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Ginkgo biloba extract EGb 761 has anti-inflammatory properties and ameliorates colitis in mice by driving effector T cell apoptosis

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Ulcerative colitis is a dynamic, chronic inflammatory condition of the colon associated with an increased colon cancer risk. *Ginkgo biloba* is a putative antioxidant and has been used for thousands of years to treat a variety of ailments. The aim of this study was to test whether the standardized *G.biloba* extract, EGb 761, is an antioxidant that can be used to prevent and treat colitis in mice. Here, we show that EGb 761 suppresses the activation of macrophages and can be used to both prevent and treat mouse colitis. Markers of inflammation (iNOS, Cox-2 and tumor necrosis factor-α) and inflammatory stress (p53 and p53-phospho-serine 15) are also downregulated by EGb 761. Furthermore, we show that EGb 761 reduces the numbers of CD4+/CD25−/Foxp3− effector T cells in the colon. Interestingly, EGb 761 drives CD4+ effector T cell apoptosis *in vitro* and *in vivo*, providing a mechanistic explanation to the reduction in numbers of this cell type in the colon. This current study is in agreement with previous studies supporting a use of EGb 761 as a complementary and alternative strategy to abate colitis and associated colon cancer.

Introduction

The use of *Ginkgo biloba* as an alternative medicine is widespread in the developed world. Leaf extracts have been used in traditional oriental medicine for several hundred years. The standardized leaf extract of *G.biloba*, EGb 761, is currently recommended to treat cerebro- and peripheral vascular deficiencies. It is also used to treat cognitive and functional symptoms associated with 'mild to moderate' dementia (1). It is widely believed that the antioxidant properties and free radical scavenging actions of EGb 761, as well as its influence on the apoptotic machinery, underlie its beneficial effects (2).

Inflammatory bowel disease (IBD) is a chronic inflammatory condition that is frequently debilitating and associated with increased colon cancer risk. The colon cancer risk increases with length and severity of the disease (3). Conventional treatment of colitis can reduce periods of active disease and help to maintain remission, but these treatments often bring marginal results, patients become refractory and there are side effects. Patients with colitis therefore often turn to unconventional treatments in hopes of abating symptoms of active disease (4,5). Here, we show that EGb 761 has anti-inflammatory properties *in vitro* and can be used to prevent and treat colitis *in vivo*. Mechanistically, we show that this is at least in part due to the ability of EGb 761 to drive CD4+ effector T cell apoptosis.

Abbreviations: DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IFN-γ, interferon-γ; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor-α.

[†]These authors contributed equally to this work.

Materials and methods

Chemicals and reagents

EGb 761 was purchased through a material transfer agreement with Ipsen (France). EGb 761 is a standardized extract of *G.biloba* leaves containing ∼24% flavonoid glycosides (primarily quercetin, kaempferol and isorhamnetin) and 6% terpene lactones (2.8–3.4% ginkgolides A, B and C and 2.6–3.2% bilobalide). Ginkgolide B and bilobalide account for ∼0.8 and 3% of the total extract, respectively. Other constituents include proanthocyanidins, glucose, rhamnose, organic acids and D-glucaric acid. The content of ginkgolic acids is kept <5 p.p.m. to avoid allergic reactions.

Cell culture and treatment

ANA-1 cells are a mouse macrophage cell line. Cells were maintained in Dulbecco's modified Eagle's medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Biofluids, Rockville, MD), 2 mM L-glutamine (Biofluids), penicillin (10 U/ml) and streptomycin (10 μg/ml, Biofluids). Experiments with EGb 761 were carried out by preincubating cells with EGb 761 dissolved in complete media for 12 h. Depending on the experiment, we used 0–200 μg/ml EGb 761 *in vitro*, which are levels probably to be achieved in blood after daily intake of 120–240 mg, representing the normal dosages for effective therapy (6). Cells were washed before exposure to 100 U/ml interferon-γ (IFN-γ) to activate ANA-1 cells. Jurkat cells are a T lymphocyte cell line derived from a 14-year-old boy with acute T cell leukemia. They were grown in American Type Culture Collection-formulated RPMI 1640 medium, also supplemented with 10% fetal bovine serum, penicillin and streptomycin as above.

Western blot analysis and antibodies

Western blots were carried out as described previously (7). Antibodies used include: iNOS (Cayman Chemical, Ann arbor, MI, 1:1000), eNOS (BD Transduction Laboratories, San Jose, CA, 1:500), Cox-2 (Cayman Chemical, 1:500), Bcl-2 (Biotechnology, Santa Cruz, CA, 1:500) and Actin (Calbiochem, San Diego, CA, 1:2000). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Amersham. Secondary antibodies were diluted at 1:2000. All antibodies were diluted in 5% milk/phosphate buffered saline +0.5% tween 20. Western blot signal was detected by Supersignal West Pico Chemiluminescent Substrate (Pierce, Rochford, IL) and developed onto Hyperfilm (Amersham, Piscatawa, NJ).

Dextran sulfate sodium mouse model of colitis

For the dextran sulfate sodium (DSS) mouse model, 8-week-old C57BL/6 mice received either water *ad libitum* or 1% DSS. All mice were on an AIN 93M diet containing double the standard iron content (90 mg/kg). The rationale for using the high iron diet has been given previously (8). EGb 761 was mixed into the chow of indicated groups at 75 p.p.m. (Research Diets, New Brunswick, NJ), which is a human equivalent dose of ∼58 mg daily for humans. This uses the body surface area normalization method (9) with the following assumptions: a typical mouse eats 3.5 g chow daily and weighs 22 g; the average adult human weighs 60 kg. Mice consumed the same amount of chow daily (on average 3.5 g) regardless of it containing EGb 761 (data not shown). To determine whether EGb 761 can treat colitis, mice were fed DSS for 1.5 cycles (where each cycle in the DSS group consisted of 1% DSS in drinking water for 7 days, followed by 7 days interval with normal drinking water) and then given EGb 761 (75 p.p.m.) in chow. Mice were then euthanized at one cycle interval. Colon samples were washed with phosphate-buffered saline (PBS), cut longitudinally, swiss-roled, then formalin fixed and paraffin embedded.

