Brief Definitive Report

Flavonoids Influence Monocytic GTPase Activity and Are Protective in Experimental Allergic Encephalitis

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Abstract

In the chronic disabling disease multiple sclerosis (MS), migration of monocytes across the blood-brain barrier is a crucial step in the formation of new lesions in the central nervous system (CNS). Infiltrating monocyte-derived macrophages secrete inflammatory mediators such as oxygen radicals, which contribute to axonal demyelination and damage, resulting in neurological deficits. Flavonoids are compounds occurring naturally in food, which scavenge oxygen radicals and have antiinflammatory properties. To investigate whether they might suppress clinical symptoms in MS, we treated rats sensitized for acute and chronic experimental allergic encephalomyelitis, an experimental model of MS, with flavonoids. We demonstrated that the flavonoid luteolin substantially suppressed clinical symptoms and prevented relapse when administered either before or after disease onset. Luteolin treatment resulted in reduced inflammation and axonal damage in the CNS by preventing monocyte migration across the brain endothelium. Luteolin influenced migration by modulating the activity of Rho GTPases, signal transducers involved in transendothelial migration. Oral administration of luteolin also significantly reduced clinical symptoms.

Key words: multiple sclerosis • luteolin • blood-brain barrier • macrophage • RhoA

Introduction

Flavonoids are a large group of polyphenolic compounds abundantly present in the human diet (1), first identified as plant pigments, but now also recognized as very potent antioxidants and immunomodulators (2). Depending on their structure, flavonoids are divided in various subclasses like flavonols, flavones, flavanones, and flavanols that differ in their biological activity (3).

In multiple sclerosis (MS), reactive oxygen species (ROS) play a crucial role in the migration process of leukocytes across the blood-brain barrier. Treatment of endothelial cells with superoxide is described to increase monocyte migration, whereas ROS scavengers impede this process (4). Besides this, ROS are involved in MS pathology by inducing oxidative damage to oligodendrocytes and neurons (5). The interaction of leukocytes with endothelial cells activates signal transduction pathways involving Rho GTPases like RhoA and Rac1, leading to the cytoskeletal rearrangements necessary for migration (6).

Previously, we described the flavone luteolin and especially the flavonol quercetin as being potent antioxidants that inhibit myelin phagocytosis by macrophages (7). Here, we report that flavonoids have a beneficial effect in acute and chronic experimental allergic encephalomyelitis (EAE). It was shown that flavonoids influence monocyte migration across the blood-brain barrier and modulate Rho GTPase activity.

Materials and Methods

EAE Induction and Flavonoid Treatment. Acute EAE (acEAE) was induced in Lewis rats (Hannover strain 230–250 g; Harlan) as described previously (8). At day 0, rats were injected subcutaneously in the left footpad with an emulsion containing 20 μg guinea pig myelin basic protein, 500 μg inactive *Mycobacterium tuberculosis* type H37RA (Difco), and 50 μl complete Freund's adjuvant (Difco) supplemented with saline (0.9% NaCl) to reach a final volume of 100 μl. Chronic EAE (crEAE) was induced in adult male Dark Agouti rats (220–260 g; Harlan) by two intradermal injections at the dorsal tail base with 75 μg rat recombinant myelin oligodendrocyte glycoprotein (MOG$_{1-125}$) emulsified in incomplete Freund's adjuvant (Difco). A total volume of 100 μl was injected. EAE induction was performed under isoflurane anesthesia. For both sensitization protocols, control animals (*n* = 3) received simi-

lar emulsions without the encephalitogenic myelin basic protein or myelin oligodendrocyte glycoprotein, respectively. Neurological aberrations of EAE rats were scored daily and graded from 1 to 5: 0, no clinical signs; 0.5, loss of tonicity in distal half of tail; 1, flaccid tail; 1.5, unsteady gait; 2, partial hind limb paralysis; 2.5, complete hind limb paralysis; 3, paralysis of the complete lower part of the body up to the diaphragm; 4, paraplegia; and 5, death due to EAE. Clinical scores were derived from 8–13 animals per group.

Quercetin and luteolin (purity > 97% HPLC grade; Kaden Biochemicals) were dissolved in 1 N NaOH. The pH was readjusted to 7.2 with 1 N HCl after which the solutions were further diluted in saline and distilled water. Flavonoids were administered at 50 mg/kg daily i.p. from day 6 to 18 during acEAE and from day 6 to 24 or day 15 to 24 during crEAE. When indicated, luteolin was administered orally (intragastrically) at 100 mg/kg from day 3 to 24. All experimental procedures were approved by the Experimental Animal Committee of the VUMC.

