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Dietary wheat amylase trypsin inhibitors exacerbate CNS inflammation in experimental multiple sclerosis

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ABSTRACT

Objective Wheat has become a main staple globally. We studied the effect of defined pro-inflammatory dietary proteins, wheat amylase trypsin inhibitors (ATI), activating intestinal myeloid cells via toll-like receptor 4, in experimental autoimmune encephalitis (EAE), a model of multiple sclerosis (MS).

Design EAE was induced in C57BL/6J mice on standardised dietary regimes with defined content of gluten/ATI. Mice received a gluten and ATI-free diet with defined carbohydrate and protein (casein/zein) content, supplemented with: (a) 25% of gluten and 0.75% ATI; (b) 25% gluten and 0.19% ATI or (c) 1.5% purified ATI. The effect of dietary ATI on clinical EAE severity, on intestinal, mesenteric lymph node, splenic and central nervous system (CNS) subsets of myeloid cells and lymphocytes was analysed. Activation of peripheral blood mononuclear cells from patients with MS and healthy controls was compared.

Results Dietary ATI dose-dependently caused significantly higher EAE clinical scores compared with mice on other dietary regimes, including on gluten alone. This was mediated by increased numbers and activation of pro-inflammatory intestinal, lymph node, splenic and CNS myeloid cells and of CNS-infiltrating encephalitogenic T-lymphocytes. Expectedly, ATI activated peripheral blood monocytes from both patients with MS and healthy controls.

Conclusions Dietary wheat ATI activate murine and human myeloid cells. The amount of ATI present in an average human wheat-based diet caused mild intestinal inflammation, which was propagated to extraintestinal sites, leading to exacerbation of CNS inflammation and worsening of clinical symptoms in EAE. These results support the importance of the gut-brain axis in inflammatory CNS disease.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS) with a prevalence that has increased over the past decades due to genetic and environmental factors.
⇒ A Western diet with high caloric intake and high salt worsened experimental autoimmune encephalitis (EAE), a mouse model that resembles human MS.
⇒ Dietary wheat, the main staple in most countries, contains amylase trypsin inhibitors (ATI), non-gluten proteins that activate intestinal myeloid cells via toll-like receptor 4.

WHAT THIS STUDY ADDS

⇒ Doses of nutritional ATI, present in normal wheat-based diet, aggravate EAE and CNS inflammation in mice.
⇒ This is not due to gluten, since gluten, de-enriched of (contaminating) ATI fails to show this EAE aggravating effect.
⇒ Myeloid cell subsets activated by ingested ATI are found in intestinal and extraintestinal organs including the CNS.
⇒ These myeloid cells activated by dietary ATI can be linked to enhanced CNS and effector lymphocytes in the CNS.
⇒ ATI enhance the immune activation of peripheral blood mononuclear cells from patients with MS and healthy controls.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study suggests that the consumption of wheat and wheat ATI may worsen human MS, and stress the relevance of gut-brain signalling in inflammatory CNS disease.
⇒ Clinical studies should be performed to confirm the beneficial effect of a wheat (ATI)-free diet as adjunctive therapy in patients with MS.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) with a prominent T cell-driven autoimmune component, which is directed towards autoantigens of the myelin sheath.[1 2] First disease manifestations usually occur between the age of 20 and 40 years with visual and sensory disturbances, muscle spasms and limb weakness, progressing to severe disability. While genetics clearly determine MS prevalence, environmental factors have emerged as important modifiers of MS manifestation and severity.[1 3]

Recently, nutritional factors have been shown to modulate disease severity in rodent models of experimental autoimmune encephalomyelitis

(EAE), which share common features with human MS, where components of the Western diet such as a high intake of dietary salt,[4–6] saturated fat[7] or refined sugars[8] promoted the infiltration of the CNS with pathogenic T helper (Th)-17 cells and macrophages,[5] enhancing EAE severity. Diet also affects the intestinal microbiota, with secondary effects on intestinal and peripheral inflammation.[9] However, the mechanisms by which these food components or the intestinal microbiota would influence peripheral immunity remain largely unknown.

Clinical nutritional intervention studies in MS often lack appropriate sample size, inclusion criteria, patient stratification and control groups.[10] There is only one observational study[11] suggesting that a gluten-free diet supplemented with vitamins and minerals might have a positive effect on pain, numbness and mobility in patients with MS. However, this report did not fulfil the criteria of a standardised clinical trial, lacking controls and valid outcome measures.

Wheat is a major food staple in most societies, mostly used to manufacture baked products, pasta or meat replacements. Wheat flour contains 9%–13% protein that can be separated into gluten and non-gluten fractions (85% and 15%, respectively). Gluten is also a ubiquitous food ingredient, often used to improve the texture of refined foods.

Wheat has been systematically grown, bred and introduced into our diet approximately 6000 years ago in central Europe.[12] Nonetheless, wheat proteins have inflammatory potential, with ~1% of most populations being affected by coeliac disease, where gluten peptides trigger an intestinal Th1 T cell response in genetically predisposed individuals, leading to small intestinal villous atrophy and a wide range of associated comorbidities.[13] Moreover, apart from respiratory and nutritional wheat allergies to gluten and non-gluten proteins,[14] many patients with irritable bowel syndrome suffer from IgE-negative wheat allergy.[15] A third inflammatory reaction to wheat is the activation of innate immunity by amylase trypsin inhibitors (ATI), a family of up to 17 structurally related non-gluten proteins that are resistant to intestinal enzymatic digestion.[16 17] ATI activate myeloid cells (monocytes, macrophages and dendritic cells) via the toll-like receptor 4 (TLR4) in vitro and after oral ingestion in vivo. Unlike the major TLR4 ligand, lipopolysaccharide (LPS), which is derived from (Gram-negative) bacteria, ATI remain active in the GI tract, where LPS is usually inactivated in the stomach by acid hydrolysis and in the gut by intestinal epithelial alkaline phosphatase.[18–21] Therefore, ATI survive GI passage in their bioactive form to stimulate lamina propria myeloid cells.[19 22 23]

Importantly, the ATI-induced mild inflammatory signal in the gut can be transmitted to the periphery via enhanced myeloid cell activation and T cell priming in mesenteric lymph nodes (MLNs) and extraintestinal organs.[19 22] This is accompanied by elevated circulating inflammatory mediators, such as interleukin (IL)-6 or CC-chemokine ligand 2 (CCL-)2, and by exacerbation of experimental IBD, intestinal/pulmonary allergies, non-alcoholic steatohepatitis and liver fibrosis.[22–27] Notably, small controlled clinical studies showed that a wheat-free versus a wheat-containing diet improved molecular and clinical features of patients with familial Mediterranean fever, UC and primary sclerosing cholangitis.[28 29] IgE-negative wheat allergy and ATI sensitivity can explain most of the symptoms of non-coeliac wheat ('gluten') sensitivity.[30 31] Importantly, food grade gluten can contain up to 5% of ATI,[17 22 31] which complicates interpretation of prior challenge studies using different gluten preparations.

Based on prior research on wheat ATI, we tested the putative disease promoting effect of dietary ATI versus gluten alone in murine EAE, focusing on clinical disease activity, cellular/molecular components of intestinal and CNS inflammation. Furthermore, we compared the inflammatory effects of wheat ATI on peripheral blood mononuclear cells (PBMCs) from patients with MS and healthy controls, confirming transferability to patients.