Quantifying inflammation

Slides were examined in a blind fashion by a trained pathologist. Inflammation was graded by extent (focal, multifocal, diffuse or extensive areas) and depth/penetration of inflammation (lamina propria, into submucosa, into mucscularis propria and into subserosa). Ulceration/erosion was assessed by the overall

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extent in the colonic tissue. Both inflammation and ulceration/erosion were then given a numerical value of 0–4, where 0 is none observed and 4 is severe inflammation and/or ulceration/erosion.

Immunofluorescence staining

Colons were harvested, fixed in freshly prepared 4% paraformaldehyde in PBS buffer (pH 7.2) and then vibratome sectioned. Tissue autofluorescence was quenched by incubation of the sections sequentially in PBS–glycine (150 mM) followed by $NaBH_4$ (1 mg/ml in PBS). CD4+ cells were labeled with a Alexa Fluor 488-conjugated rat anti-mouse CD4 monoclonal antibody (Abcam, Cambridge, MA, cat# 557667, 1:100). CD8+ cells were labeled with a rat anti-mouse CD8 monoclonal antibody (Abcam, cat# ab3081, 1:100) and visualized with Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, cat# 712-165-150, 1:100); Foxp3+ cells were labeled with a mouse Foxp3 monoclonal antibody (Abcam, cat# ab22510, 1:100) using a Vector mouse on mouse (M.O.M) detection kit using a fluorescein-tagged secondary antibody. Tumor necrosis factor-α (TNF-α) was labeled with anti-TNF-α (rabbit polyclonal, Santa Cruz, 1:100) and visualized with Cy3-conjugated donkey anti-rabbit IgG. Nuclei of all cells were labeled with 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA, D3571, 1:5000). Finally, rhodamine phalloidin (Invitrogen, Carlsbad, CA, cat# R415, green, 1:50) or Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA, cat# A12379, red, 1:50) was used to stain for F-actin (1:100 dilution; Molecular Probes, Eugene, OR).

Immunohistochemical staining

For immunohistochemical staining, serial sections of mouse colon tissues (processed as described above) were incubated with antibodies against iNOS (Mouse monoclonal, clone 5D5-H7, cat# MC-5245; diluted 1 in 10 000, Research and Diagnostic Antibodies, North Las Vegas, NV), Cox-2 (Rabbit polyclonal, cat# 160126; diluted 1 in 20 000, Cayman Chemical), TNF-α (Mouse monoclonal, clone P/T2, cat# ab9579; diluted 1 in 50 000, Abcam), p53 (Mouse monoclonal, clone Pab 122, cat# X1494; diluted 1 in 1 000 000, Exalpha Biologicals, Maynard, MA) or p53-phospho-serine 15 (Mouse monoclonal, Anti-Phospho-Serine 15-53, clone 16G8, cat# 9286S; diluted 1 in 20 000, Cell Signaling, Danvers, MA). To ensure even staining and reproducible results, sections were incubated by slow rocking overnight in primary antibodies (4°C) using the Antibody Amplifier™ (ProHisto, LLC, Columbia, SC). Following incubation with primary antibody, sections were processed with EnVision+ System-HRP kits (DakoCytomation, Carpinteria, CA) according to the kit protocols. The chromogen was diaminobentidine and sections were counterstained with 1% methyl green. The positive control tissue was colon cancer sections. These sections were highly positive for iNOS, Cox-2, TNF-α, p53 and p53-phospho-serine 15. Immunohistochemistry was quantified using the ACIS® III Automated Cellular Imaging System (DakoCytomation). ACIS® III consists of an automated bright-field microscope with image and proprietary processing analysis software for evaluating tissue sections on glass microscope slides. For each slide, the entire tissue section was scanned, and results are represented as the percentage of cells staining positive.

Examination of T cell populations by flow cytometry

We carried out experiments similar to that outlined for prevention experiments (Table I). However, after 2.5 cycles of DSS or water ± EGb 761 (75 p.p.m.), we collected single-cell suspensions of colons from seven mice in each group. To accomplish this, colons were flushed out with 1× PBS, opened longitudinally and cut into two pieces. Colons were incubated in 10% fetal bovine serum/5 mM ethylenediaminetetraacetic acid in Ca^{2+}/Mg^{2+}-free PBS for 15 min at room temperature. Colon tissues were then shaken to dislodge the epithelial layer into single-cell suspensions. Cell viability was checked by trypan blue exclusion and >95% cells were viable. The remaining lamina propria was added to an enzyme cocktail consisting of collagenase I (Sigma-Aldrich, St.Louis, MO; 0.1 mg/ml) and dispase (EMD, Gibbstown, NJ; 0.2 mg/ml) for 75 min at 37°C in a shaking water bath. The suspension was further mechanically separated by pipetting and examined for trypan blue exclusion. Again, >95% of the single-cell suspension was viable. For Treg staining (CD4/CD25/Foxp3), the mouse regulatory T cell staining kit #3 was utilized according to the protocol provided by the manufacturer (eBioscience, San Diego, CA). For CD4/CD8 staining profiles, colon-derived single-cell suspensions were first treated with ice-cold ACK lysis buffer (Quality Biological, Gaithersburg, MD) for 10 min to lyse any erythrocytes. Cells were subsequently washed twice in dulbecco's phosphate buffered saline + 1% fetal calf serum (Atlanta Biologicals, Norcross, GA) and incubated with 4 μg/10^6 cells rat anti-mouse CD16/CD32 antibody (2.4G2, BD Pharmingen, San Diego, CA) for 30 min on ice, washed twice in dulbecco's phosphate buffered saline + 1% fetal calf serum and stained for 30 min on ice with fluorescein isothiocyanate-conjugated rat anti-mouse CD4 (L3T4, BD Pharmingen) at 0.5 μg/10^6 cells and phycoerythrin-conjugated rat anti-mouse CD8a (Ly-2, BD Pharmingen) at 0.25 mg/10^6 cells. Appropriate isotype controls were also used for staining as negative controls. Cells were then washed twice in dulbecco's phosphate buffered saline + 1% fetal calf serum and analyzed on a FACS Caliber flow cytometer (Cytomics FC 500, Beckman Coulter, Miami, FL).