Immunohistochemistry. Rats were killed at various time points after acEAE and crEAE induction. After perfusion of the animals with 4% formalin, brains and spinal cords were dissected, postfixed in 4% formalin for 1 wk, and embedded in paraffin. 8-μm thick sections were dewaxed, endogenous peroxidase activity was blocked with 1% H_2O_2 in methanol, and then sections were microwaved for 10 min in 0.1 M citric acid, pH 6. Sections were incubated with the mAb ED1 (Serotec) raised against the macrophage-specific CD68 antigen, the mAb W3/13 (Serotec) raised against the pan–T cell marker CD43, or the mAb 348 (Chemicon) raised against amyloid precursor protein (APP). Binding of primary antibodies was revealed by avidin-biotin-peroxidase as described previously (9), with reagents supplied by Vector Laboratories. Cellular infiltrates and APP$^+$ dots were quantified in 20–30 pictures per rat, taken with a digital camera at 10× objective throughout the spinal cord, using the digital image analysis program AnalySIS (Soft Imaging System GmbH).

Monocyte Migration. The migratory capacity of monocytes over a brain endothelial cell monolayer was assayed by time-lapse video microscopy, as described previously (4). In brief, brain endothelial monolayers were established using the well-characterized, immortalized Lewis rat brain endothelial cell line GP8/3 (4, 10). 5 × 10^5/ml monocytes were added to the endothelial cell monolayers and the number of migrated monocytes was assessed after 4 h. The migration assay was conducted with or without the presence of 50 μM luteolin. The effect of luteolin on monocyte migration was determined using the rat NR8383 monocytic cell line, which resembles primary monocytes in its migratory behavior (11) and V14Rho-NR8383 cells, which overexpress constitutively active RhoA (12). The migration capacity of monocytes derived from control rats and vehicle- or luteolin-treated crEAE animals was also established. Peripheral blood monocytes were purified as described previously (10). Mononuclear cells (PBMCs) were obtained from a concentrated cell pool obtained after perfusion of rats and centrifugation on a Ficoll-Paque gradient. Subsequently, B and T cells were removed using immunomagnetic separation and granulocytes and remaining blood cells were removed using FACS sorting on their size and granularity. 20–30 × 10^6 monocytes were isolated from one rat. 5 × 10^5/ml monocytes and 50% serum of the same animals were added to brain endothelial cell monolayers to determine monocytic migration behavior.

