Prevention of colitis-associated carcinogenesis with infliximab

Running title: Chemoprevention of colitic cancer with infliximab

Yoon Jae Kim\textsuperscript{a}, Kyung Sook Hong\textsuperscript{b}, Jun Won Chung\textsuperscript{a}, Ju Hyun Kim\textsuperscript{a}, Ki Baik Hahm\textsuperscript{a,b,*}

\textsuperscript{a} Department of Gastroenterology, Gachon Graduate School of Medicine, Incheon, Korea
\textsuperscript{b} Laboratory of Translational Medicine, Gachon University Lee Gil Ya Cancer and Diabetes Institute, Gachon University of Medicine and Science, Incheon, Korea

*Corresponding author. Address: Prof. Ki-Baik Hahm, Laboratory of Translational Medicine, Lee Gil Ya Cancer and Diabetes Institute and Department of Gastroenterology and Gachon Graduate School of Medicine, Gil Medical Center, 7-45 Songdo-dong, Yeonsu-gu, Incheon 406-840, South Korea.
Tel: +82 32 899 6055; fax: +82 32 899 6054
E-mail address; hahmkb@gachon.ac.kr (K-B, Hahm)

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Abstract
The emergence of infliximab was epochal event in the treatment of inflammatory bowel disease (IBD). Since colitis-associated cancers arose in the setting of chronic inflammation, during which “inflammation-dysplasia-carcinoma” prevails and anti-inflammatory agents can prevent carcinogenesis, we hypothesized whether infliximab can prevent colitic cancer in animal models, for which C57BL/6 mice were exposed to 15 cycles of dextran sulfate sodium (DSS) with each cycle consisting of 0.7% DSS for 1 week followed by sterilized water for 10 days. Infliximab, 4mg/kg iv, was given on 1st, 3rd, and 7th week or 25th, 27th, and 31th week of cycle according to “step-up” versus “top-down” strategy. Molecular change about inflammation and carcinogenesis were compared between groups. Multiple colorectal tumors developed in 75-80% of control mice, whereas only 16.7% of mice treated with 1st, 3rd, and 7th weeks of infliximab developed colon tumors. Significant decreases in TNF-α level, mast cell number, and the expression of inflammatory cytokines were observed in “top-down” strategy using infliximab. The expression and activity of MMP-9 and MMP-11 were significantly decreased in mice treated with infliximab accompanied with attenuated numbers of “β-catenin accumulated crypts”. In animal group where infliximab was administered at later stage of 25th, 27th, and 31th weeks, no reduction in tumorigenesis was noted. These biological effects of infliximab were further explored in in vitro experiment using Raw264.7 and Jurkat T cells. Conclusively, earlier and intensive therapy with infliximab should be considered for either mitigating clinical course or preventing ultimate development of colitic cancer in high risk IBD patients.

INTRODUCTION
Individuals with IBD have a 10-to 40-fold increased risk of developing colorectal cancer (CRC) compared to general population, supporting the fact that colitis-associated cancer develops from chronic persistently inflamed mucosa and progresses through dysplasia to adenocarcinoma, taking the “inflammation-dysplasia-carcinoma sequence” in contrast to “adenoma-carcinoma sequence” of sporadic CRC. Therefore, efficacious anti-inflammatory treatment can reduce or retard the development of colorectal dysplasia and cancer in IBD (1-4) and the earlier intervention of potent anti-inflammatory strategy or agents can be an effective way to rescue from colitis-associated cancer, colitic cancer (5).
The development of effective biological therapy in the treatment of IBD has given rise to a clinical debate about “step-up” versus “top-down” strategy. Step-up therapy refers to the classical therapeutic approach, namely progressive intensification of treatment as disease severity increases, so called “therapeutic pyramid”, whereas top-down strategy refers to the early intervention of intensive therapies including biological agents or immunosuppressive agents (6-8). This concept has evolved by the emergence of infliximab, novel anti-tumor necrosis factor-α (TNF-α) biologics, which has been effective for patients who are refractory to conventional step-up treatments.