Table I. Effects of EGb 761 on the prevention of inflammation and ulceration in the colon induced by DSS

Treatment group	Score					Number of mice
	0	1	2	3	4	
Inflammation score						
Water	7 (100)[a]	0 (0)	0 (0)	0 (0)	0 (0)	7
DSS	0 (0)	0 (0)	3 (14)	10 (48)	8 (38)	21
DSS + EGb 761	0 (0)	7 (30)	8 (35)	9 (35)	0 (0)	23
Ulceration score						
Water	7 (100)[a]	0 (0)	0 (0)	0 (0)	0 (0)	7
DSS	0 (0)	3 (14)	6 (29)	9 (43)	3 (14)	21
DSS + EGb 761	1 (4)	9 (39)	9 (39)	4 (18)	0 (0)	23

[a]Percentage of mice.

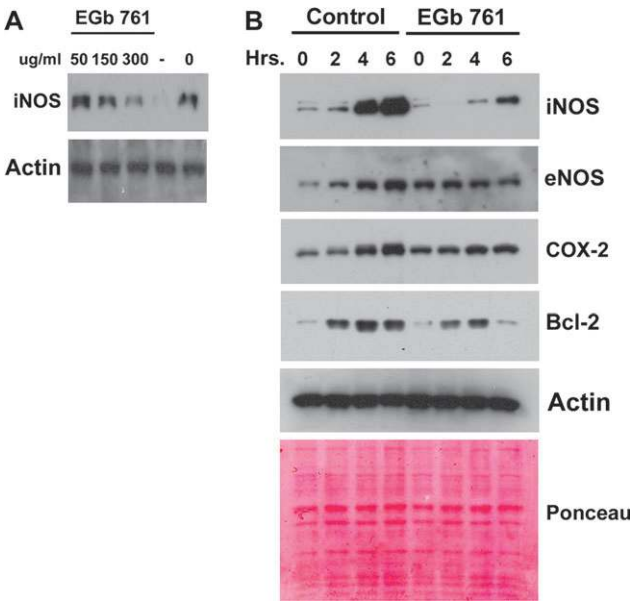

Fig. 1. EGb 761 suppresses the activation of key inflammatory players. (**A**) ANA-1 mouse macrophages were preincubated for 12 h with indicated concentrations of EGb 761. EGb 761 was washed off and then media containing IFN-γ to stimulate cells were added. Following 6 h, cells were harvested for protein and examined for a response with iNOS being the inflammation marker. The negative control (−) is ANA-1 cells not exposed to IFN-γ. (**B**) Using 100 μg/ml EGb 761, we repeated the experiment except IFN-γ was added for indicated times. Results show preincubation with EGb 761 attenuates the induction of iNOS, eNOS, Cox-2 and Bcl-2, an inhibitor of apoptosis.

Apoptosis in T cells

To determine if EGb 761 induced apoptosis in primary T cells, T cells from C57BL/6 mice were purified from the spleens using nylon wool columns (Polysciences, Warrington, PA) followed by depletion of B cells and macrophages. The purity of T cells was >90% as determined by flow cytometry (Cytomics FC 500, Beckman Coulter). CD4+ T cells were then isolated using a separation column (MACS® separation, no. 130-090-860, Miltenyi Biotec, Germany), by depletion of non-CD4+ T cells (negative selection). About 1×10^6 CD4+ T cells, or the Jurkat T cell line, were cultured in six-well plates overnight, then either unactivated or activated [Con A (2.5 μg/ml) for 12 h], and were treated with vehicle (media) or indicated concentrations of EGb 761 dissolved in media for 24 h. Apoptosis in unactivated or activated T cells post-EGb 761 exposure was determined by performing Annexin V (BD Bioscience, San Jose, CA) or TUNEL assays (fluorescein isothiocyanate–deoxyuridine triphosphate nick end labeling) using an *in situ* cell death detection kit (Roche, Indianapolis, IN) following company protocols. To determine if EGb 761 induced apoptosis in CD4+ T cells *in vivo*, T cells from

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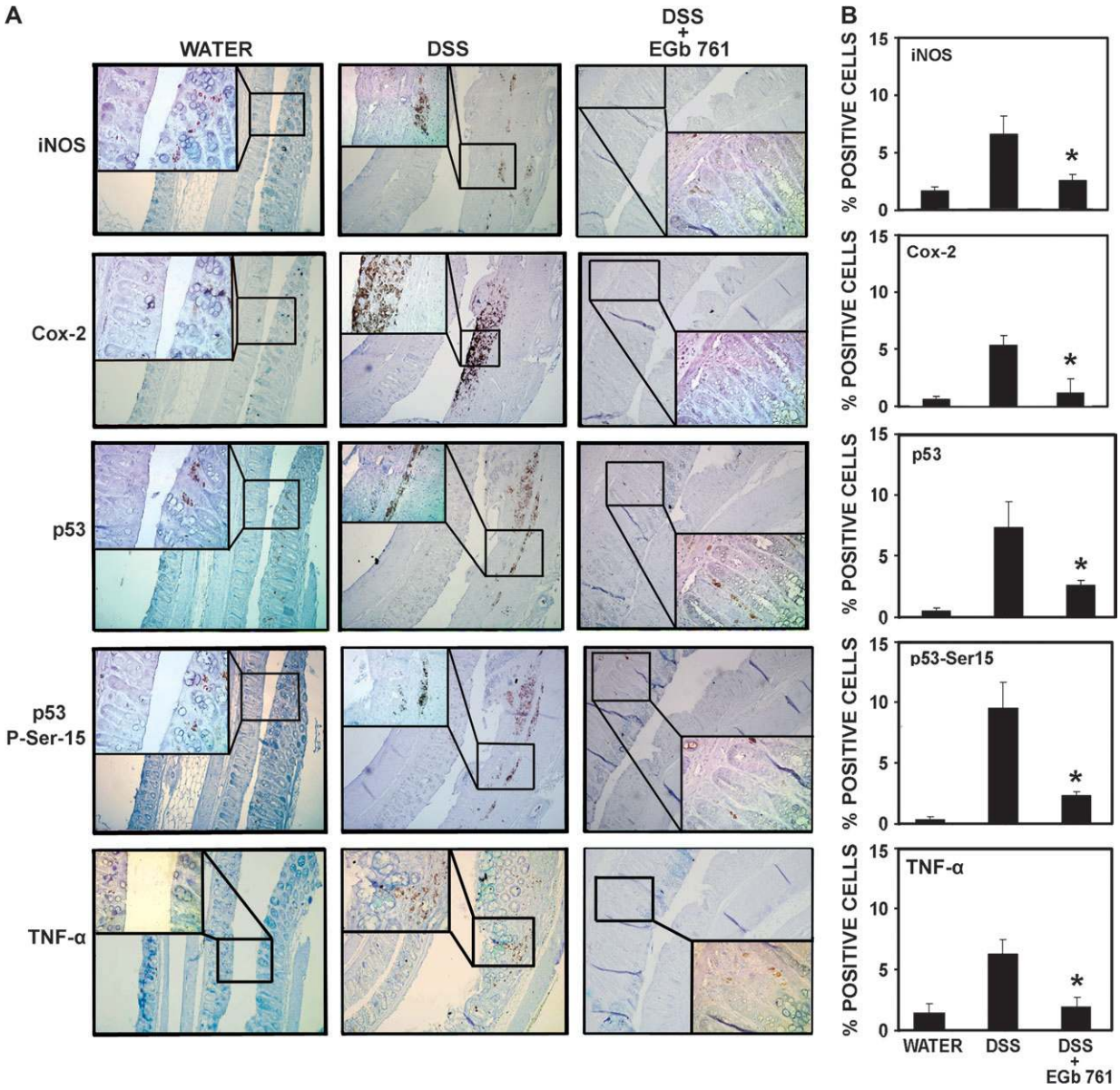