RhoA and Rac1 Activity Assays. RhoA and Rac1 activity assays were performed as described previously (4, 13, 14). RhoA and Rac1 activities were determined in 5 × 10^6 or 20 × 10^6 NR8383 cells, respectively, after treatment with 50 μM luteolin for 1 h. Cells were washed in ice-cold PBS and lysed. Cleared lysates were incubated with either bacterially produced GST-RBD (Rhotekin) bound to glutathione-agarose beads purified from bacterial lysates as described previously (13), or PAK-CRIB-bio (provided by J.G. Collard, The Netherlands Cancer Institute, Amsterdam, Netherlands) purified with streptavidin-agarose beads. Beads were washed
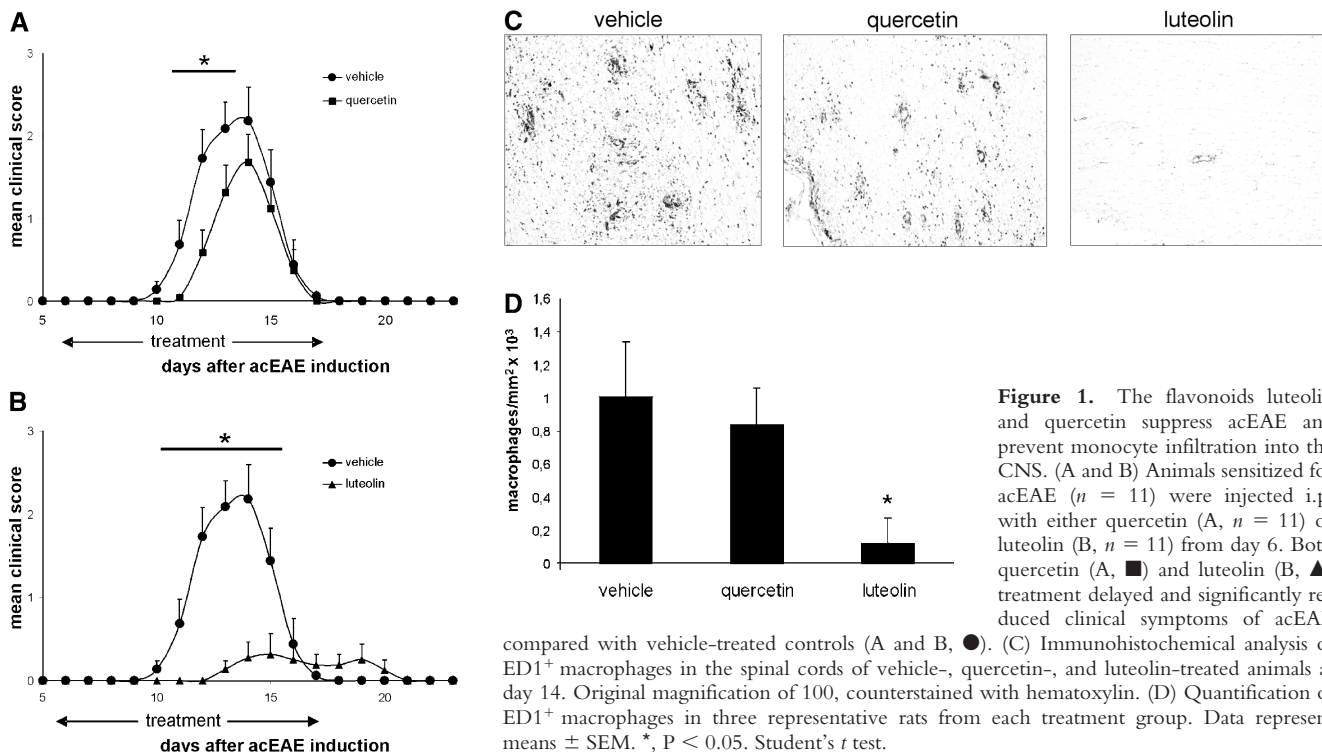

Figure 1. The flavonoids luteolin and quercetin suppress acEAE and prevent monocyte infiltration into the CNS. (A and B) Animals sensitized for acEAE (n = 11) were injected i.p. with either quercetin (A, n = 11) or luteolin (B, n = 11) from day 6. Both quercetin (A, ■) and luteolin (B, ▲) treatment delayed and significantly reduced clinical symptoms of acEAE compared with vehicle-treated controls (A and B, ●). (C) Immunohistochemical analysis of ED1$^+$ macrophages in the spinal cords of vehicle-, quercetin-, and luteolin-treated animals at day 14. Original magnification of 100, counterstained with hematoxylin. (D) Quantification of ED1$^+$ macrophages in three representative rats from each treatment group. Data represent means ± SEM. *, P < 0.05. Student's t test.

three times with lysis buffer, and bound proteins were eluted in SDS sample buffer and analyzed by Western blotting using anti-RhoA (Santa Cruz Biotechnology, Inc.) or anti-Rac1 (clone 23A8; Upstate Biotechnology) mAbs. Luteolin treatment did not affect total RhoA or Rac1 content of the cells as assessed by Western blotting of total lysates (not depicted).

Statistics. Data were analyzed statistically by means of analysis of variance, Student's *t* test, and Spearman's rank correlation coefficient.

Results and Discussion

Flavonoids Suppress acEAE by Preventing Leukocyte Infiltration. The potential beneficial effect of flavonoids in MS was determined in the acEAE model. acEAE animals were injected daily i.p. with either one of the flavonoids (50 mg/kg quercetin or luteolin) from day 6 after induction. All vehicle-treated acEAE animals developed neurological symptoms from day 10, with a maximum score at day 14, and were recovered by day 18 after disease induction (Fig. 1, A and B). In all rats that had been treated with quercetin, disease was initiated, although its onset was significantly delayed by at least 1 d (P < 0.05), and clinical symptoms were slightly though significantly reduced (P < 0.05; Fig. 1 A). Treatment with luteolin substantially suppressed the development of acEAE (P < 0.01). Disease incidence was observed in only 36% of these animals. Within this treatment group, the mean maximum disease score was 0.4 compared with 2.3 for vehicle-treated animals, and disease onset was delayed with a mean of 6 d (P < 0.01; Fig. 1 B). Upon termination of luteolin administration at day 17, no increase in the clinical symptoms of diseased animals occurred, and animals were completely recovered by day 21. Histological examination at the peak of regular disease progression (day 14) revealed reduced numbers of infiltrated T cells (not depicted) and macrophages in quercetin- and luteolin-treated animals. Macrophage numbers were reduced by 17 and 88% (P < 0.05), respectively, compared with vehicle-treated animals (Fig. 1, C and D). These data demonstrate that luteolin significantly reduces the development and severity of acEAE, and prevents leukocyte infiltration into the central nervous system (CNS). Luteolin was found to be more potent than quercetin, indicating the importance of the lack of a hydroxyl group at the C–3 position (7), the only structural difference between the two flavonoids.