TNF-α is a prototype member of the TNF family of ligand that binds the corresponding TNF receptor family. Normally, TNF-α and other pro-inflammatory cytokines are maintained in balance by anti-inflammatory factors (9-10), but this balance is shifted in favor of the pro-inflammatory cytokines in IBD, during which TNF-α is steeply generated by immune and even epithelial cells and contributes to tissue inflammation and destruction. The introduction of infliximab was an epoch-making event especially in the treatment of complicated Crohn’s disease (CD) such as fistula and obstruction. In addition to refractory patients to conventional diverse therapy, this agent has been shown to be effective for active IBD with rapid onset of mucosal healing, steroid-sparing properties, inducing closure of perianal fistulas, and even maintaining remission. The therapeutic effect infliximab is mediated by TNF-α neutralization since infliximab can bind to both soluble and trans-membrane receptor (11-12).

Based on the results of several investigators that inflammation-induced cytokines may have a pivotal role in initiation, promotion, and progression of colon carcinogenesis (13) and the blocking TNF-α significantly decreased colon tumor development in a mouse model established with two stage carcinogenesis of azoxymethane (AOM) administration followed by DSS (14), we hypothesized that the early interventions of infliximab can afford definite prevention of colitic cancer beyond designed mechanism of TNF-α blocking. Our study might be the first to elucidate the in vivo anti-tumor effect of infliximab and to emphasize the usefulness of “top-down” strategy for cancer preventive purpose especially in high risk patients presenting with longstanding and extensive involvement of IBD.

Materials and Methods
Animal model for colitis-associated cancer (colitic cancer) Six week old female C57BL/6 mice (Charles River Japan, Tokyo, Japan) were fed sterilized commercial pellet diets (Biogenomics, Seoul, Korea) and sterilized water ad libitum, and housed in an air-conditioned biohazard room at a temperature of 24°C. One group composed of 24 mice. Normal control group (Group 1) was administered ordinary tap water throughout the experiment, whereas disease control group (Group 2) was exposed to 15 cycles of DSS (dextran sulfate sodium (DSS), molecular weight 40,000, ICN Pharmaceuticals, Costa Mesa, CA) containing sterilized water, with each cycle consisting of 7 days of 0.7% DSS w/v in the drinking water, followed by 10 days of sterilized ordinary tap water (Fig. 1a & 1c). Group 3 was administered with additional daily intraperitoneal injection of infliximab (4 mg/kg in 200 μl volume), Remicade®, Shering Pharma., Seoul, Korea) on the basis same as Group 2. Animals were handled in an accredited animal facility in accordance with the AAALAC International Animal Care Policies and Center for Animal Care and Utilization (CACU, Gachon University of Medicine and Science). According to the interventional schedule of infliximab injection, the disease control group was set twice, once for Group 3, a group treated with infliximab at 1st, 3rd, and 7th weeks of DSS commencement, but the other for Group 5, a group administered infliximab at rather later stage of 25th, 27th, and 31th weeks.

Histopathological evaluation. Following sacrifice, the whole colons were removed. The tumors were counted on gross examination (Fig. 1a) and their sizes were measured, respectively. Tumor incidence was calculated as the number of tumor-bearing mice divided by the total number of mice and tumor multiplicity was calculated as the number of tumors divided by the number of tumor-bearing mice. The pathology of each tumor was evaluated by two pathologists unknown to group. Each dissected colon was spread onto a plastic sheet, fixed in 10% buffered formalin for 4hr, and prepared for paraffin tissue slides. The paraffin sections were stained with hematoxylin and eosin. Colitis severity was graded on a scale of 0 to 3, with 0 for absence of inflammation, 1 for focal inflammatory cell infiltration, 2 for gland loss with inflammatory cell infiltrations, and 3 for ulcerations and colitis-associated colon tumors were analyzed microscopically and diagnosed as low grade dysplasia, high grade dysplasia and adenocarcinoma.