Fig. 2. EGb 761 suppresses the players (14) involved in inflammation (iNOS, Cox-2 and TNF-α) and inflammatory stress (p53 and p53-phospho-serine 15). Tissues from experiments performed for Figure 2 were examined for iNOS (dilution: 1 in 10 000), Cox-2 (dilution: 1 in 20 000), p53 (dilution: 1 in 1 000 000) and p53-P-ser-15 by immunohistochemistry, using the Antibody Amplifier^{TM} (ProHisto, LLC) rocked on a laboratory rocker to ensure even staining and reproducible results. (**A**) Representative staining of indicated end points in serial sections from water, DSS and DSS + EGb 761 groups. The inset is ×200 magnification of the indicated area of the ×100 magnification photomicrograph. (**B**) Quantification of indicated end points. All five markers were elevated in the DSS-treated group and suppressed when the DSS-treated group was fed EGb 761 (75 p.p.m.). *Indicates significant reduction in % positive cells in the EGb 761 + DSS group compared with the DSS group.

water-, DSS- and DSS + EGb 761-treated C57BL/6 mice were isolated from the spleens of five separate mice, as above. Because apoptotic cells are quickly recognized and engulfed by phagocytes as a means to clear them from the body *in vivo* (10), it was necessary to culture 1×10^6 CD4+ T cells in complete media for 24 h before examination of apoptosis by Annexin V or TUNEL methods as described above.

Statistical analysis

For prevention and treatment studies with inflammation and ulceration as an end point, a chi-square contingency table analysis was done on the DSS and DSS + EGb 761 groups to determine if there is a statistically significant difference in their inflammation and ulceration scores. For immunohistochemical quantification, mean differences between groups were compared by one-way analysis of variance with Scheffe multiple comparison tests. The *P*-value chosen for significance in this study was 0.05.

Results

EGb 761 attenuates proinflammatory protein marker in vitro

The standardized, well-characterized *G.biloba* extract, EGb 761, is a putative anti-inflammatory agent. To test whether EGb 761 abates inflammation *in vitro*, we examined its ability to attenuate inflammatory players. Figure 1A shows that IFN-γ activates iNOS in ANA-1 mouse macrophages and that this is attenuated when ANA-1 cells are preincubated with EGb 761 (12 h) in a dose–response manner. Figure 1B shows that iNOS, eNOS and Cox-2 in ANA-1 cells are inhibited by 100 μg/ml EGb 761 in a time–response manner. In all cases, EGb 761 was washed off prior to activation with IFN-γ to avoid the possibility that EGb 761 would sequester IFN-γ in the media. As indicated earlier, 100 μg/ml are levels probably to be achieved in blood

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Table II. Effects of EGb 761 on treating inflammation and ulceration in the colon induced by DSS

Treatment group	Score					Number of mice
	0	1	2	3	4	
Inflammation score						
DSS, 1.5 cycles	0 (0)[a]	0 (0)	2 (20)	2 (20)	6 (60)	10
DSS, 2.5 cycles	0 (0)	0 (0)	0 (0)	5 (50)	5 (50)	10
DSS + EGb 761, 2.5 cycles	0 (0)	0 (0)	2 (28.5)	2 (28.5)	3 (43)	7
DSS, 3.5 cycles	0 (0)	0 (0)	0 (0)	5 (36)	9 (64)	14
DSS + EGb 761, 3.5 cycles	0 (0)	0 (0)	5 (72)	1 (14)	1 (14)	7
Ulceration score						
DSS, 1.5 cycles	0 (0)[a]	1 (10)	6 (60)	2 (20)	1 (10)	10
DSS, 2.5 cycles	0 (0)	0 (0)	4 (40)	5 (50)	1 (10)	10
DSS + EGb 761, 2.5 cycles	0 (0)	2 (28.5)	2 (28.5)	3 (43)	0 (0)	7
DSS, 3.5 cycles	0 (0)	2 (14)	3 (21)	5 (36)	4 (29)	14
DSS + EGb 761, 3.5 cycles	1 (14)	2 (29)	3 (43)	1 (14)	0 (0)	7

[a]Percentage of mice.

after daily intake of 120–240 mg, representing the normal dosages for effective therapy (6).