Luteolin Blocks Monocyte Migration across Brain Endothelial Cells by Influencing Rho GTPase Activity. The effect of luteolin in a well-established in vitro migration model was assessed to further investigate its mechanism in inhibiting cellular migration across the blood-brain barrier. ROS are required for the diapedesis of monocytes across brain endothelium, and the migration process can be inhibited by antioxidants (4). As anticipated, luteolin impaired migration of the rat monocytic cell line NR8383 across a brain endothelial cell monolayer by 71% as determined by video time-lapse microscopy (P < 0.01; Fig. 2 A). Adhesion of NR8383 cells to brain endothelial cells as well as the viability of both cell types, assessed as described previously (4, 7), remained unaffected (not depicted). However, we observed

a clear reduction in monocyte motility and the formation of monocytic membrane protrusions when treated with luteolin. During luteolin treatment, monocytes showed less membrane protrusions as analyzed by the video images of the migration experiments (Fig. 2 B). Upon quantification, 49% of luteolin–treated monocytes revealed no clear membrane protrusions compared with 100% of untreated cells, indicating that the flavone may affect cytoskeletal regulatory components such as the family of the Rho GTPases. Activation of RhoA is particularly essential for migration and the completion of diapedesis of monocytes (12, 15). Therefore, the effect of luteolin on RhoA activity was assessed. Incubation of NR8383 cells with 50 μM luteolin for 1 h indeed inhibited RhoA GTP loading by 64% (P < 0.01; Fig. 2 C). Overnight incubation of monocytes with 10 μM luteolin also decreased levels of active RhoA by 60%.

To exclude regulation of other signaling pathways by luteolin, we determined the influence of luteolin on migration of monocytes that express constitutively active RhoA (12). Luteolin did not inhibit the migration capacity of these monocytes, indicating that constitutively active RhoA in monocytes overcomes luteolin–induced decrease in transmigration (Fig. 2 D). As Rac1, a close family member of RhoA and part of the NADPH–oxidase complex, is described to down–regulate RhoA (16), we also determined Rac1 GTP loading upon luteolin treatment. Rac1 activity was increased by 144% upon luteolin treatment for 1 h (P < 0.05; Fig. 2 E) and by 50% after 4 h, suggesting that luteolin may directly regulate RhoA activity via Rac1. Conversely, Rac1 is reported to inhibit RhoA by activation of the NADPH–oxidase complex (17), which is not in line with our earlier observations that luteolin is an effective scavenger of ROS (7). Therefore, we assume that other signaling pathways must be involved in the effects mediated by luteolin. Our data are consistent with previous reports that have demonstrated that Rho GTPase inhibitors are protective in acEAE through prevention of leukocyte infiltration into the CNS (18). Interestingly, the family of the cholesterol-lowering drugs, for instance lovastatin, is also beneficial in EAE and is reported to inhibit the activation of RhoA (19–20). Thus, observed diminished monocyte migration and infiltration into the CNS of EAE animals by luteolin treatment might be mediated by inhibition of RhoA activity.