Serum and tissue levels of TNF-α. To detect the colitis activity, the levels of TNF-α in serum and colon tissues were measured in all samples. All samples were measured for their individual levels and each sample was analyzed in triplicate manner, taking the mean of the three determinations. ELISA was applied to detect the titer of the TNF-α, for which kits from R&D Systems (Abington, UK) were used.
RT-PCR for expressions of inflammatory cytokines, real-time PCR for quantitating inflammatory cytokines, and RT-PCR for MMP expressions

Total RNA were extracted tissues using an RNeasy mini kit (Qiagen Inc., Valencia, CA). Primer used for inflammatory cytokines were as follows, 5'-TCT CTT CAA GGG ACA AGG CTG-3' and 5'-ATA GCA AAT CGG CTG ACG GT-3' for TNF-α, 5'-TGG GTG GGA TGT AGC TAG TTC C-3' and 5'-AGT TGC CTT GAC CCT GAA GCC-3' for IL-8, 5'-GTG TTC CAC CAG GAG ATG TTG-3' and 5'-CTC CTG CCC ACT GAG TTC GTC TGC-3' for iNOS, 5'-GGA GAG ACT ATC AAG ATA GT-3' and 5'-ATG GTG GTC AGT AGA CTG TTA CA-3' for COX-2, 5'-TTG TTG CCA TCA ATG ACC CC-3' and 5'-TGA CAA AGT GGT GG-3' for GAPDH. Primers for MMPs, MT-MMPs, and TIMPs were as follows, 5'-AGA TCT TCT TCT TGA ACC GGT T-3' AND 5'-GCC TGG TCA GTG GGT A'Three' for MMP-2, 5'-GAT TCT TTC ATT TTG GCC ATC CCT TC-3' and 5'-CTT CCA GTA TTT GTC CTC TAC AAA GAA-3' for MMP-3, 5'-TAC TGG ACT GAT GAT GAG AA-3' and 5'-AGC ACA AGG AAG AGG AC-3' for MMP-7, 5'-GTT TTT GAT GCT CCT CTC AAC ATC-3' and 5'-CCC ACA TTT GCG ACT AGA TAG ATC TTG TCT T-3' for MMP-11, 5'-ATG ATC TTC TTT AAA GAC AGA TCT CCG TCC-3' and 5'-TTG GAT AAC CTT CCA GAA TGT CAT AA-3' for MMP-13, 5'-CTG ACC ATC CCG GGT GGA ACT AAA CCC CAG AGT CC-3' and 5'-CTG AAG AAC GCA CGC GTC GAT AG-3' for MT1-MMP, 5'-CGC CAA GAC GTG CGT TTC TTT T-3' and 5'-GGA CTC CAG ATG TCT GGG TCC-3' for MT2-MMP, 5'-GCA GTG AAG AGT TTC TCA CTG GGC CCC AAG GGA TCT C-3' for TIMP-1, 5'-GCA GGA AAG GCA GAA GGA GAT-3' and 5'-TTA CGG GTC CTC GAT GTC AAG-3' and 5'-GCA GAT GAA GAT GTA CCG AGG-3' for TIMP-2. The amplifications were performed in 50-µl reaction volumes containing 10x reaction buffer (Promega Korea, Seoul), 1.5 mM MgCl₂, 200 mΜ deoxynucleotide triphosphates (dNTPs), 1 mM of each primer, and 2.5 units of Taq DNA polymerase (Promega, Madison, WI) using a Perkin-Elmer GeneAmp PCR system 2400. Each cycle consists of denaturation at 95°C for 1 min, annealing at 55°C for 45 secs, and amplification at 72°C for 45 secs. The RT-PCR–derived DNA fragments obtained by 25 PCR cycles were subjected to electrophoresis on a 1.5% agarose gel. The results were also scanned and quantified by densitometry. Quantitative real-time PCR was performed in a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) with the FastStart DNA Master SYBR Green I kit (Roche Diagnostics), and results were analyzed with the LDCA software supplied with the machine. Each 50 µL PCR contained 1/50th of the original
cDNA synthesis reaction, 7 μL (25 mM) MgCl₂, 0.8 μL (20 pM) of each primer, 1 μL (10 mM) dNTP, 1 μL SYBR Green I, 0.5 μL (5 U/μL) Taq polymerase and 5 μL Buffer10 X. 50 cycles of amplification were performed: after 94°C, 3 min, the annealing temperature was reduced from 94°C, 30 s, to 57°C, 30 s, then to 72°C, 30 s. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. Primer used for inflammatory cytokines were as follows; 5′-GAA GTC TTT GCT GTG CCT G-3′ and 5′-GTC TGC TGG TTT GGA ATA GTT GC-3′ for COX-2, 5′-GGA GCG AGT TGT GGA TTG TC-3′ and 5′-GTG AGG GCT TGG CTG AGT GAG-3 for iNOS, and 5′-GTG GAA CTG GCA GAA GAG GC-3′ and 5′-AGA CAG AAG AGC GTG GTG GC-3′ for TNF-α.