MATERIALS AND METHODS

Diet preparation

We prepared a gluten and ATI-free (GAF) diet as customised pellets (Ssniff, Soest, Germany) with a defined carbohydrate content and either milk casein or maize zein as main protein source (~22% of dry weight) as described before.[22–24] Based on the GAF diet, 3 diet preparations were manufactured, namely: the gluten and ATI (GA) diet (5.5% of the food dry weight and 25% of the zein is supplemented with gluten, which contains 0.75% associated ATI, equivalent to 0.165% of food dry weight); the gluten (G) diet de-enriched of ATI (5.5% of the food dry weight and 25% of the zein is supplemented with gluten, which contains 0.19% ATI that remains associated with the gluten after de-enrichment, equivalent to 0.042% of food dry weight); the ATI (A) diet (0.15% of the food dry weight) . Wheat flour contains ~0.4% ATI, and humans consume ~200 g of wheat flour per day, equivalent to 0.8 g ATI daily=11.4 mg/kg/day (body weight 70 kg). Mice consume ~3 g chow per day, and with the 0.15% pure ATI diet an amount of 3.75 mg per 25 g body weight=150 mg/kg/day. Since the division factor for pharmacological dosing (body surface based) for mouse versus man is 12, the human equivalent of the mouse pure ATI dose is 150 mg/12=12.5 mg/kg/day. For dietary compositions, see online supplemental table 1.

Isolation of ATI and ATI bioactivity assays of enzymatically digested food pellets

ATIs were extracted and purified from commercial hexaploidy wheat flour (Diamant). Prominent ATI species were dimeric 0.19>tetrameric CM3>dimeric 0.28, and tetrameric CM2, CM16 and CM17.[32 33] Briefly, wheat or gluten was extracted using ammonium bicarbonate buffer, dialysed, sterile filtrated and lyophilised. ATI were isolated by fractional salt precipitation using ammonium sulfate. Further purification of ATI by fast protein liquid chromatography (FPLC) was used only for in vitro experiments.[22] Food pellets were enzymatically digested as described[22] and bioactivity tested by adding samples reconstituted in phosphate-buffered saline (PBS) to TLR4/MD-2/CD14/IL-8 Prom/LUCPorter HeLa cells (Novus Biologicals, Wiesbaden, Germany) at a concentration of 50–250 µg protein/mL of ATI extracts and incubated for 6 hours. Luciferase activity was then analysed 5 min after addition of a luciferase reporter assay reagent using a luminometer (Tecan, Männedorf, Switzerland).

Mice

Female C57BL/6J mice, aged 6–8 weeks, were purchased from Harlan (An Venray, The Netherlands) and maintained on standard chow (Ssniff, Soest, Germany) before being placed on a defined GAF diet for 4 weeks. Two days before EAE induction, mice continued a GAF diet or changed to a GA, G or A diet (see 'Diet preparation' section).

Induction of EAE and assessment of EAE severity

EAE was induced and assessed as previously described.[34] Mice were immunised subcutaneously at the tail base with 50 µg of myelin oligodendrocyte glycoprotein peptide (MOG_{p35-55}), emulsified in 100 µL complete Freund's adjuvant, supplemented with

8 mg/mL of heat-inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories, Schwerte, Germany). Mice also received 200 ng pertussis toxin (Sigma-Aldrich, Schnelldorf, Germany) intraperitoneally, on the day of immunisation and 2 days later. Clinical signs of disease were monitored as described,[34] based on progressive deterioration of motor skills (from mild to total paralysis), using the following staging criteria: 0, healthy; 1, limp tail; 2, partial hindlimb weakness/ataxia; 3, paralysis of at least one hindlimb; 4, complete hindlimb paralysis; 5, partial forelimb paralysis and 6, moribund/dead. CNS and peripheral organs were collected for further evaluation.

Cell preparation, flow cytometry staining and acquisition

CNS-infiltrating cells were isolated from mice at the peak and at onset of disease as described by us,[35] stained for surface and intracellular markers (online supplemental methods) and analysed using a FACSCanto device, FACSDiva (BD Biosciences, Heidelberg, Germany) and FlowJo software (Tree Star, Ashland, Oregon, USA).

Histology and immunohistochemistry

Six μm formalin-fixed small intestinal sections were stained with an antibody to F4/80+ (BM4007, diluted 1:100, Acris, Herford, Germany) to identify resident macrophages. Immunofluorescence staining on intestinal cryosections was performed with the TSA Cy3 System (NEL704A001KT, PerkinElmer, Waltham, Massachusetts, USA) and a fluorescence microscope (IX70; Olympus, Tokyo, Japan) using primary antibodies against CD11c (550283, BD, New Jersey, USA, dilution 1:200) and F4/80 (BM8 eBioscience, dilution 1:1000) as described by us[36] (online supplemental methods).

Quantitative real-time PCR

RNA was extracted from duodenum, ileum and colon (Roboklon, E3598-02, Berlin, Germany) and reverse-transcribed (Bio-Rad, Hercules, USA). Quantitative PCR was performed using exon-exon boundary-spanning primer sequences for keratinocyte chemoattractant (KC, murine IL-8), monocyte chemoattractant protein 1 (MCP-1), IL-6 and IL-15 (online supplemental table 2), and the SYBR Green methodology on a Step One Plus sequence amplification system (Applied Biosystems, Foster City, USA). The relative messenger RNA expression of the tested gene relative to glyceraldehyde-3-phosphate dehydrogenase expression was calculated using the $2^{-\Delta C_P}$ method, as described.[22]

Human peripheral blood mononuclear cells

PBMCs were isolated from six healthy donors (HD) and six patients with relapsing-remitting MS (online supplemental table 3) using Lymphoprep gradient (STEMCELL Technologies, Cologne, Germany). Monocytes were isolated by negative selection immunomagnetic cell separation (monocyte isolation kit II, Miltenyi Biotec, Bergisch Gladbach, Germany) as described.[37]

Cell culture and stimulation of human monocytes

Human PBMCs were cultured and stimulated with ATI/LPS, cell supernatant cytokine/chemokines measured (online supplemental methods) using ELISAs (Ready-Set-Go, eBioscience, Darmstadt, Germany) and transcripts from monocytes (online supplemental table 2) were quantified using real-time PCR (Bio-Rad).

Statistical analyses

Data were statistically analysed using GraphPad Prism V.9.40 (GraphPad Software, San Diego, California, USA). Multiple comparisons were performed by one-way or two-way analysis of variance. Differences among selected experimental groups with p values <0.05 were considered statistically significant.

RESULTS

Dietary ATI aggravate CNS autoimmunity in mice

Mice were kept on a defined GAF diet for 4 weeks, before studying the immunogenic effect of dietary ATI on CNS inflammation. After this period, mice were either placed on a GA diet (25% of total protein as crude gluten containing 0.75% ATI) or continued on a GAF diet. Both groups of mice were immunised with MOG_{P35-55} and pertussis toxin for the induction of EAE and clinical scores were evaluated until day 22, when intestinal and CNS tissues were collected for evaluation (figure 1A). The pro-inflammatory activity of ATI in the food pellets was tested in a cell-based TLR4 reporter assay after quantitative extraction,[22 38] confirming that GA pellets had a fourfold higher TLR4-stimulating activity than GAF pellets (figure 1B).