Luteolin Is Protective in crEAE When Administered before and after Disease Onset and by Oral Treatment. The therapeutic potential of luteolin was assessed in a chronic relapsing-remitting model for MS. crEAE rats were treated daily with 50 mg/kg luteolin i.p. from day 6 after induction. Vehicle-treated crEAE animals developed clinical symptoms at day 7, with a peak at day 12. These animals then temporarily recovered around day 16, whereafter a second clinical episode developed from which the animals did not recover (Fig. 3, A and B). Luteolin administration greatly ameliorated clinical crEAE symptoms as previously observed with the acEAE model (Fig. 1 B). Administration of luteolin be-
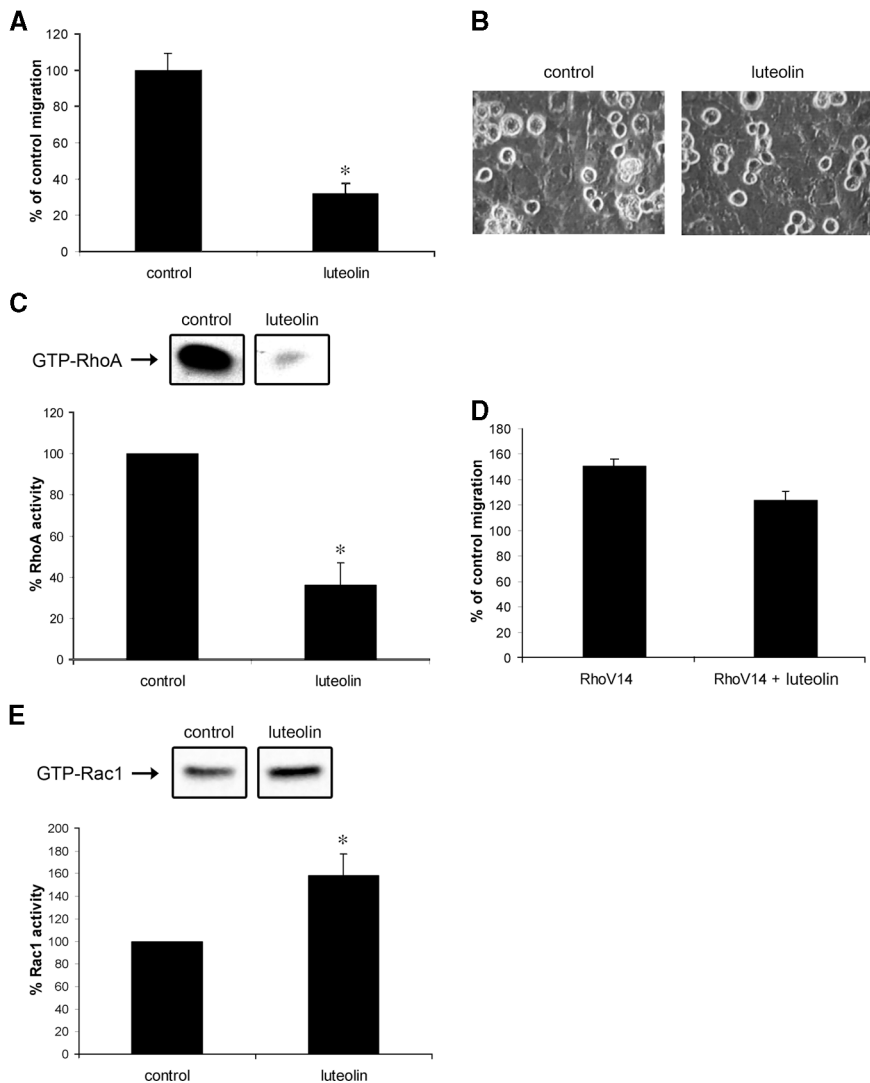
Figure 2. Luteolin reduces monocyte migration across brain endothelial cells by modulating Rho GTPase signaling. (A) Luteolin inhibited migration of the monocytic cell line NR8383 across a brain endothelial cell layer. Migration of untreated monocytes was set at 100%. (B) During luteolin treatment, monocytes showed less membrane protrusions as compared with control cells as analyzed by video microscopy. (C) Luteolin treatment (50 μM) for 1 h inhibited RhoA activity in NR8383 cells. The amount of RhoA in untreated cells was set at 100%. Lanes are derived from different parts of the same gel. (D) RhoV14 monocytes (NR8383 monocytes overexpressing constitutively active RhoA) had an increased migration capacity compared with control monocytes. 50 μM Luteolin did not significantly affect migration of RhoV14 monocytes. (E) In contrast to RhoA, luteolin treatment for 1 h promoted Rac1 activity in NR8383 cells. The amount of Rac1 in untreated cells was set at 100%. Lanes are derived from different parts of the same gel. Data represent at least three independent experiments and are expressed as the means $\pm$ SEM. *, $P < 0.05$. Student's t test.

fore disease onset, from day 6, almost completely abolished the first clinical episode ($P < 0.01$, no disease symptoms in 91% of animals). In addition, the second, more chronic phase was also significantly suppressed ($P < 0.05$, 69% reduction of clinical symptoms; Fig. 3 A). Ending luteolin treatment at day 24 led to an incline in clinical symptoms that became as severe as those of vehicle-treated crEAE animals at day 28 ($n = 5$; not depicted).