Zymography. To detect the gelatinolytic activity in colon tissue, the samples were analyzed by gelatin. Briefly, the extracts of each mouse colon mucosa were dissolved in 2×SDS sample buffer and subjected to electrophoresis in a 10% polyacrylamide gel embedded with 0.8 mg/ml gelatin without reducing regent. After electrophoresis the enzymes were renatured by incubation with 3% Triton X-100, and the enzymatic reaction was allowed to proceed at 37 °C for 36 hr. After incubation, the gel was stained with 0.5% Coomassie blue in 30% methanol and 10% glacial acetic acid. Gelatin digestion was visualized as transparent bands against the dark blue background.

RNase protection assay (RPA) The ribonuclease protection assay (RPA) is a highly-sensitive and specific method for the detection and quantitation of mRNA species. Gene expressions of inflammatory cytokines and chemokines including IL-12, IL-1β, IFN-γ, MIF, IP-10, TCA-3, etc and gene expressions of apoptotic executors including caspase-8, FAS, FADD, TRAIL, TRADD, etc were examined by multi-probe detection RiboQuant RPA kits (mCK-5, mCK-2, mAPO-3, BD PharMingen, San Diego, CA) with two housekeeping gene products L32 and GAPDH. Total RNA was extracted from the colon mucosa using the Trizol method (Life Technologies, Gibco BRL, Gaithersburg, MD) according to the manufacturer's protocol and quantified spectrophotometrically by absorption at 260 nm and 280 nm. Total RNA (20 μg) was labeled with [α-32P] UTP (Amersham Pharmacia Biotech, Buckinghamshire, UK), using T7 RNA polymerase according to the manufacturer's description, and hybridized at 42°C overnight with 1×10⁵ cpm of riboprobe and then digested with an RNaseA cocktail. The reaction products were resolved on 5% acrylamide gel and analyzed after 72 hr by autoradiography.

Immunohistochemical stainings. After paraffin blocks were dewaxed and rehydrated with graded alcohol, these tissue sections were heated in pressure jars filled with 10mM citrate buffer using microwave for 10mins.
After that, slides were cooled in water for 15 mins, then, washed in phosphate buffer saline. The slides were incubated overnight with the primary antibody. The primary antibodies were specific rabbit polyclonal antibody of tryptase and β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA). Each antibody was diluted 1:100. After incubation, the subsequent reaction was formed using an Envision kit (DAKO Cytomation, Carpinteria, CA). Finally, the slides were incubated with 3’, 3’-diaminobenzidine (DAKO Cytomation) and counterstained with hematoxylin (Sigma, St Louis, MO). Numbers of tryptase positive mast cells were counted on x200 magnification field in each pathology specimen and averaged according to groups. Accumulation of β-catenin proteins, termed β-catenin accumulated crypt (BCAC), a valuable biomarker for colon cancer bioassay, was counted by a single reader who was blind to the treatment group. The mean numbers of BCAC noted in x100 magnification field were counted, calculated as mean numbers, and compared among group.

TUNEL (Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling) staining for apoptosis.

Apoptosis was visualized with terminal deoxynucleotidyl transferase (TdT) FragEL DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA). After routine deparaffinization, rehydration, and washing in 1x PBS (pH 7.4), tissues were digested with proteinase K (20 μg/mL in 1x PBS) for 20 minutes at room temperature and washed. After then tissues were incubated in equilibration buffer for 10 minutes and were treated with terminal deoxynucleotidyltransferase (TdT) enzyme at 37°C for 1 hour. To determine the apoptotic index (AI) in each group, we first scanned TdT-nick end labeling (TUNEL)-immunostained sections under low power magnification (x100) to locate the apoptotic hot spots. The AI at x400 field was then scored by counting the number of TUNEL-positive cells. At least five hot spots in a section were selected and average count was determined. Data were expressed as a mean percentage of total cell numbers.