The mean EAE clinical score as well as the cumulative score (area under the curve) over 22 days were significantly higher in the group of mice consuming the GA versus GAF diet, reaching significance 14 days post-EAE induction (figure 1C and D), while body weight and small intestinal length were comparable in both groups (online supplemental figure 1A–B). With GA feeding, transcripts for key innate inflammatory mediators (KC, keratinocyte chemoattractant or Murine IL-8; monocyte chenmmoattracrctant protein 1, MCP-1) were significantly upregulated in three representative sections of the intestine (duodenum>-terminal ileum and mid-colon) at day 22, with highest expression seen in the duodenum (figure 1E), in line with increased numbers of duodenal and ileal macrophages (figure 2A and B), with a trend for upregulation of IL-6 and IL-15 (online supplemental figure 1C).

Notably, FACS analysis of inflammatory cell infiltration in the CNS revealed a significant increase of $CD4^+$ T cell counts (figure 2C). CNS-infiltrating cells were stimulated with MOG_{P35-55} and thereafter gated on MOG-specific $CD44^+CD40L^+$ followed by quantification of T cells as previously described by us.[39] These MOG-specific encephalitogenic $CD4^+$ T cells, and particularly the numbers of interferon (INF)γ^+ $IL17a^+$ $CD4^+$ T cells were significantly elevated in the GA compared with the GAF group (figure 2C). No difference between both groups was observed for $CD8^+$ T cells and their subsets (online supplemental figure 1D).

Dietary gluten de-enriched of ATI fails to promote EAE

To separate the effect of ATI from a potential effect of dietary gluten in worsening EAE, we prepared a diet with 25% gluten (G) de-enriched of ATI (~0.19% ATI of total protein) and compared it against a GA (~0.75% ATI) and control GAF (0% ATI) diet, using the same conditions as in the first experiment (figure 3A). ATI de-enrichment was also confirmed in G pellets, demonstrating a significantly reduced TLR4-stimulating bioactivity, comparable to GAF pellets (figure 3B). Importantly, the clinical symptom score observed over 22 days in all three study groups correlated with the amount of bioactive ATI present in each diet, that is, the GAF group generated a lower disease score, comparable to the G group, and both groups (GAF and G) showed a significantly lower cumulative clinical score than the GA group, indicating significant worsening of EAE by the ATI component (figure 3C and D). As before, there was no significant change in body weight between the three experimental groups (online supplemental figure 1E). Again, key inflammatory genes (IL-6,

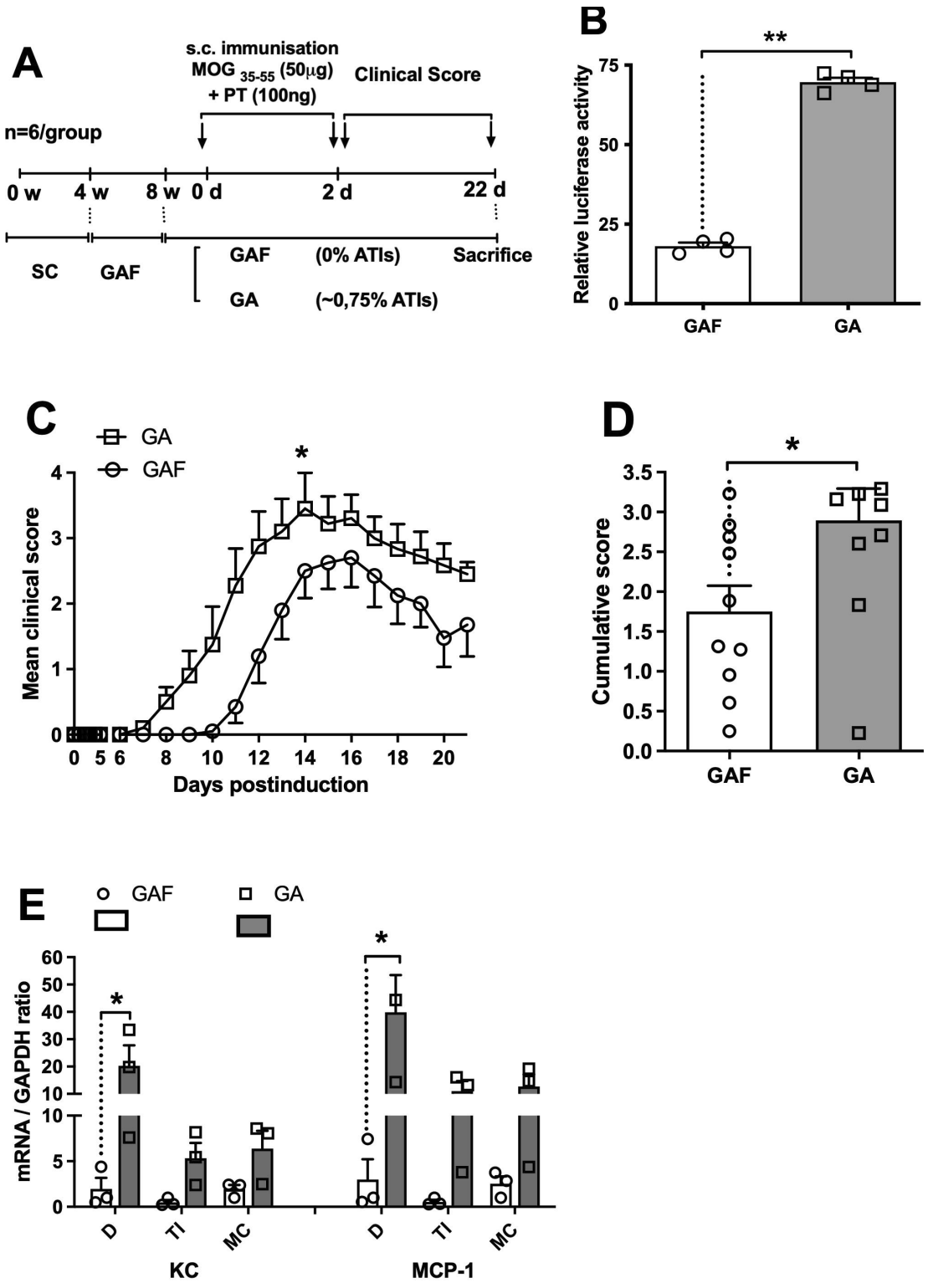

Figure 1 Dietary amylase trypsin inhibitors (ATI)-containing gluten worsens experimental autoimmune encephalitis (EAE). (A) Schematic view of experimental layout: C57BL/6J mice aged 6 weeks raised on standard chow (SC) were placed on the gluten and ATI-free (GAF) diet for 4 weeks prior to myelin oligodendrocyte glycoprotein (MOG)-immunised. Forty-eight hours prior of EAE induction, mice were randomly divided into two groups, which either continue receiving GAF dietary regime or changed to a gluten and ATI (GA) diet containing 25% gluten and 0.75% ATI (n=6 per group). (B) Lipopolysaccharide (LPS)-free toll-like receptor 4 (TLR4)-stimulating bioactivity of GAF and GA pellets, quantitatively extracted for ATI and expressed as relative luciferase activity of indicator cells. (C) EAE mean clinical scores, 22 days post-EAE induction. (D) EAE mean cumulative clinical score. (E) KC (IL-8) and MCP-1 (CCL-2) gene expression in duodenum (D), terminal ileum (TI) and mid-colon (MC), of mice subjected to GAF or GA dietary regiments, as detected 22 days post-MOG immunisation. Results are expressed as mean±SEM (n=6/group, except for figure E having n=4/group). Results are representative of three independent experiments (B and D) or one representative experiment (E). Statistical significance was determined by one-way or two-way analysis of variance. *P<0.05, **p<0.01. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA.