The effectiveness of luteolin in diminishing clinical symptoms in crEAE rats after its onset was evaluated by treating crEAE animals with 50 mg/kg luteolin after the first clinical episode, from day 15 onwards. Clinical symptoms were completely abolished within 5 d of luteolin administration, whereas vehicle-treated crEAE animals suffered a severe relapse (Fig. 3 B). Upon ending treatment at day 24, luteolin-treated rats remained free of clinical symptoms until day 27, after which disease developed and progressed to become as severe as that in control crEAE animals ($n = 5$; not depicted). This indicates that the continuous presence of luteolin will be required to maintain suppression of crEAE.

As shown for the acEAE model (Fig. 1, C and D), luteolin treatment reduced the infiltration of leukocytes into the CNS in crEAE when treatment was started both at day 6 and at day 15. Significantly reduced numbers of macrophages ($P < 0.05$; Fig. 3 C) and T cells ($P < 0.05$; not depicted) were found in the spinal cords of luteolin-treated rats. Axonal damage, as indicated by an accumulation of APP, was also reduced ($P < 0.05$), and this correlated with the number of infiltrated macrophages ($P < 0.01$; Fig. 3 D).

As luteolin directly influences monocyte migration in vitro (Fig. 2 A), we evaluated whether monocytes derived from luteolin-treated crEAE rats were affected in their migratory behavior. Blood monocytes were isolated from luteolin- and vehicle-treated animals and tested in vitro for their capacity to migrate across a brain endothelial cell layer. Monocytes derived from luteolin-treated animals indeed showed reduced migration, at both phases of disease (Fig. 3 E; $P < 0.05$), revealing that inherent monocyte migratory behavior is affected by luteolin in vivo.