In vitro experiment for validating the anti-inflammatory and apoptotic actions of infliximab. Raw264.7 cells and Jurkat T cells were cultured in culture media. As preliminary study to decide the time point when LPS challenge provoked highest levels of inflammatory cytokines, the mRNA levels of TNF-α, IL-8, iNOS, and COX-2 and protein levels of apoptosis executors were decided with RT-PCR at 2, 4, 8, 16, and 24 hrs of 1mg LPS. Raw264.7 cells showed highest induction after 4 hrs and it was 16hrs in Jurkat T cells. Next, we compared the expressions of these inflammatory mediators according to LPS alone, LPS + infliximab 1μg (24 hrs), and infliximab 1μg alone (24 hrs). These experiments were repeated according to the different doses of infliximab, 1,
Western blotting was repeated to check the changes of apoptotic executors, cytochrome c, caspase-3, caspase-9, FAS, TRADD, and PARP according to treatment group.

**Antibodies and Western blotting** Primary antibodies specific for cytochrome c, caspase-3, caspase-9, Fas, TRADD, and PARP were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Total proteins were extracted from control, LPS-treated, LPS + infliximab, and infliximab-treated cells, electrophoresed on SDS-PAGE gels, and transferred to PVDF membranes using a semidry transfer system (Hoeffer Pharmacia Biotech). Nonspecific binding sites were blocked by incubation with 5% non-fat dry milk in TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20 v/v) at room temperature for 1 hr. The membranes were incubated overnight at 4°C with a 1:500 dilution of primary antibody in blocking solution, followed by incubation with 1:1,000 dilution of horseradish peroxidase-conjugated secondary antibody. The immunocomplexes were detected using an ECL detection kit (Amersham Biosciences) and auto-radiographed onto x-ray film.

**Statistical analysis.** Results are expressed as the mean ± SD. The data were analyzed by one-way analysis of variance (ANOVA), and the statistical significance between groups was determined by Duncan’s multiple range test. Statistical significance was accepted with a p<0.05.

**Result**

**Establishment of animal model for colitis associated cancer and prevention with earlier intervention of infliximab.** Mice were randomized into five groups as follows; Group 1, normal control group; Group 2, repeated DSS administration group for 37 weeks; Group 3, same as with Group 2, but further treated with infliximab three times at 1\(^{st}\), 3\(^{rd}\), and 7\(^{th}\) weeks \(iv\) (Fig. 1a), Group 4, repeated DSS administration group for 37 weeks as the control group for Group 5, and Group 5, same as with Group 4, but further treated with infliximab three times at 25\(^{th}\), 27\(^{th}\), and 31\(^{th}\) weeks \(iv\) (Fig. 1c). The basic difference between Group 3 and Group 5 is the intervention timing of infliximab administration, the former Group 3 as earlier administration and the latter Group 5 as later administration of infliximab. As result, repeated induction of mild colitis with DSS in drinking water led to significant development of colorectal tumor after total 225days (37 weeks). The tumor incidence at sacrifice was 75.0% (9/12) in Group 2 and 80.0% (8/10) in Group 4, respectively. The tumor multiplicity was 1.57 ± 0.5 in Group 2 and 1.85 ± 0.31 in Group 4. Therefore, repeated inductions of mild colitis with 0.7% DSS in drinking water (totally 15cycles, 1cycle is 17days composed of 7 days administration of 0.7% DSS in drinking water led to significant development of colorectal tumor after total 225days (37 weeks).
drinking water followed with 10 days of sterilized tap water drinking) led to significant development of colorectal tumor, confirming the hypothesis that chronic colitis with alternating relapse and remission course is the pathway connecting between chronic inflammation and carcinogenesis under the no administration of carcinogen. Compared to other animal model of colitic cancer, in which tumorigenesis induced by azoxymethane and DSS treatment where the tumor developed mostly on anal side and spread into proximity (14), tumors developed in the whole colon with the features of mass forming type without any prevalent locations in our model of colitic cancer (Fig. 1a). Very interesting differences in tumorigenesis were came from the results of Group 3 and Group 5, both of which administered with same dosing of infliximab, but differed only in the timing of administration. When infliximab was administered at 1st, 3rd, and 7th week after the commencement of repeated DSS cycle in Group 3, colon tumors were developed only in 2/12 (16.7%, Fig. 1b), whereas when infliximab was administered at 25th, 27th, and 31th week in Group 5, colon tumors were noted in 7/10 (70%, Fig. 1d), suggesting that only early intervention of infliximab imposed significant cancer preventive roles in colitis-associated tumorigenesis, whereas no tumor preventive effect in late intervention of infliximab. Conclusively, infliximab could play efficient cancer prevention only in case of early intervention, but late stage of administration of infliximab did not afford this benefit.

