thus, we sought to determine whether chronic hypothalamic inflammation in this metabolic model might have an effect on the central regulation of adaptive immunity. To do so, we subjected mice with diet-induced obesity (obese mice) and mice fed normal chow ('normal' mice) to daily central injections of a low dose of TNF (10 pg) for 3 d. In agreement with published reports^{30,31}, we noted that the number of lymphocytes in the fat of obese mice was higher than that in 'normal' mice (Fig. 8a–d). However, in response to hypothalamic injection of a low dose of TNF, the number of adipose T cells and B cells in these obese mice did not become greater than that in vehicle-injected obese mice, whether the calculations were based on cell numbers per fat pad of epididymal fat

(Fig. 8a–d) or per gram of epididymal fat (data not shown). We also studied splenic lymphocytes in these mice and found that there was a lack of responsiveness to the hypothalamic injection of TNF in obese mice (Fig. 8e–h). To gain insight into the underlying basis of this, we analyzed the hypothalamus for the expression of various TNF-induced genes encoding inflammatory molecules, including *Nfkb*, *Il1b*, *Il6* and *Socs3*. We found that under basal conditions, expression of mRNA from these genes was generally higher in the hypothalamus of obese mice than in that of 'normal' mice (Fig. 8i–l). In response to the hypothalamic injection of TNF at the low dose, the abundance of mRNA from these genes in the hypothalamus increased substantially in 'normal' mice but only slightly or negligibly in obese mice (Fig. 8i–l). This reduction in hypothalamic responsiveness to TNF might explain the observed impairment of adaptive immunity in obesity, which would indicate that a responsive hypothalamic environment for innate immunological signals such as TNF is necessary for the brain to acutely regulate the adaptive immune response. Thus, while acute induction of brain TNF provided an important aid for the initiation of adaptive immune responses, the sensitivity of this function was diminished under obesity-associated chronic hypothalamic inflammation and contributed to impaired adaptive immunity in obesity and related diseases (Supplementary Fig. 8).

DISCUSSION

On the basis of results obtained with *in vitro* and *in vivo* models, it is known that cytokines such as TNF are produced from cells of the innate immune system, such as macrophages, and have a critical role in conveying innate immunity to adaptive immunity⁶. However, as an adaptive immune response typically requires 4–7 d to develop, it is a considerable limitation for an organism to fight against an invasion that becomes overwhelming soon after initial attack^{4,5}. Therefore, study of the early processes and regulation of the adaptive immune response is important, and understanding of the underlying basis of this may help in the development of new strategies by which lymphocytes can be promoted in a rapid fashion to aid the adaptive immune response. Here we discovered that the brain and, in particular, the hypothalamus quickly responded to the innate immunological signal TNF and used the brain-fat axis to initiate increases in the

abundance of T cells and B cells in peripheral tissues, including the fat and spleen. The brain is best known for its fast action in regulating physiology, but possible relevance of this feature to immunology has not been explored much. However, it has been shown that the brain can respond to cytokines of the innate immune system to alter activities of the sympathetic nerve system, and TNF is a cytokine that can rapidly induce neural synaptic changes in experimental autoimmune encephalomyelitis³². Also, as observed clinically and experimentally, brain injuries are often associated with impairments of the immune system and high susceptibility to infection, and it has been suggested that the hypothalamus is a factor that mediates systemic depression of the immune system³³. Notably, activation of certain neuronal pathways can downregulate peripheral responses of the innate immune system^{34–37}, although the hypothalamus has not been studied in this context. Thus, in conjunction with our findings here, we suggest that the hypothalamus-periphery axis has an unappreciated role in regulating immunological homeostasis by improving the sensitivity and efficiency of adaptive immunity on the one hand and preventing excess innate immune responses on the other hand.