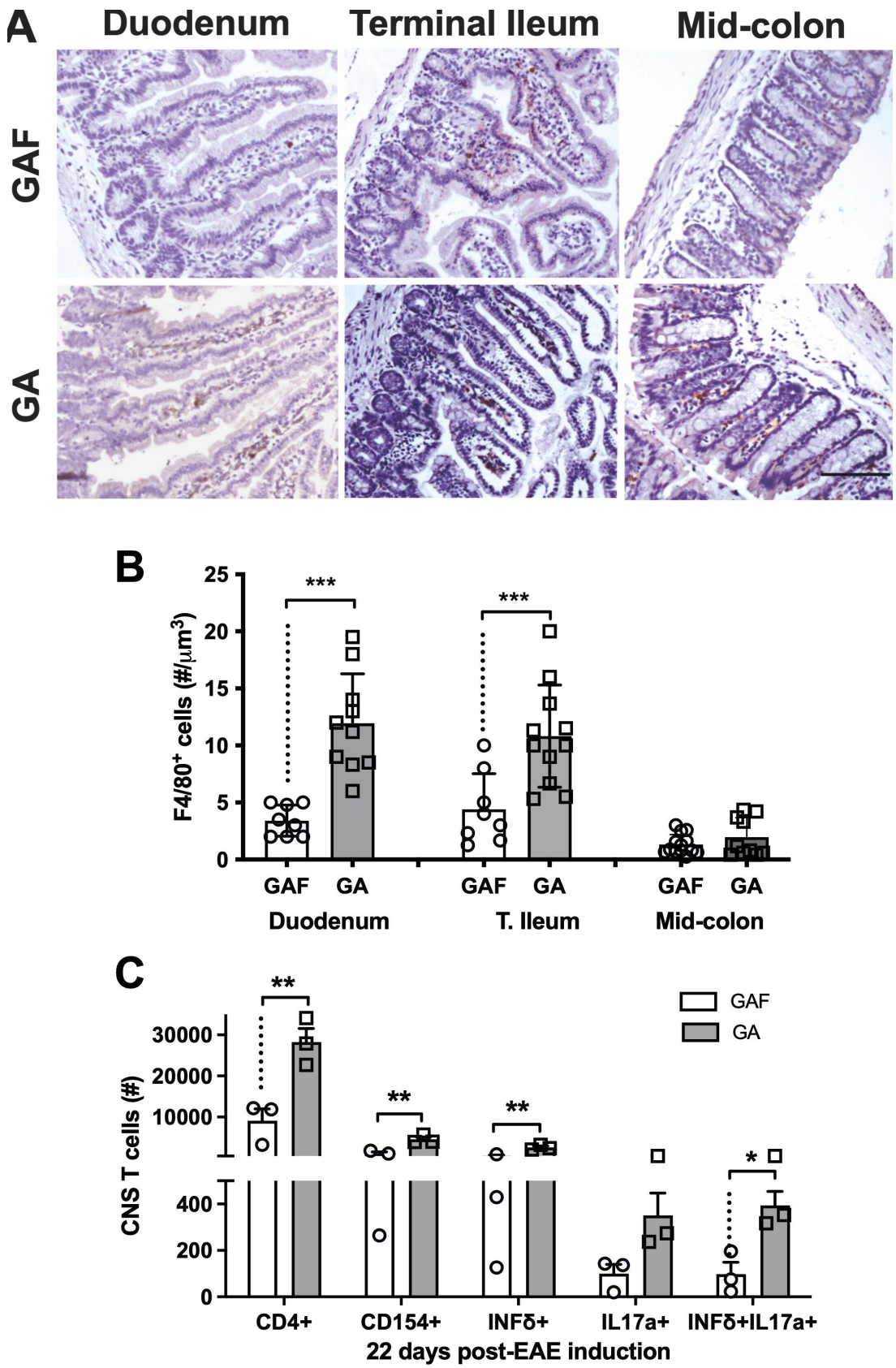

Figure 2 Dietary amylase trypsin inhibitors (ATI)-containing gluten promotes intestinal and central nervous system (CNS) inflammation. Analyses were performed at day 22 postexperimental autoimmune encephalitis (post-EAE) induction in mice on a gluten and ATI-free (GAF) and gluten and ATI (GA) diet. (A) Representative intestinal sections stained for F4/80$^+$ macrophages. (B) F4/80$^+$ cell densities in the villi. (C) Inflammatory cells infiltrating the CNS, including CD4$^+$, CD154$^+$, interferon (INF)γ^+, interleukin (IL)-17a$^+$ and double positive INFγ^+IL-17a$^+$ cells as quantified by flow cytometry (FACS) analysis (means±SEM; n=4/group; representative of two independent experiments). Analysis by one-way or two-way analysis of variance. *P<0.05, **p<0.01, ***p<0.001.