Footprint of inflammation on colitis-induced tumorigenesis and attenuated levels of inflammatory mediators with infliximab treatment. The serum level of TNF-α was markedly increased in Group 2 compared with Group 1 (p<0.01), but infliximab treatment significantly decreased the serum level of TNF-α (Fig. 2a). Tissue levels of TNF-α were also significantly decreased in infliximab treatment group compared with Group 2. Pooled levels of TNF-α mRNA in colon tissues from 10 non-tumorigenic mucosa nearby tumor were similar between Group 1 and Group 2, but the mean expression of TNF-α in non-tumorous mucosa of Group 3 was significantly decreased compared to Group 2 (p<0.01) (Fig. 2b). Since mast cells are known to be one of major source for producing TNF-α, we counted the numbers of mast cell according to group with tryptase antibody staining. Mast cells were predominantly found in the tumors arising from chronic colitis, whereas the mean number of mast cell was significantly decreased in Group 3 (p<0.001, Fig. 2c). These results suggested that attenuated tumorigenic outcomes of Group 3 were based on efficient anti-inflammatory actions of infliximab presenting with attenuated levels of TNF-α far beyond blocking of TNF-α. Next, we performed multiplex RNase protection assay (RPA) using RNA extracted from pooled samples of mucosal homogenates in
order to know whether anti-tumorigenic effects of infliximab were related with suppressing other kinds of cytokines and chemokines beyond TNF-α blocking. As shown in Fig. 2d, migration inhibitory factor (MIF), IL-12p40, and IL-1β, cytokines reported to be principally involved in initiation or progress of ulcerative colitis, were significantly attenuated in pooled data of Group 3 compared to Group 2. In a similar way, the expression of macrophage inflammatory protein (MIP)-1β, MIP-1α, MIP-2 and T-cell activation gene (TCA)-3 mRNA was increased after repeated DSS administration, but top-down strategy of infliximab significantly decreased these expressions of RANTES, MIP-1α, MIP-2, IP-10, and TCA-3 mRNA.

**Increased canvas of apoptosis in colitic cancer tissues treated with with infliximab.** Attenuated or abolished apoptotic footprint in tumor section is general finding observed in colitic cancer model as well as sporadic colon cancer because deranged apoptotic activities might be fundamentally prerequisite mechanisms for tumorigenesis as for opportunity of tumor expansion (15). Using TUNEL staining, we evaluated the apoptotic activity of colitic tumor tissues and neighbor non-tumorous tissues according to group. As result, attenuated apoptotic activities were also noted in tumor tissues of colitic cancer as seen in Fig. 3a, whereas the increased apoptotic activities were detected even in tumor tissues of Group 3, signifying that infliximab might afford considerable levels of apoptotic activities in tumor tissue. Collectively, apoptotic index (AI) was significantly increased in Group 3 compared to either Group 1 or Group 2 (p<0.05). If we analyzed AI only in tumor tissue, the significance might be higher, but since only three tumors were found in Group 3, we presented mean AI countered in whole colon comprising of tumor and tumor neighbor tissue together in Fig. 3b. RNase protection assay showed caspase-8, FAS, FADD (FAS associated protein with death domain), TNFRp55, and TRAIL (TNF related apoptosis-inducing ligand), TRADD (TNFR1 adaptor proteins), and RIP (Receptor interacting protein) were increasingly expressed in Group 3 compared to either Group 1 or Group 3 (Fig. 3c), suggesting that apoptosis induction of infliximab could be one of anti-tumorigenic mechanisms of top-down strategy of infliximab.