EGb 761 prevents DSS-induced colitis

There is increasing evidence that EGb 761 targets many key players in inflammation (11). Ulcerative colitis is a high colon cancer risk, chronic inflammatory disease associated with overactive inflammatory cells infiltrating the colon. Based on this information, as well as *in vitro* data described for Figure 1 here, we tested the hypothesis that EGb 761 may inhibit the onset of colitis. Table I indicates that the DSS group consuming 75 p.p.m. EGb 761 has a statistically significant lower inflammation and ulceration (Table I) score compared with the group consuming DSS alone. The *P*-value associated with the observed chi-square value, under the hypothesis of no differences between the two groups, is 0.00057 for the inflammation score and 0.0158 for the ulceration score (Table I).

Mouse colon length shrinks with stress, inflammation and ulceration. Therefore, as an additional indicator of inflammation and inflammatory stress, mouse colon lengths were measured upon euthanasia. Results indicate that compared with the colon lengths of the control (water)-treated group (7.1 ± 0.1 cm), the length was significantly reduced in the DSS group (5.9 ± 0.1 cm). Mice consuming DSS + EGb 761 had a non-statistically different colon length (6.8 ± 0.12 cm) to that of the water-treated group. Finally, a complete white blood cell count was carried out to monitor systemic inflammation. The water-treated group had an average circulating white blood cell count ($\times 10^6$ cells/ml) of 7.5 ± 0.8. The DSS and DSS + EGb 761 groups had an average count of 15.7 ± 2.4 and 10 ± 1.2, respectively, indicating less systemic stress with the consumption of EGb 761. Supplementary Figure 1 (available at *Carcinogenesis* Online) shows representative Hematoxylin & Eosin sections from the experiment. Because TNF-α plays a key role in colitis (12), we probed tissues for TNF-α as another end point of colon inflammation. Supplementary Figure 1 (available at *Carcinogenesis* Online) also shows that mice consuming EGb 761 have lower levels of TNF-α than that observed in DSS-treated mice. Iron content is doubled in the chow of mice for this model because high iron diets are often encouraged for ulcerative colitis patients, and this stimulates a Fenton reaction so that less DSS is needed for generating colitis. We therefore examined the iron content of tissues and confirmed that all tissues had similar iron content (data not shown), eliminating the possibility that EGb 761 sequesters iron as a mechanism toward tempering colitis.

To further quantify the impact of EGb 761 on inflammatory markers *in vivo*, we examined iNOS, Cox-2 and TNF-α. Because p53 is activated due to phosphorylation at serine 15 during inflammatory stress (13), we also probed tissue sections for these markers. Immunohistochemical staining was accomplished by rocking slides using the Antibody Amplifier™ (ProHisto, LLC) to ensure even, consistent, sensitive and reproducible staining. Figure 2A shows representative sections of end points as indicated. Figure 2B shows quantification of staining using the ACIS® III Automated Cellular Imaging System (DakoCytomation). Results indicate that all five end points of inflammation (iNOS, Cox-2 and TNF-α) and/or inflammatory stress (p53 and p53-serine 15 phosphorylation) are elevated in DSS-treated mice, but significantly reduced to normal levels when DSS-treated mice are fed EGb 761 (75 p.p.m.). Supplementary Figure 2 (available at *Carcinogenesis* Online) shows a higher magnification (×400) of the DSS-treated mice. It appears that non-epithelial cells (mostly immune and connective tissue cells in these pictures) are staining positive for all markers. This is consistent with the appearance of these end points of inflammation in areas of higher inflammation and/or ulceration. Interestingly, the staining patterns of iNOS, TNF-α, p53 and p53-serine 15 phosphorylation are similar. Cox-2 staining is mostly toward the luminal areas of the mucosa and is specific to ulcerated areas (supplementary Figures 2 and 3, available at *Carcinogenesis* Online).

EGb 761 extract reverses active colitis

To examine whether EGb 761 extract can be used to treat colitis, we repeated the DSS experiment with timing modifications. Here, mice were given 1% DSS for 1.5 cycles (7 days DSS, 7 days water and 7 days DSS) and then fed either EGb 761 (75 p.p.m.) or standard AIN 93M chow for the duration of the experiment. Table II indicates that the DSS group consuming 75 p.p.m. EGb 761 has a statistically significant lower inflammation score compared with the group consuming DSS alone. The *P*-value associated with the observed chi-square value, under the hypothesis of no differences between the two groups, is 0.0098 for the inflammation score (Table II). Although the ulceration score (Table II) was lower in the mice consuming EGb 761, the differences were not significant ($P = 0.16$). Supplementary Figure 4 (available at *Carcinogenesis* Online) shows Hematoxylin & Eosin staining of representative sections from each group. Overall, it appears that EGb 761 reverses inflammation in the colon, consistent with the hypothesis that this extract has the potential to be used to treat active colitis in patients with this disease.

EGb 761 reduces the CD4+ effector T cell population in the colons of DSS-treated mice

To identify mechanisms of amelioration of colitis by EGb 761, the cellular populations that affect colitis progression were examined. We carried out experiments similar to that outlined for Table I. However, after 2.5 cycles of DSS or water ± EGb 761 (75 p.p.m.), we collected single-cell suspensions of colons from seven mice in each group. Results indicate that the colonic CD4+/CD25−/Foxp3− T cell numbers are elevated in DSS-treated mice and reduced when DSS-treated mice are fed EGb 761 (Figure 3). The CD4+/CD25+/Foxp3+ cell population (reflecting regulatory T cells) is slighty elevated in the DSS treated group, but EGb 761 does not reduce this. The CD8+ T cell population is reduced with DSS and further reduced when these mice are fed EGb 761.