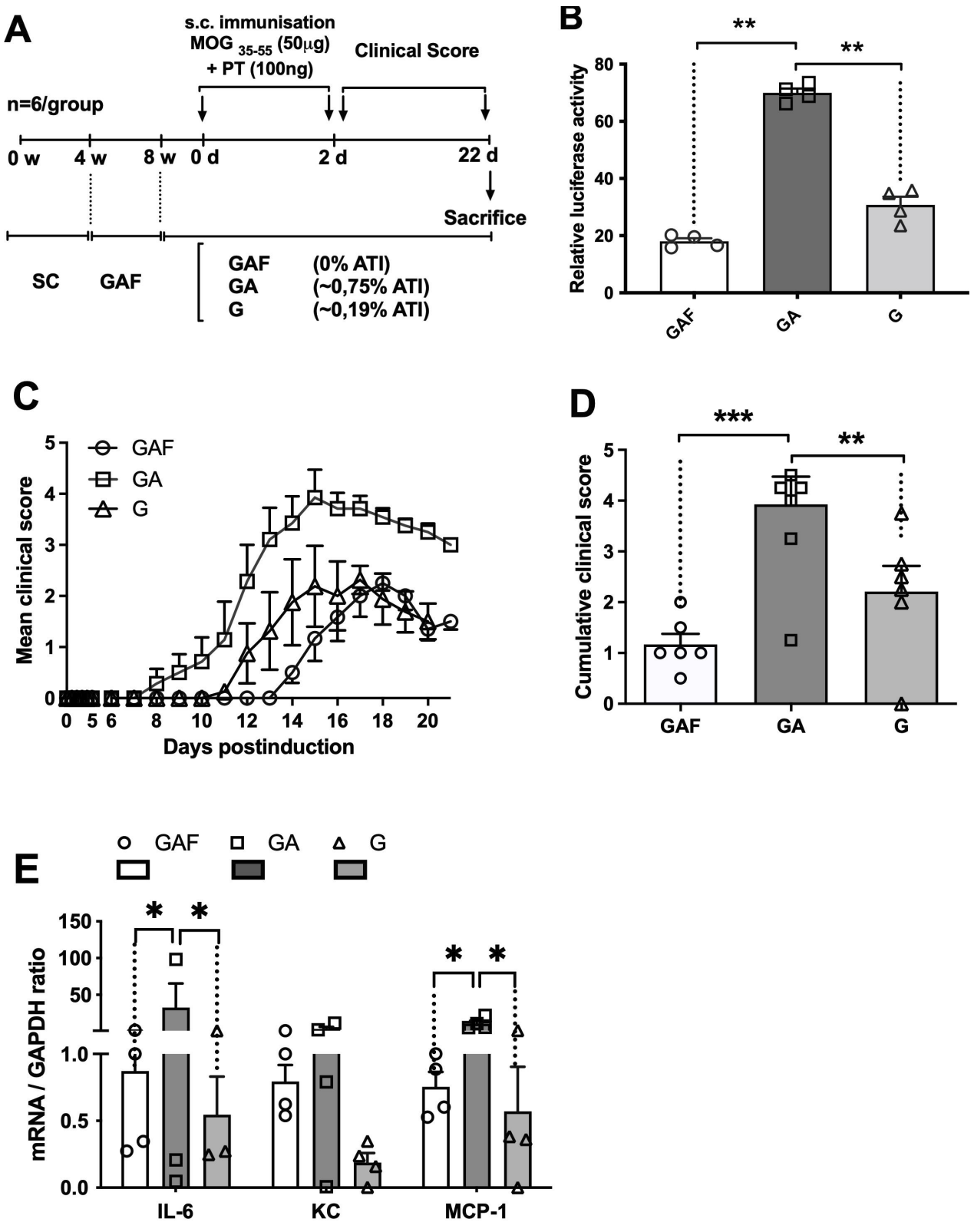

Figure 3 Dietary gluten depleted of amylase trypsin inhibitors (ATI) fails to worsen experimental autoimmune encephalitis (EAE). (A) C57BL/6 mice (n=6 per group) were raised on standard chow (SC), myelin oligodendrocyte glycoprotein (MOG)-immunised, placed on a control gluten and ATI-free (GAF) diet for 4 weeks and divided in three groups: (1) continued GAF; (2) changed to gluten and ATI (GA) (25% gluten and 0.75% ATI) or (3) changed to gluten (G) (25% gluten and 0.19% ATI). (B) Lipopolysaccharide (LPS)-free toll-like receptor 4 (TLR4)-stimulating bioactivity of quantitative extracts of pellets from the three dietary regimes, expressed as relative luciferase activity of indicator cells. (C) Mean clinical scores (22 days postinduction). (D) Mean cumulative clinical scores. (E) Interleukin (IL)-6, KC and MCP-1 gene expression in duodenal samples analysed after 22 days post-EAE induction (means±SEM; n=6/group, except for (E) with n=4/group). Results are representative of two to three independent experiments for (B), (C) and (D). Analysis by one-way or two-way analysis of variance. *P<0.05, **p<0.01, ***p<0.001. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA.

MCP-1 and KC) were upregulated in the duodenum of mice in the GA group compared with the G and GAF groups (figure 3E), which was paralleled by increased duodenal numbers of F4/80$^+$, CD11c$^+$ and F4/80$^+$CD11c$^+$ macrophages and dendritic cells (figure 4A and B). In the CNS, total infiltrating CD4$^+$ T cells, and more importantly the number of encephalitogenic, MOG-specific INFγ$^+$IL-17A$^+$ CD4$^+$ T cells was significantly increased in the GA group and only marginally increased in the G and GAF groups, in alignment with the ATI content and intestinal innate inflammation (figure 4C). Mice on the GAF group had higher ratios of CNS CD4$^+$ Foxp3$^+$ regulatory T (Treg) cells (online supplemental figure 1F), indicating that a gluten and ATI containing diet shifts the composition of CNS-infiltrating encephalitogenic CD4$^+$ effector Th1/17 vs suppressive Treg cells.

Dietary ATI increase CD45$^+$CD11b$^+$ pro-inflammatory myeloid cells infiltrating the CNS and decrease Foxp3$^+$CD25$^+$ Treg cells in mesenteric lymph nodes

To better capture myeloid cell populations in the CNS and to confirm the effects of ATI in worsening EAE symptoms, we set mice on three dietary regimes: GAF (0% ATI), GA (gluten containing 0.75% ATI) and A (1.5% purified ATI) using the same conditions as in the initial experiments but ending the experiment at day 15 post-EAE induction, that is, at the peak of disease (figure 5A). As observed before (figures 1C and 3C), ATI induced an earlier onset (day 10) of clinical symptoms that continued to increase until day 15, resulting in a significantly elevated cumulative disease score compared with mice kept on GAF diet (figure 5B, online supplemental figure 2A). We isolated and analysed the different cell populations infiltrating the CNS for whole spinal cord/full brain without isolating specific parts of these organs, following EAE induction, using the gating strategy outlined in online supplemental figures 3 and 4, with primary selection for non-myeloid (CD11b$^-$) and myeloid (CD11b$^+$) CD45$^+$ cells (figure 5C,D and F). ATI feeding increased dose-dependently (A>GA) T cells infiltrating the CNS (figure 5C), and MHC-II$^+$ cDC2 (CD11c$^+$CD11b$^+$) and macrophages (CD11c$^{+/-}$CD11b$^+$) (figure 5F). Moreover, CNS-infiltrating myeloid cells showed a dose-dependent increase of MHC-II^{int} inflammatory monocytes and enhanced activation of CNS-resident myeloid cells (ie, microglia) (figure 5D). In line with the aggravated CNS inflammation, we also observed enhanced neutrophil infiltration (MHC-II$^-$Ly6G$^+$), although the numbers of CNS-resident microglia remained unaltered (figure 5E). Such increase in largely pro-inflammatory myeloid cells infiltrating the CNS was demonstrable both in percentage (data not shown) and as total cell counts.

The intestinal inflammatory milieu in mice on GA-containing diets (figures 1E and 2A–B and online supplemental figure 1C) could also promote gut-associated Treg cell accumulation. To further explore whether dietary ATI may modulate the number of Treg cells, we performed a short-term comparative study using the three dietary regimes (GAF, GA and G) for 9 days in mice with MOG-peptide immunisation versus PBS-sensitised controls (online supplemental figure 2B). Expression of key innate inflammatory genes (IL-6 and MCP-1) in the duodenum showed only a trend of higher levels in MOG-immunised mice kept on a GA diet compared with the GAF or G diet (online supplemental figure 2C). Although total numbers of CD4$^+$Foxp3$^+$ Treg cells in the spleen and Peyer's patches were elevated on EAE induction (day 9), the different dietary regimens had no additive effect on peripheral Treg cells (online supplemental figure 2D–E). Nevertheless, within the intestine-draining MLNs, the numbers of Treg cells were significantly elevated in MOG-sensitised and ATI-consuming mice compared with those consuming ATI-free dietary regimes (GAF and G). At the same time, the reverse was observed in the mock-treated control groups exposed to the same dietary regimes (online supplemental figure 2F). This is in line with the context-dependent immune adjuvant role of nutritional ATI in already established inflammatory diseases.[22 24–26]

ATI promote the release of innate cytokines and chemokines in peripheral blood mononuclear cells from patients with MS

To translate our findings in the rodent model to patients with MS, we isolated PBMC from fifteen patients with MS (ten females) and fifteen healthy controls (HC) (ten females). The PBMCs were cultured and stimulated with ATI and LPS (as a major alternative TLR4 ligand) for 16 hours (figure 6A). The release of CCL-2 (MCP-1), IL-6 and tumour necrosis factor (TNF)α was highly increased by exposure to ATI and comparable to levels achieved on LPS stimulation, both in cells from both patients with MS and HC (figure 6B–D). Furthermore, gene expression of the same cytokines and chemokines (IL-6, CCL-2, TNFα) and of IL-8 was equally upregulated in both patients with MS and HC after stimulation with ATI or LPS. While there was a trend for higher values in the patients with MS, this did not reach significance (figure 6E–F). The absence of a major difference in ATI-responsiveness between patients with MS and healthy controls is in line with the general innate immune-stimulatory activity of ATI.