**Inhibition of MMPs and attenuated formation of BCAC (β-catenin accumulated crypt) in colitic cancer tissue treated with with infliximab.** Since MMPs are known to play core function in either inflammation propagation or carcinogenesis (16), we checked the expression and activity of MMPs according to group. Increased expressions of MMP-3, MMP-9 and MMP-13 were noted in Group 2 compared to Group 1 (Fig. 4a), whereas significant reduction in increased MMPs activities of group 2 was noted in Group 3 documented by
zymography (Fig. 4b). Taken together, anti-tumorigenic action of infliximab treatment was associated with attenuated expression and activity of MMP-2, MMP-9, and MMP-11. The expression β-catenin was further evaluated by immunohistochemical staining. As shown in Fig. 4c, β-catenin was stained mostly in membrane of colonocytes in non-transformed mucosa whereas the β-catenin was more definitely stained in nucleus in accordance with increased expressions in cytoplasm and membrane in transformed glands. Definitely different expression patterns of β-catenin were noted in non-tumorous mucosa and cancerous mucosa (left and right figure of Fig. 4c). Important finding regarding β-catenin in colitic cancer came from Fig. 4d, so called, the changes of BCAC. Previously the numbers of ACF (aberrant crypt foci) were counted with 5 to 10 times magnified scopy after the staining with methylene blue in order to reflect the potentiality of premalignant lesion. However, BCAC has been acknowledged as more specific marker for potential cancerous risk than ACF because of some debates about the real significance of ACF. When we counted the mean numbers of BCAC in x100 magnified filed of microscopy according to group, statistically significant increases in mean BCAC numbers were noted in Group 2. On the other hand, the mean numbers of BCAC were significantly decreased in Group 3 compared to Group 2 (p<0.01, Fig. 4d), elucidating that anti-tumorigenic activities of infliximab might be based on their anti-mutagenic actions. Taken together of all documented results, infliximab imposed significant levels of anti-inflammatory actions in addition to apoptosis execution and hindrance of β-catenin accumulation, by which results significant attenuation in colitic tumor formations were achieved.

**In vitro validation of anti-inflammatory action of infliximab.** In order to validate the findings noted from animal model as shown in Fig. 2, in vitro assay was done in macrophage and T cells. The expression of TNF-α was significantly increased after stimulation by *E. coli* LPS in these cells, but the time for peak TNF-α induction was differed according to target cell, 4 hr in *Raw* 264.7 macrophage cells and 16 hr in *Jurkat* T cells. The reasons why we stimulated the cells with LPS rather than DSS as relevance of animal study were that DSS provoked COX-2 expression only in challenged cells and was toxic to the cells (see supplementary fig. 1). Instead, colon epithelial cells were not good target to LPS challenge (see supplementary fig. 2). TNF-α expressions were significantly decreased after infliximab treatment in *Raw* 264.7 macrophage cells and *Jurkat* T cell (Fig. 5a). Further investigations whether infliximab could exert change inflammatory mediators including IL-8, iNOS and COX-2 in *Raw*264.7 and *Jurkat* T cell lines were performed. 4 hrs after LPS challenge in *Raw* 264.7 cells, strong induction of inflammatory mediators such as TNF-α, IL-8, iNOS and COX-2 was observed.
but infliximab treatment attenuated TNF-α, iNOS and COX-2 except IL-8, of which results were further documented with real-time PCR. Similar attenuated expressions of IL-8, iNOS, COX-2 in addition to TNF-α were also observed in Jurkat T cells stimulated with 16 hr LPS in the presence of infliximab. We extended the measurements of these inflammatory mediators after different dosings of infliximab (Fig. 5b). Infliximab showed a dose dependent attenuation of IL-8, COX-2, and iNOS expression in addition to TNF-α. Real-time PCR clearly showed that infliximab in the presence of LPS challenge exerted decreases of COX-2, iNOS, and TNF-α expression in Raw cells and IL-8, iNOS, and TNF-α expression in Jurkat T cells in a dose-dependent manner.