The hypothalamus uses pathways of the innate immune system to exert metabolic changes^{7–11}, and hypothalamic actions from cytokines of the innate immune system have considerable effects on the metabolic state of fat^{38,39}. Since the blood-brain barrier surrounding the MBH is partially permeable^{14,15}, this hypothalamic region represents a critical site for studying the involvement of brain-periphery communications in physiology, including systemic immunity⁴⁰. In this work, we sought to determine whether the brain might work through a hypothalamus-fat axis to coordinate innate immunological signals with adaptive immunity. This question is provocative, because unlike the operation of innate immunity, the operation of adaptive immunity is orchestrated by specialized organs of the immune system such as the spleen, while fat tissue is not classically viewed as a component of adaptive immunity. However, fat tissue has appreciable numbers of cells of the immune system, including not only those of the innate immune system, such as macrophages, but also those of the adaptive immune system, including T cells and B cells^{29,41,42}. While cells of the innate immune system in the fat have often been related to the development of metabolic disorders such as type 2 diabetes^{43,44}, the biological function of cells of the adaptive immune system in fat tissue is still unknown. It has been reported that the memory function of CD8⁺ T cells can be enhanced by the modulation of fatty acid metabolism in mice²⁴, which might indicate a role for fat tissue, via lipid metabolism, in adaptive immunity. Here we found that the fat was an organ with a function in supporting the initiation of adaptive immunity and that the hypothalamus was responsible for ensuring this function. Indeed, it is well documented that severe lack of fat in the body, such as lipodystrophy, is extremely injurious to adaptive immunity⁴⁵. However, fat excess in disorders such as obesity is also detrimental to adaptive immunity, and we have provided evidence suggesting that obesity-associated chronic neural inflammation might blunt the hypothalamic regulation of the adaptive immune response. Together our findings indicated that a homeostatic and responsive hypothalamus-fat axis was necessary for adaptive immunity. Moreover, since fat tissue is distributed throughout the body as widely as lymphoid tissue is, our findings might lead to the viewpoint that components of adaptive immunity can be extended to include fat tissue.

In this work, we also initially investigated the route by which the brain-fat axis works to increase the abundance of lymphocytes in the periphery. Our findings suggested that the hypothalamus used the sympathetic nervous system to induce lipolysis and the release of long-chain

fatty acids and thus increase adipose and splenic lymphocytes. The sympathetic induction of lipolysis is rapid, which would fit with the fast action of the brain-fat axis in promoting cells of the adaptive immune system. Consistent with that finding, it has been reported that changes in fatty acid metabolism affect CD8⁺ T cells²⁴, and palmitate has been reported to increase the proliferation of T lymphocytes⁴⁶. In this context, we also considered that lipolysis itself might be induced in conditions (for example, starvation) that may be unrelated to immunity. However, infection-induced lipolysis has several characteristics (for example, it is associated with the release of adipokines such as leptin) that might act cooperatively with fatty acids in increasing the abundance of lymphocytes. Indeed, unlike starvation, during which the release of leptin is suppressed, infection leads to rapid and robust release of leptin from the fat and, furthermore, leptin has been shown to enhance the function of organs of the immune system²⁹. Here we obtained results suggesting that the hypothalamus-induced release of fatty acids might require the simultaneous release of leptin to induce or optimize the effects on systemic lymphocytes. If so, a subsequent question is whether there are other secretory adipose factors that mediate the effects of hypothalamus-induced lipolysis on adaptive immunity. Clearly, additional research is needed to identify these molecules in the central regulation of adaptive immunity by the brain-fat axis.