EGb 761 drives apoptosis of Jurkat T cells and CD4+ effector T cells in vitro and in vivo

Because apoptosis of inflammatory cells is a key mechanism regulating many chronic inflammatory and autoimmune diseases (15,16), and the CD4+/CD25−/Foxp3 T cell population is reduced in EGb 761-treated mice (Figure 3), we next asked whether EGb 761 drives T cell apoptosis. The first indication that apoptosis is driven by EGb 761 is shown in Figure 1. EGb 761 inhibits the induced expression of the apoptosis inhibitor, Bcl-2, in IFN-γ-treated ANA-1 mouse macrophages. Figure 4A and B shows that EGb 761 induces apoptosis in both unstimulated and Con A-stimulated Jurkat T cells in

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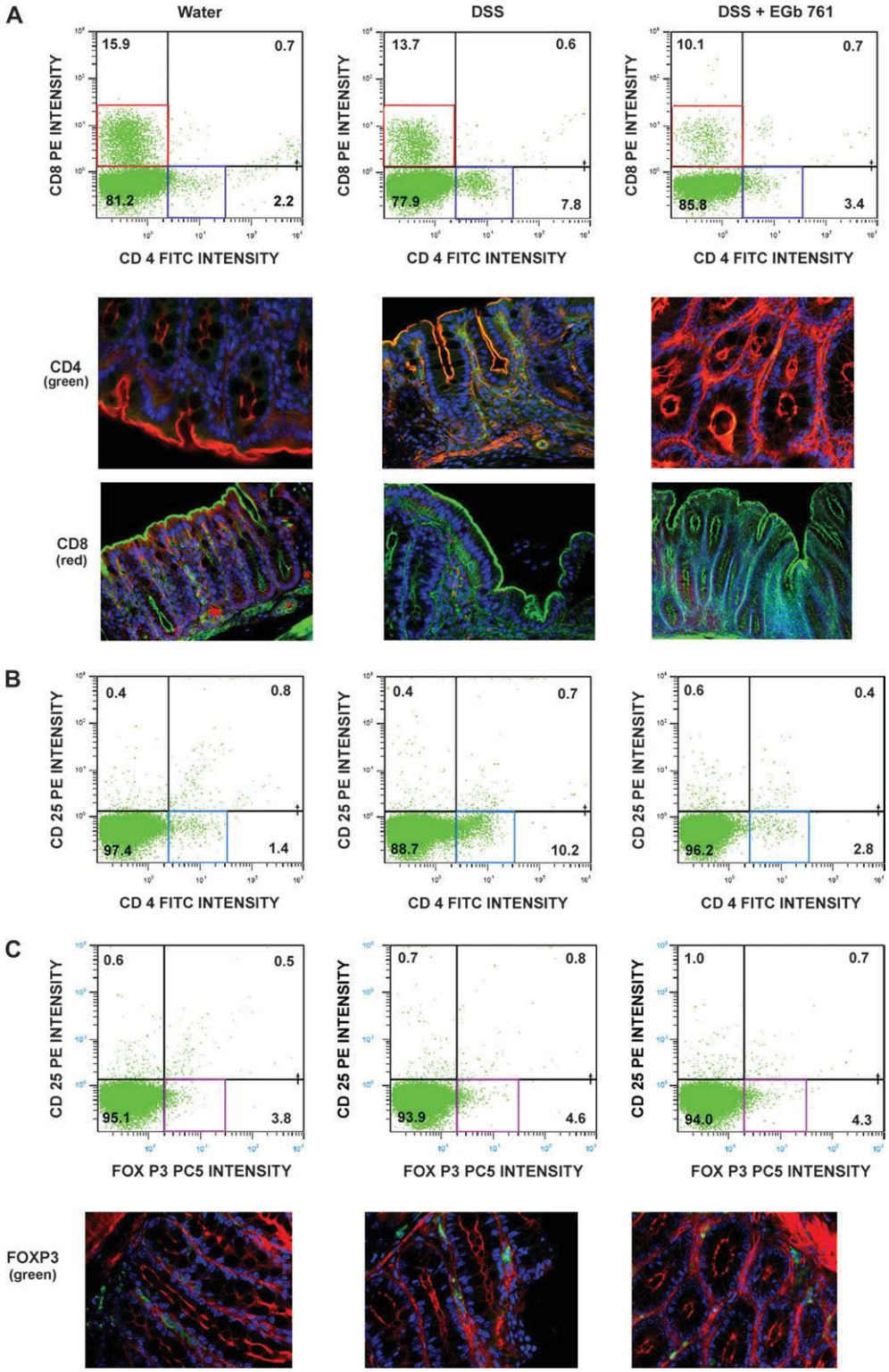

Fig. 3. EGb 761 (75 p.p.m.) reduces the CD4+/CD25−/Foxp3− effector T cell population in colons of DSS-treated mice. Mice were treated as described previously for Figure 2. After euthanasia, seven colons from seven mice in each group were harvested for single-cell suspension as described in the text. Single-cell suspensions were then fluorescently labeled with indicated antibodies and examined by FACS. (**A**) Scatterplots representing the percentage of CD4/CD8−, CD4+, CD8+ and CD4+/CD8+ T cells. To confirm this result, we also carried out immunofluorescent staining. Representative photomicrographs are shown, indicating that the CD4+ effector T cell population is elevated in DSS treated, but not in DSS-treated mice supplemented with EGb 761. (**B**) Scatterplots representing the percentage of CD4−/CD25−, CD4+, CD25+ and CD4+/CD25+ T cells. Results confirm that in (A), indicating an increase in the CD4+/CD25− T cell population in DSS treated, but not in DSS-treated mice supplemented with EGb 761. (**C**) Scatterplots representing the percentage of CD25/Foxp3−, CD25+, Foxp3+ and CD25+/Foxp3+ T cells. Immunofluorescent staining confirmed that the Foxp3 population does not change with DSS or with DSS + EGb 761. Overall, data are consistent with the hypothesis that EGb 761 reduces colitis by targeting CD4+/CD25−/Foxp3− T cells.