DISCUSSION

Dietary wheat ATI that activate TLR4 on myeloid cells (dendritic cells, monocytes, macrophages) residing in the intestinal lamina propria[19 22 23 26 27] promotes CNS inflammation and clinical symptoms of murine EAE, an established animal model of human MS. Our experimental diet containing gluten and ATI (25% and 0.75% of protein, respectively), corresponds to the average gluten/ATI content in a standard Western diet (15–20 g of gluten and 0.7–1.5 g of ATI/person/day).[22] We compared this diet with a control gluten and ATI-free diet, and with dietary regimes containing mostly gluten (25% gluten and only 0.19% ATI), or ATI (0% gluten and 1.5% ATI). Mice consuming high dietary ATI (1.5% and 0.75%) developed significantly more severe EAE and CNS inflammation than those consuming a low ATI (0.19%) or an ATI-free diet. Notably, gluten alone, did not promote CNS inflammation. Moreover, ATI exerted a dose-dependent effect on innate immune activation, which consecutively had an impact on CNS inflammation.

Body weight was not significantly affected, indicating a comparable food consumption independent of dietary regime or CNS inflammation. Small intestines of mice on the high ATI versus low or ATI-free diet controls, showed the highest expression of innate inflammatory genes (KC, MCP-1, IL-6 and IL-15) and the highest number of macrophages and dendritic cells (F4/80$^+$, CD11c$^+$ and F4/80$^+$CD11c$^+$), particularly in the duodenum. This increased intestinal inflammatory signal significantly correlated with increased numbers of MOG-responsive, IFNγ-producing and IL-17A-producing Th1 and Th17 cells and, consequently, a higher cumulative EAE clinical score. These inflammatory/encephalitogenic Th cells are key drivers of EAE pathogenesis, as they are one of the first pathogenic cells trans-migrating across the blood-brain barrier and infiltrating the brain parenchyma.[40]

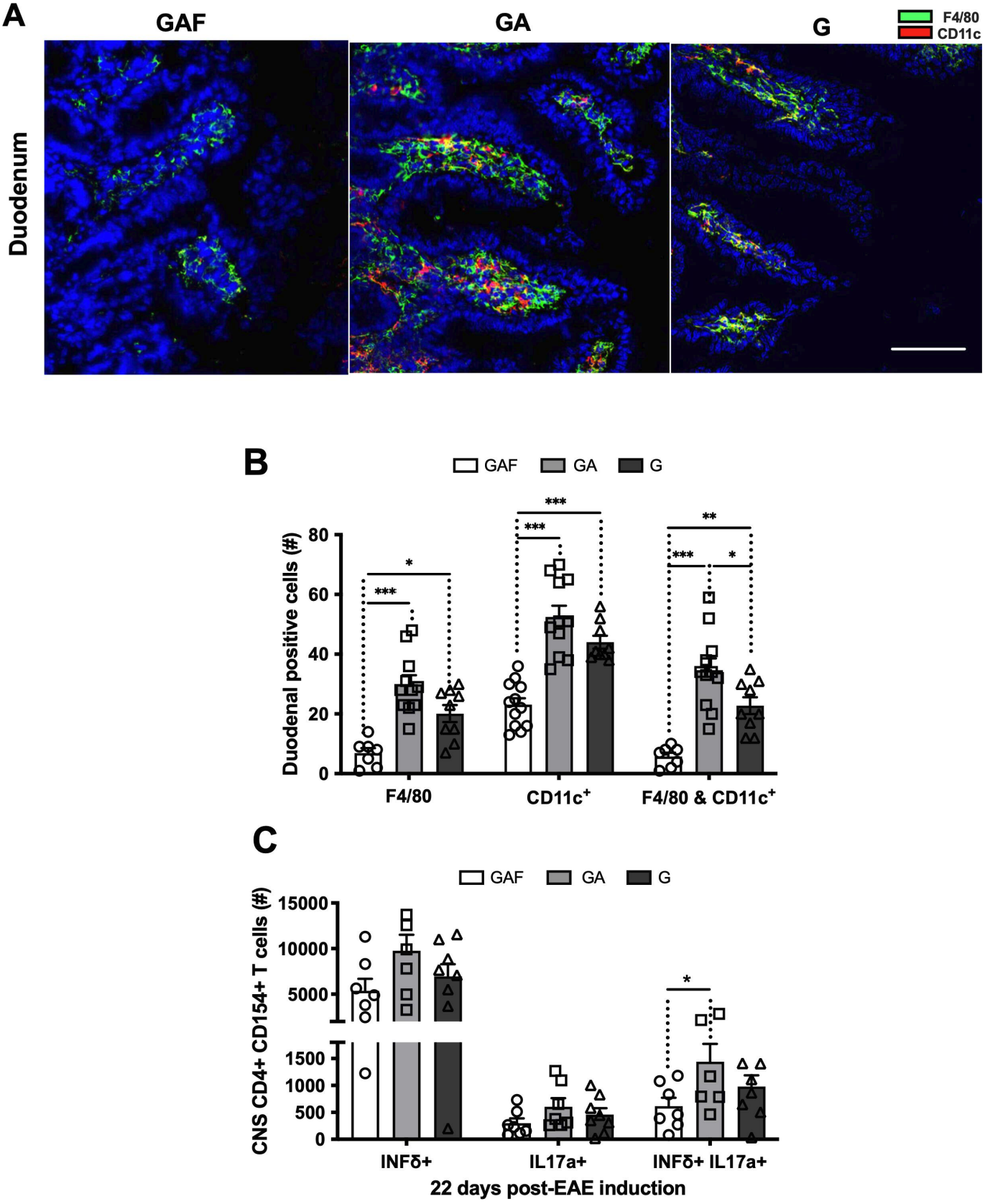

Figure 4 The amylase trypsin inhibitors (ATI) component in dietary gluten promotes intestinal and central nervous system (CNS) inflammation. (A) Representative duodenal sections from the experimental groups in figure 3 stained for F4/80 and CD11c. (B) Numbers of duodenal F4/80$^+$ and CD11c$^+$ cells. (C) CNS-infiltrating inflammatory CD4$^+$ T cells, INFγ^+, IL1a$^+$ and double positive INFγ^+IL17a$^+$ cells, as determined by FACS analysis 22 days postexperimental autoimmune encephalitis (post-EAE) induction. Results are expressed as means±SEM (n=4/group in figure A and B, n=6/group in figure C). Results are representative of two independent experiments. Statistical significance was determined with one-way or two-way analysis of variance. *P<0.05; **p<0.01; ***p<0.001. G, gluten; GA, gluten and ATI; GAF, gluten and ATI-free.