Thus, the brain represents a critical participant in conveyance of the innate immunological signal TNF to the adaptive immune response. In this conceptual model, the brain action of TNF is sufficient to initiate the increase in the abundance of peripheral T cells and B cells and that this effect is mediated by the hypothalamic TNFR pathway. This function of hypothalamic TNF in initiating adaptive immunity is mediated critically through hypothalamus-induced lipolysis. In conclusion, we propose, on the basis of our results, that the hypothalamus-fat axis has an important role in rapidly linking signals from the innate immune system to adaptive immune responses.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.S.K. co-designed and performed all experiments, prepared all figures and provided writing assistance; J.Y. did viral injection, histology and immunostaining; W.W. contributed to animal generation, sample preparation and flow cytometry; G.Z. co-designed and did preliminary experiments for **Figures 1** and **7**; Y.Z. did viral cloning and generated viruses; M.S.K. and D.C. performed data analysis; D.C. conceived of the hypothesis and designed the project and structure of experiments and wrote the paper; and all authors participated in discussions.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals and peripheral treatment. *Tnfrsf1a^{tm1lm}/Tnfrsf1b^{tm1lm}*/J mice and wild-type C57BL/6 mice were from the Jackson Laboratory. *Pomc*-Cre mice (which express Cre recombinase from the gene encoding pro-opiomelanocortin- α) were produced as described⁴⁷. All mice were maintained on the C57BL/6 background. Mice were housed in standard, pathogen-free conditions with 12 h–12 h light–dark cycles and were maintained on a normal chow diet; adult male mice (3–5 months of age) were used in experiments. C57BL/6 mice with diet-induced obesity were generated through 15 weeks of being fed a high-fat diet, and age-matched chow-fed C57BL/6 mice were used as controls. For pharmacological injections, mice were given intraperitoneal injection of TNF, palmitic acid, linoleic acid, cerulenin (Sigma), recombinant mouse leptin and/or neutralizing polyclonal antibody to mouse leptin (AF498; R&D Systems) at the appropriate dose for the necessary duration. For bacterial injection, mice were given intravenous injection of *L. monocytogenes* (ATCC) at a dose of 5×10^4 colony-forming units. Mice were killed at the appropriate times after treatment, and various tissues were collected for subsequent analysis. The Institutional Animal Care and Use Committee at the Albert Einstein College of Medicine approved all the procedures.

Cannulation of and injection into the hypothalamic third ventricle. The hypothalamic third ventricle was cannulated as described^{8,9,11}. We used an ultraprecise small animal stereotactic apparatus (Kopf Instruments) to implant a 26-gauge guide cannula (Plastics One) at the midline coordinates of 1.8 mm posterior to the bregma and 5.0 mm below the bregma. Mice were given 1 to 2 weeks for complete surgical recovery, and were given injection, via the cannula, of the appropriate doses of TNF (Sigma) or WP9QY (AnaSpec) dissolved in 0.5 μ l of artificial cerebrospinal fluid, over a duration of 5 min. Injection of artificial cerebrospinal fluid alone served as the vehicle control. Intra-MBH injection, which targeted mainly the arcuate nucleus, was performed as described¹¹. Injections were directed on an ultra-precise stereotactic apparatus at coordinates of 1.5 mm posterior to bregma, 5.8 mm below the surface of skull and 0.3 mm lateral to midline. Purified lentiviruses suspended in 0.2 μ l artificial cerebrospinal fluid were injected over 10-minute period via a 26-gauge guide cannula and a 33-gauge internal injector connected to a 5- μ l Hamilton Syringe and infusion pump (WPI Instruments).