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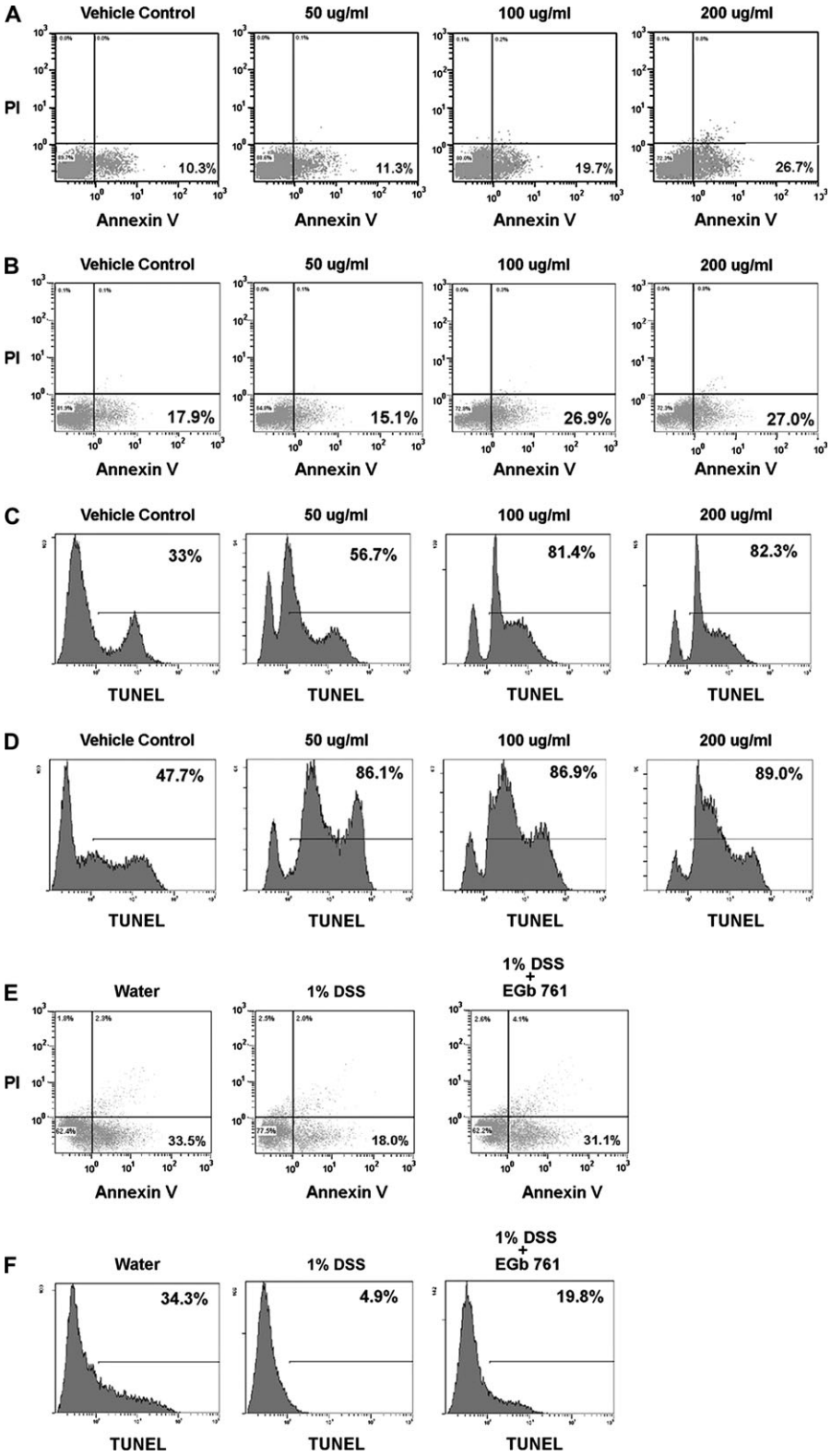

Fig. 4. CD4+ T lymphocytes exposed to EGb 761 undergo apoptosis *in vitro* and *in vivo*. To initiate experiments on the effects of EGb 761 on T cell apoptosis, either unactivated (**A**) or Con A-activated (**B**) Jurkat cells (a T lymphocyte cell line) were treated with EGb 761 at indicated doses. Apoptosis (assessed by Annexin V staining) was induced in both unactivated and activated Jurkat T cells by EGb 761. To examine apoptosis in the CD4+ T cell population, CD4+ T lymphocytes were isolated from the spleens of untreated C57BL/6 mice. Purified (>90% T cell purity), non-activated (**C**) or Con A-activated (**D**) T lymphocytes (1×10^6) from C57BL/6 mice were exposed to vehicle (media) or indicated doses of EGb 761 dissolved in media for 24 h. TUNEL assays were performed to determine apoptosis posttreatments and analyzed using flow cytometry. Percentage of apoptotic cells is depicted in each histogram. (**E** and **F**) CD4+ T lymphocytes were isolated from the spleens of water, DSS-treated or DSS-treated mice supplemented with EGb 761 (75 p.p.m., as outlined for Figure 2). After culturing for 24 h in complete media, cells were examined for apoptosis by Annexin V (E) and TUNEL (F). DSS-treated mice supplemented with EGb 761 had increased apoptosis compared with DSS alone treated, indicating that EGb 761 drives apoptosis of this cell type *in vivo* upstream of the colon.

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a dose–response manner. Doses are within non-toxic ranges, as assessed by the CytoTox-ONE Assay (Promega, Madison, WI), which is a rapid, fluorescent measure of the release of lactate dehydrogenase from cells with a damaged membrane (supplementary Figure 5, available at *Carcinogenesis* Online). To test whether the CD4+ T cell population in particular is susceptible to apoptosis to EGb 761, we isolated primary CD4+ T cells from spleens of untreated C57BL/6 mice. Figure 4C and D shows a dose–response induction of CD4+ T cell apoptosis as assessed by a TUNEL assay. Finally, to investigate the *in vivo* effects of EGb 761 on CD4+ T cells, we carried out experiments as outlined for Table I and Figure 3. Following treatment, CD4+ effector T cells were isolated by antibody columns from single-cell suspensions of spleens, cultured for 24 h and then examined for apoptosis by Annexin V and TUNEL staining. Figure 4E (Annexin V) and 4F (TUNEL) shows that there is a reduction in CD4+ effector T cell apoptosis by DSS. However, DSS-treated mice consuming EGb 761 had levels of apoptosis similar to that of water-treated mice. These results indicate that EGb 761 drives apoptosis of activated CD4+ effector T cells upstream of the colon, providing a mechanism for the protection from this disease.