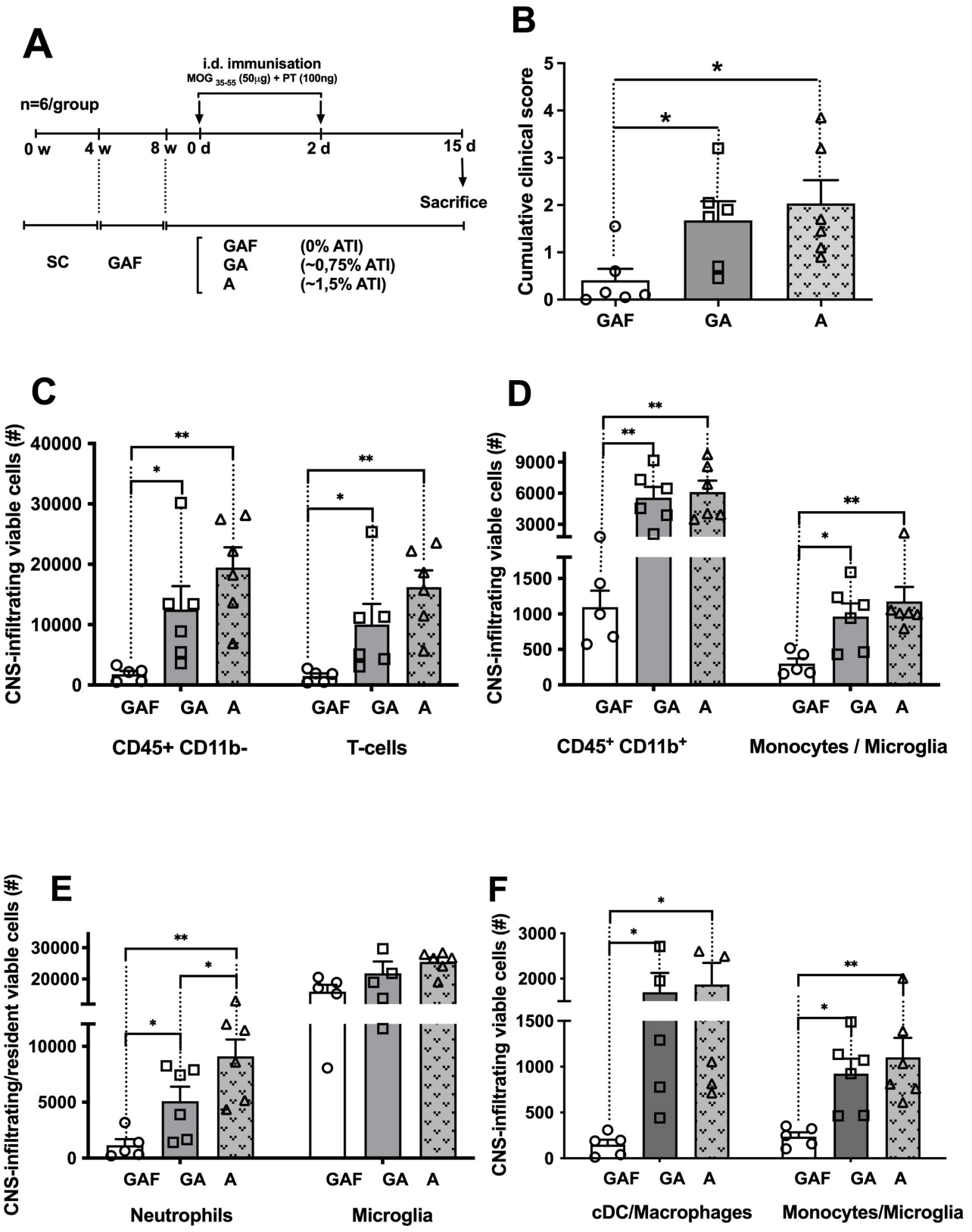

Figure 5 Amylase trypsin inhibitors (ATI) dose-dependently increase pro-inflammatory myeloid CD11b– and CD11b$^+$ cells in the central nervous system (CNS) of experimental autoimmune encephalitis (EAE) mice. (A) C57BL/6 mice (n=6 per group) were raised on standard chow (SC), myelin oligodendrocyte glycoprotein (MOG)-immunised, placed on a gluten and ATI-free (GAF) diet for 4 weeks and divided in three groups: (1) continued GAF, (2) change to gluten and ATI (GA) (25% gluten and 0.75% ATI) or (3) change to ATI (1.5% ATI). Mice were sacrificed at day 15 postexperimental autoimmune encephalitis (post-EAE) induction. (B) Mean cumulative clinical score. (C) Total numbers of CNS-infiltrating CD45$^+$CD11b– (left) and T cells (right). (D) CD45$^+$CD11b$^+$ cells (left) and MHCII^{lo}CD11c$^-$CD38$^+$ inflammatory monocytes/activated microglia (right). (E) Lineage– CD45$^+$CD11b$^+$Ly6G$^+$ neutrophils (left) and CNS-resident F4/80^{hi}CXCR1^{hi} microglia (right). (F) CNS-infiltrating MHC-II$^+$CD11c$^+$CD38$^+$ cDC/macrophages (left) and MHC-II$^+$CD11c–CD38$^+$ macrophages/microglia (right). For gating strategy, see online supplemental figure 2. *P<0.05, **p<0.01.