Lentivirus and histology. Using a procedure similar to those described before^{7–11}, we cloned cDNA encoding TNFR1 (Sigma) into a lentiviral expression system driven by the promoter of the gene encoding synapsin, and cloned sequence encoding shRNA targeting mRNA encoding TNFR1 or TNFR2 into a Cre-dependent conditional lentiviral shRNA system (Addgene). Lentiviral vectors containing shRNA targeting mRNA encoding TNFR1 or TNFR2 were obtained from Sigma. Lentiviruses were generated and amplified through the use of HEK293T human embryonic kidney cells and then were purified as described¹¹. Brain histology was analyzed with brain sections and immunostaining. Mice under anesthesia were transcardially perfused with 4% PFA and brains were removed, fixed and post-fixed in 4% PFA, and infiltrated with 20–30% sucrose. Tissue sections at 20 μ m thickness were cut and blocked, then were penetrated with 0.2% Triton-X 100, treated with the primary antibody rabbit polyclonal anti-TNFR1 (1:1,000 dilution; ab19139; Abcam), mouse monoclonal anti-TNFR2 (1:1,000 dilution; L-20; Santa Cruz), or mouse monoclonal anti-BrdU (1:1,000 dilution; Bu20a; Cell Signaling), followed by reaction with the secondary antibody Alexa Fluor 488–conjugated goat polyclonal antibody to mouse (1:1,000 dilution; A-10608; Invitrogen). For BrdU staining, mice were given injection of BrdU (5-bromodeoxyuridine; Invitrogen) at a dose of 30 mg per kg body weight 2 h before perfusion of mice. Staining with DAPI (4,6-diamidino-2-phenylindole) was used to reveal all cells in the sections. Images were captured by confocal microscopy.

Denervation of epididymal fat. Epididymal fat was denervated as described²⁸. In this procedure, mice were anesthetized, a small skin incision on the lower abdominal portion was made to invade the peritoneal cavity and, subsequently, the vascular strand innervating the epididymal fat pad was identified by laparotomy. The nerve bundle from the vessels was carefully dissected and cut with scissors, followed by local application of phenol. The tissue was cleaned with sterile saline and skin was closed with sterile sutures.

Flow cytometry. Each spleen was minced with DMEM, filtered through a 100- μ m cell strainer and, after centrifugation, was incubated for 5 min at 25 °C with lysing buffer (BD Pharm Lyse) for cell isolation via centrifugation. The epididymal fat was carefully dissected, minced with a scalpel, digested for 20 min at 37 °C with Liberase TM Research Grade (Roche), filtered through a 100- μ m cell strainer and pelleted by centrifugation for cell suspension. Blood was collected and incubated for 5 min at 25 °C with lysing Buffer (BD Pharm Lyse), and single cells were prepared by centrifugation. Single-cell suspensions of these tissues were washed twice, and cell yields and viability were assessed by trypan blue staining. Cell suspensions were adjusted to a density of 1×10^6 cells and nonspecific binding was blocked by incubation for 10 min at 4 °C with antibody to CD16-CD31 (Fc Block; BD Pharmingen) for flow cytometry. Single-cell suspensions were incubated for 20 min at 4 °C with the following antibodies: fluorescein isothiocyanate–anti-CD4 (GK 1.5; eBioscience), allophycocyanin–anti-CD8 (53-6.7; BD Pharmingen), phycoerythrin–anti-CD3 (145-2c11; eBioscience), phycoerythrin–indotricarbocyanine–anti-B220 (RA3-6B2; Biolegend), allophycocyanin–anti-CD25 (PC61; BD Pharmingen), allophycocyanin–anti-F4/80 (BM8; eBioscience), fluorescein isothiocyanate–anti-CD11b (M1/70; BD Pharmingen), phycoerythrin–anti-CD11c (HL3; BD Pharmingen), fluorescein isothiocyanate–anti-CD278 (anti-ICOS) (7E.17G9; eBioscience), phycoerythrin–indotricarbocyanine–anti-CD279 (anti-PD-1) (J43; eBioscience), phycoerythrin–anti-CD274 (anti-PD-L1) (MIH5; eBioscience), Brilliant Violet 421–anti-CD183 (anti-CXCR3) (CXCR3-173; BD Horizon) and Alexa Fluor 647–anti-CD196 (anti-CCR6) (140706; BD Pharmingen). Intracellular staining with Alexa Fluor 488–anti-Foxp3 (FJK-16s; eBioscience) was achieved with BD Cytotfix/Cytoperm Plus (BD Pharmingen). Lymphocyte subpopulations incubated with those flow cytometry antibodies were analyzed with an LSR II (BD Bioscience) and then data were analyzed with FlowJo software (BD Bioscience).