Because flavonoids are dietary compounds, their effectiveness on disease severity after oral administration was
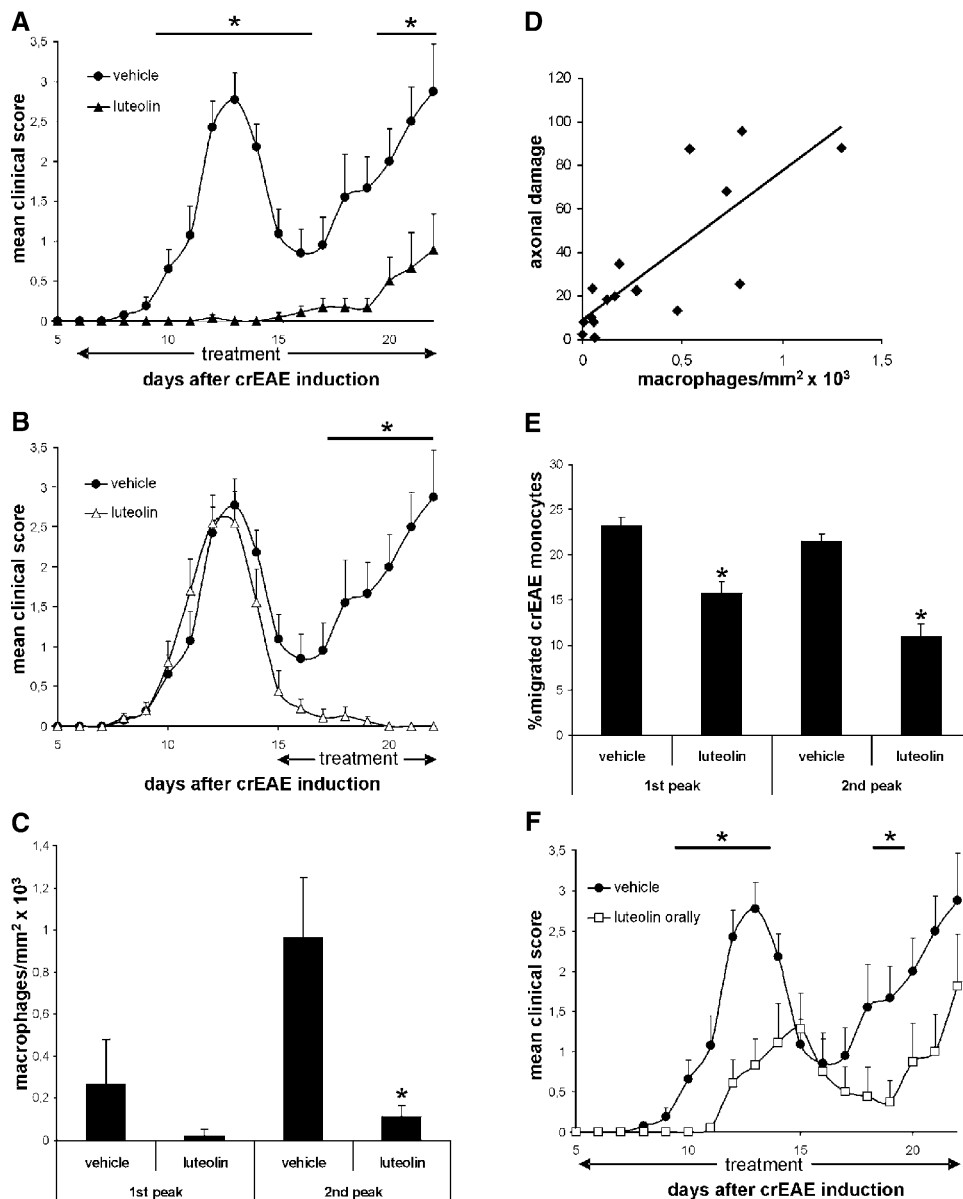
Figure 3. Luteolin abolishes clinical symptoms in crEAE. (A) crEAE animals treated i.p. with luteolin ($n = 13$, ▲) from day 6 show reduced clinical signs compared with vehicle-treated crEAE controls ($n = 13$, ●). (B) Treatment of crEAE animals with 50 mg/kg luteolin ($n = 10$, △) i.p. after disease onset (from day 15) rapidly abolished clinical symptoms compared with vehicle-treated animals ($n = 13$, ●) that developed a severe relapse. (C) Luteolin-treated animals showed reduced numbers of ED1+ macrophages in their spinal cords. Macrophage infiltrates were quantified in three representative rats from each treatment group. (D) Numbers of macrophages present in the spinal cords of vehicle- and luteolin-treated rats correlated with the presence of axonal damage as detected by APP accumulation and assessed by the Spearman's rank correlation coefficient ($P < 0.01$). (E) Peripheral blood monocytes isolated at days 12 and 22 from crEAE animals treated with luteolin from days 6 and 15, respectively, were impaired in their capacity to migrate across brain endothelial cells in vitro compared with monocytes derived from crEAE control animals. Experiments were conducted in the presence of 50% serum derived from the same animals (A and B). (F) Oral treatment with luteolin ($n = 10$, □) from day 3 onwards delayed disease onset and reduced clinical symptoms compared with control crEAE animals (●). Data are expressed as the means ± SEM. *, $P < 0.05$. Student's t test.

tested. crEAE animals were treated orally from day 3 after induction with 100 mg/kg luteolin. This treatment also resulted in a delayed onset ($P < 0.05$; 4 d) of both disease phases and significantly inhibited the first phase ($P < 0.01$; Fig. 3 F) in a manner similar to observed i.p. treatment (Fig. 3 A). However, at day 24, luteolin-treated animals started to develop clinical scores similar to control crEAE animals (not depicted).

Flavonoids are poorly absorbed from the digestive tract, but their metabolites might be functionally active (21). After oral administration of luteolin in rodents, serum concentrations of unmodified luteolin and its conjugates were detected (22), which were functionally active in reducing proinflammatory cytokine production (23) and present at a similar concentration range to that used in our in vitro assays. Luteolin is present in large quantities in vegetables and herbs like artichokes, celery, and parsley. Additionally,

other flavonoids are abundantly present in our diet in fruits, vegetables, herbs, seeds, and plant-derived products like red wine and tea (USDA database: http://www.nal.usda.gov/fnic/foodcomp). The total dietary flavonoid intake is estimated to be several hundreds of milligrams per day (21). Therefore, a luteolin- or flavonoid-enriched diet might be sufficient to reach intake levels that are beneficial in MS. Luteolin might be especially beneficial together with structurally similar flavonoids like apigenin and other possibly protective dietary compounds like vitamin D, lipoic acid, and curcumin (24–26). Taken together, these data stress the potential impact of nutritional components on the progression of MS.

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