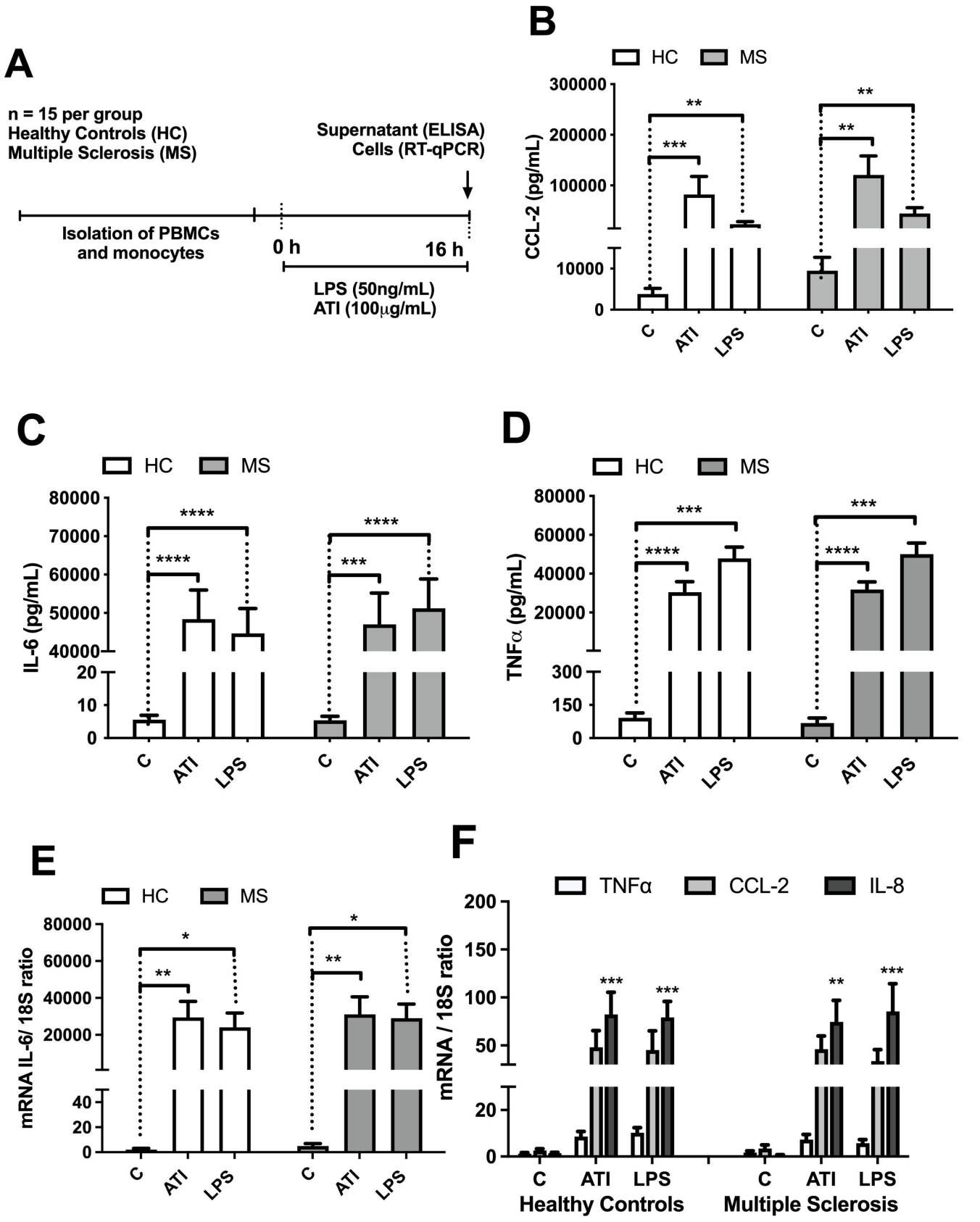

Figure 6 Amylase trypsin inhibitors (ATI) induce expression of inflammatory mediators in monocytes from patients with multiple sclerosis (MS) and healthy controls (HC). (A) Blood monocytes were isolated from peripheral blood mononuclear cells (PBMCs) from HC and patients with MS (each n=15), challenged with lipopolysaccharide (LPS) or ATI, or left unstimulated (Con) for 16 hours. (B–D) CCL-2, interleukin (IL)-6 and tumour necrosis factor (TNF)α released into the medium. (E) IL-6 and (F) TNFα, CCL-2 and IL-8 gene expression in monocytes of all groups. Results are expressed as means±SEM. Significance was determined with one-way or two-way analysis of variance. *P<0.05, **p<0.01, ***p<0.001, ****p<0.0001. mRNA, messenger RNA.

The small intestine has been identified as a key site for the activation of effector Th17 cells with relevance for MS pathophysiology,[41] and in our prior study we provided evidence that the small intestinal signal of ATI-induced innate immune stimulation was quickly transmitted to the MLNs, with a highly enhanced expression of myeloid/dendritic cell activation markers.[22] While an interaction of ATI-primed myeloid cells with already MOG-specific encephalitogenic T cells at the level of MLN lymph nodes is plausible, additional mechanisms may be operative; a further migration of these myeloid cells to peripheral lymph nodes or even the CNS; a general adjuvant role of elevated cytokine/chemokine levels originating from the intestine or the generation/activation of CNS homing Th17 cells within the intestine. In the context of CNS autoimmunity, small intestinal dendritic cells activated by segmented filamentous bacteria have enhanced the pro-inflammatory phenotype of MOG-specific (Th17) T cells, facilitating CNS immune activation.[42] We previously showed that mice consuming ATI showed a selective increase of segmented filamentous bacteria,[23] which were implicated to favour a pro-inflammatory intestinal environment to drive intestinal, joint and CNS autoimmunity.[23 43] In patients with MS, an expansion of intestinal Th17 cells was linked to similar microbial changes, but the mechanism remains ill-defined.[41]

To determine the early effects of dietary ATI on CNS inflammation, we compared the 1.5% ATI group against the 0.19% low ATI and the control ATI-gluten-free group (0% ATI). Fifteen days post-MOG immunisation, an ATI-dependent worsening of clinical symptoms accompanied by increase numbers of pro-inflammatory MHC-II^{int}, and MHC-II^{hi} myeloid cells as well as Ly6G^{+} neutrophil infiltrating the CNS was clearly observed, indicating that the (mild) ATI-induced intestinal inflammation affects the differentiation and acquisition of (peripheral) T cell pathogenicity.

Furthermore, dietary ATI reduced the number of CD4^{+}CD25^{+}Foxp3^{+} Treg in the MLN during the early stage of EAE (9 days postsensitisation), suggesting an early suppression of immune regulation by nutritional ATI. Treg cells are essential for the inhibition of Th1, Th2 and Th17 cell-mediated inflammatory responses, modulated by the complex plasticity of helper T cells.[44 45] Here, IL-10-mediated Stat3 activation in Treg regulates Th17 cells,[46] whereas local IL-6 drives differentiation of the common RORγt precursors to Th17 instead of Treg cells.[47] In line with the established roles of these cytokines in Treg cell differentiation and the suppression of Treg cells in ATI-fed EAE mice, we previously found that IL-10 was downregulated and IL-6 prominently upregulated after incubation of myeloid cells with ATI in vitro and after exposure of mice to ATI in vivo.[22] In this line, serum IL-6, as well as serum MCP-1 (CCL2) and IL-8 were increased up to fourfold 2–4 hours after a gavage of ATI.[22]

To translate some of our findings in mice to humans, monocytes from patients with MS and healthy controls were challenged with ATI, which triggered the expression and release of a range of pro-inflammatory chemokines and cytokines (CCL-2, IL-6, TNFα and IL-8) in both groups. This indicates that, as expected, (dietary) ATI can directly enhance pro-inflammatory monocyte-macrophage responses via activation of TLR4 in general,[19] while the extent of this response was not different in patients with MS versus (healthy) controls. Circulating immune cells cannot be expected to reflect the state of activity of an autoimmune disease, especially in a low activity and stable CNS disease, as in our patients. However, these results suggest that dietary ATI may promote the severity of murine EAE, and of human MS, downstream of modest monocyte-macrophage activation in the gut.

Notably, a diet containing 25% of protein as gluten and a low amount of ATI (0.19%) only weakly promoted intestinal and CNS inflammation and neurological symptoms (comparable to the gluten-free and ATI-free), whereas a diet with crude gluten and a higher amount of ATI (0.75% ATI), or purified ATI without gluten (1.5% of total protein), significantly increased intestinal innate immune activation and worsened all features of EAE. This clearly confirms that (1) dietary gluten itself, even at high concentrations, can be excluded as trigger of this immune activation, and (2) the immune adjuvant effect of ATI is dose-dependent, unlike the other two immune-mediated wheat sensitivities (coeliac disease and wheat allergy), where even low amounts of wheat protein can cause disease.

Early studies evaluating the effects of a gluten-free diet on patients with MS,[48 49] showed a positive effect on MS-related outcomes such as expanded disability scale score, lesion activity on MRI, mood, fatigue and quality of life when patients strictly avoided gluten-containing foods. However, these studies were not well-controlled or observational. In addition, dietary compliance, placebo/nocebo effects and the lack of end points other than symptoms further limit the plausibility of these studies which were performed before the pro-inflammatory activity of ATI had not been discovered. Considering the dose-dependent effect of dietary ATI and our finding that gluten does not activate the intestinal immune system nor worsens EAE, future dietary recommendations for dietary studies with patients with MS may not require a complete wheat-free diet but rather a reduction of around 90% of usual wheat ('gluten') intake. Based on these insights, it may be worthwhile to select wheat products that are low in ATI and therefore safe for patients with MS.[50 51]

Limitations of our study are that in vivo data were obtained exclusively in the EAE mouse model of MS, and that human functional data were based on peripheral PBMC that have a limited predictive value for MS as a CNS disease. Moreover, a clinical study of patients with well-defined MS on a wheat (ATI)-based versus a wheat (ATI)-free diet remains to be done.

To conclude, we provided evidence that nutritional wheat ATI dose-dependently exacerbate CNS autoimmunity in murine EAE. The immune activation is driven by activated myeloid cells from the gut and from (mesenteric) lymph nodes. Moreover, ATI can suppress Treg cell numbers, and results are transferable to myeloid cells from patients with MS and controls. Further studies to specify the exact trajectory of signal transduction from the gut to the periphery including the CNS are on the way. Our results justify a clinical trial of an ATI-free versus the standard ATI-containing diet in patients with MS, including both immunological readouts and patient-related outcome measures.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Consent obtained directly from patient(s).

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