Biochemical assays. Free fatty acids in serum were assessed biochemically with acyl-CoA synthetase–acyl-CoA oxidase (ACS-ACOD) with the NEFA-HR (non-esterified fatty acids) reagent according to the manufacturer's instructions (Wako). TNF concentrations in the serum and CSF of mice were determined with a Mouse TNF ELISA kit according to the manufacturer's instructions (eBioscience). Serum leptin concentrations were measured with a Mouse Leptin ELISA Kit (Crystal Chem). CSF was collected as described⁴⁸: an anesthetized mouse was fixed on the stereotactic apparatus with the head placed to an angle of $\sim 135^\circ$ relative to the body, then a sagittal incision in the neck skin was applied to inferior to the occiput, followed by penetration of a capillary tube through the dura mater into the cisterna magna to draw the CSF. For metabolomics analysis, ^2H -labeling of fatty acid was assessed as described⁴⁹. After labeling of ^2H , fatty acids were derivatized with diazomethane. Fatty acid methyl esters were formed by dissolution of the extracted fatty acids in 50 μ l of methanol and the addition of 300 μ l of ether-diazomethane. The sample was allowed to react for 45 min at 25 °C. The fatty acid methyl esters were then dissolved in 100 μ l of chloroform and were analyzed by gas chromatography–electron ionization mass spectrometry. To account for possible differences in the ionization efficiency of each fatty acid, the profile was compared with standards prepared by mixture of known quantities of each fatty acid.

Quantitative RT-PCR. Tissues were homogenized, and RNA was extracted from them with TRIzol (Invitrogen). Complementary DNA was synthesized with a M-MLV Reverse Transcriptase kit (Promega) and was subjected to amplification by PCR with SYBR Green PCR Master Mix (Applied Biosystems) and primers with the following sequences: *Lipe*, 5'-TGTTGGGGTGACTCTAACGC-3' and 5'-GTCTTCTGCGAGTGTCACCA-3'; *Lpl*, 5'-CCAGCTGGGCCTAACTTTGA-3' and 5'-AACTCAGGCA GAGCCCTTTC-3'; *Acs1l*, 5'-GCCTCACTGCCCTTTTCTGA-3' and 5'-GGCTGATGATTTCCACCCCA-3'; *Nfkb1a*, 5'-TGAAGGACGAGG AGTACGAGC-3' and 5'-TTCGTGGATGATTGCCAAGTG-3'; *Il1b*, 5'-GCTCAGGGTCAACAAGAAACC-3' and 5'-CATCAAAGCAATGTGCTGGT-3'; *Il6*, 5'-CCAGAGATACAAAGAAATGATGG-3' and 5'-ACTCCAGAA GACCAGAGGAAAT-3'; *Socs3*, 5'-ATGGTCACCCACAGCAAGTTT-3' and 5'-TCCAGTAGAATCCGCTCTCCT-3'; *Actb*, 5'-CCAACTGGGA CGACATGGAGA-3' and 5'-CATGGCTGGGGTGTGTAAGG-3'; and

Tbp, 5'-ACCCTTCACCAATGACTCCTATG-3' and 5'-TGACTGCAGCAAATCGCTTGG-3'. PCR results were normalized to those of the control genes encoding TATA box-binding protein (*Tbp*) or β -actin (*Actb*).

Statistical analysis. ANOVA and Tukey's *post-hoc* test were used for comparisons involving more than two groups. Two-tailed Student's *t*-test was used for comparisons only involving two groups. A *P* value of <0.05 was considered statistically significant. Sample sizes were designed with adequate power according to the literature and our previous studies. Animals were randomly assigned to experimental groups, and the investigators were not blinded to

group allocations. The data met normal distribution with similar variance between groups being compared.

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