Discussion

Although there have been inroads into the treatment of IBD (17,18), these treatment strategies often bring side effects with marginal results and have population-specific efficacy. Many patients therefore turn to alternative treatment (4). The frequency of IBD patients taking EGb 761 is unknown. In one small pilot study, 33% of patients with ulcerative colitis treated with a ginkgo extract (Cedemin) achieved disease remission (19). Although there is only one study of the effects of ginkgo extracts in human colitis, several more recent studies have found a protection of either *G.biloba* (20,21) or EGb 761 (22) against rat colitis induced by acetic acid or 2,4,6-trinitrobenzene sulfonic acid. In these studies, *G.biloba* or EGb 761 was given at 30–200 mg/kg body wt by oral gavage or intraperitoneally. Although *G.biloba* is considered safe, reaching toxicity only at hundreds of times the recommended daily dose (23,24), we also used levels of EGb 761 consistent with doses currently recommended to humans as supplements (120–240 mg daily). We found that doses (75 p.p.m. in chow) equivalent to ~58 mg daily in humans successfully prevented and, more importantly, caused a remission of established murine colitis. Pathologically, EGb 761 reduced inflammation and ulceration associated with DSS. *In vitro* results (Figure 1), as well as immunofluorescent and immunohistochemical results (Figure 2 and supplementary Figures 1–3, available at *Carcinogenesis* Online), indicate that inflammatory markers such as TNF-α, iNOS and Cox-2 are also reduced by EGb 761. This is consistent with the known antioxidant and anti-inflammatory properties of EGb 761. As well, markers of inflammatory stress (p53 and phosphorylation of p53 at serine 15) are reduced. Interestingly, there was remarkable consistency in staining patterns between these two end points, as well as with iNOS and TNF-α (Figure 2 and supplementary Figures 2 and 3, available at *Carcinogenesis* Online). This is consistent with the understanding that TNF-α drives the induction of nitric oxide, which causes serine 15 phosphorylation of p53, which drives wild-type p53 stabilization and activation (13,25).

IBD is a heterogenous, chronic, relapsing, inflammatory condition that is mediated by an overactive immune system (26,27). Immune responses begin when either cytotoxic T lymphocyte CD8+ cells or CD4+ effector T cells in the intestinal lumen recognize a foreign antigen. The importance of CD4+/CD25− effector T cells is highlighted by findings in animals that adoptive transfer of CD4+/CD25− effector T cells into immune-compromised animals causes moderate to severe colitis (28–31). An immunological cascade, including cytokine production and the infiltration of innate immune cells, responsible for removing the antigenic material, is then initiated. Normally, following removal of the antigen, the activity of innate and adaptive immune cells, such as macrophages and effector T lymphocytes, is suppressed through a number of mechanisms, including apoptosis. Failure to regulate these responses in the colonic mucosa leads to a sustained immunologic reaction and results in mucosal damage. A key mechanism for immune suppression is apoptosis of overly aggressive effector T cells and defects in mucosal T cell apoptosis are likely to be critical in the pathogenesis of colitis (26,27). We have shown here that the CD4+/CD25−/Foxp3− effector T cell population is reduced in the colons of DSS-treated mice consuming EGb 761 compared with mice consuming only DSS (Figure 3). The finding that the regulatory T cell population does not change indicates that these cells are not required for the onset of colitis or a target of EGb 761 in this particular mouse model of colitis. The finding that the CD8+ population is reduced by DSS treatment and further reduced by combination treatment with EGb 761 and DSS cannot at this time be explained. However, studies have found the CD8+ population to be less critical to colitis than the CD4+ population in this and other animal models of colitis (31,32).

Mechanistically, we show that EGb 761 can suppress the activation of macrophages (Figure 1). Consistent with this, others have shown EGb 761 to decrease the expression of iNOS, release of nitric oxide, as well as inhibit other markers of inflammation such as Cox-2 and nuclear factor-kappa B (11,33,34). Such results are consistent with our *in vivo* immunohistochemistry results here, indicating that such markers are suppressed by EGb 761 (Figure 2 and supplementary Figures 1–3, available at *Carcinogenesis* Online). Importantly, we show for the first time that EGb 761 drives apoptosis of the CD4+ effector T cell population (Figure 4). Recent studies have found that EGb 761 can induce apoptosis in oral cavity cancer cells (35). Interestingly, key neuroprotective effects of EGb 761 are thought to be associated with its ability to inhibit free radical-driven apoptosis (36–40). These results indicate possible selective and divergent effects of EGb 761 on different cell types. Tang *et al.* (41) recently showed that effects of EGb 761 on T lymphocyte populations were dependent on concentrations. They also showed that EGb 761 drives T cell apoptosis, consistent with our findings. Significantly, here, we show that apoptosis is not only stimulated by EGb 761 *in vitro* but also EGb 761 stimulates CD4+ T cell apoptosis *in vivo*. Although the apoptotic machinery driving this process is currently unknown, we show here an effect on Bcl-2 (Figure 1), hinting at an intrinsic mechanism in lymphoblastoid cells. Interestingly, p53 has also been shown to be stabilized by EGb 761 in hepatocytes, which undergo apoptosis upon exposure to EGb 761 (42). In PC12 neuronal cells, reactive oxygen species cause an increase in proapoptotic molecules, such as Bax and p53, whereas ginkgo extracts inhibit this increase. In addition, EGb 761 can protect PC12 cells from neurotoxicity by increasing Bcl-2, again suggesting that it is neuroprotective in these cell types (43,44). In the colon, EGb 761 appears to inhibit colitis by exacerbating an apoptotic effect on cells that drive this disease. To this end, it is intriguing that p53 and its phosphorylation occur mainly in immune and non-epithelial cells in DSS-induced colitis (supplementary Figure 2, available at *Carcinogenesis* Online). The hypothesis that stabilization of p53 in inflammatory cells is a key to the effects of EGb 761 on treating DSS-induced colitis is currently being explored. These and other experiments are ongoing to delineate the apoptotic machinery targeted by EGb 761 in effector CD4+ T lymphocytes, as a prerequisite to explore the possibility of using EGb 761 as a therapeutic agent for the treatment of IBD and possibly other high cancer risk, chronic inflammatory diseases.

Supplementary material

Supplementary Figures 1–5 can be found at http://carcin.oxfordjournals.org/

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