**In vitro validation of apoptotic action of infliximab in tumors.** Since mucosal immune derangement such as the resistance of lamina propria T cells to apoptosis plays key role in persistence and perpetuation of inflammatory response in IBD, we hypothesized that one of anti-tumorigenic mechanisms of infliximab might be induction of apoptosis of engaging T cells or macrophages in IBD. As shown in Fig. 5c, increased expression of apoptosis executors, including caspase-3, a key effector caspase that degrades cellular proteins, FAS, TRADD (TNFR1 adaptor proteins), PARP cleavage was noted in Raw macrophage cells, signifying that both extrinsic and intrinsic apoptotic pathway was activated with infliximab, whereas cytochrome c release, that is, intrinsic apoptosis pathway associated with cytochrome c release from mitochondria, was noted to be main mechanism of infliximab on Jurkat T cells.

**DISCUSSION**

The main goal of IBD treatment is the induction and maintenance of disease remission. While the standard medical approach is guided by tracing clinical improvement with concurrent therapy (‘step-up’ approach), a new evolving concept for IBD treatment adopting earlier and aggressive intervention with anti-TNF therapy and immunosuppressive agents (‘top-down’ approach) is raising. Usually infliximab is reserved for CD patients refractory to conventional therapies or complicated as fistulous or stenotic diseases, but randomized controlled trial demonstrated that “top down” approach seems to be superior to conventional “step up” strategy in inducing higher remission rates and avoiding corticosteroid side effects (8), raising the advocacy that infliximab can be preferentially used for the aggressive induction of remission in order to, finally, retard the progression of chronic inflammation to dysplasia or carcinoma (17). In this point, current study might be the first to document the
rationale that “top-down” strategy should be preferred to high risk patients suffered from either pancolitis or longer duration of illness in wax and wane tendency to achieve the ultimate prevention of colitis-associated cancer, emphasizing that “top-down” strategy should be considered than “step up” approach in order to block “inflammation-dysplasia-carcinoma” sequence (Fig. 6).

In normal condition, TNF-α plays important roles in the development of the immune system and host protection against infectious pathogens, after which TNF-α has been called “a sentinel cytokine”. However, the role of TNF-α is quite different in pathologic condition, the generation of either inflammation or carcinogenesis in addition to dysregulated immune responses, leaving double-edged sword action of TNF-α and associated NF-κB (18-21). Mast cells are an important source of TNF-α, by which mast cells are involved in epithelial carcinogenesis (22-23). Mucosal immune derangement, which is characterized by the resistance of lamina propria T cells to apoptosis, decreased production of anti-inflammatory cytokine like IL-10 or TGF-β, and increased production of TNF-α by local macrophages or monocytes, are reported to be the major contributors to the development of IBD (24-25). Even though a lot of the molecular alterations that are responsible for sporadic CRC also play a role in colitis-associated tumorigenesis, but the final consequence of molecular alterations makes clear the difference between sporadic CRC and colitis-associated carcinogenesis (see supplementary fig. 3). For instance, the loss of APC (adenomatous polyposis coli) gene, which is a common early event in sporadic CRC, is less frequent and usually occurs very late in the colitis-associated dysplasia-carcinoma sequence. As another important alteration, p53 mutations occur late in sporadic colon carcinogenesis, whereas p53 mutations occur early and frequently detected in mucosa before the appearance of dysplasia or indefinite dysplasia in colitis patients. In contrast to sporadic CRC where dysplastic lesions or adenomatous polyps arise as one or two focal lesions in the colon, the premalignant changes have a tendency to be multi-focal in colitis-associated tumorigenesis (26-31). All of these differences are due to that colitis-associated cancer arises in the oncogenic setting based on chronic inflammation. Clinically, colitis-associated CRC affects younger individuals than sporadic CRC and commonly have mucinous or signet ring cell pathology. All of these characteristics were identified in our colitis-associated carcinogenesis.

Chronic inflammation plays a critical role in GI carcinogenesis. As examples, H. pylori-induced gastritis, hepatitis due to several etiologies including virus, toxin, alcohol, liver flukes induced cholangitis, Barrett’s metaplasia, and inflammatory bowel disease are well known inflammatory conditions or triggers that predispose...
to cancer development in gastroenterology (32). Chronic inflammation after *H. pylori* infection induced repopulation of the stomach with bone marrow-derived cells and these cells subsequently progress to cancer through metaplasia and dysplasia, which model provided the first molecular evidence how chronic inflammation exerted its role on tumor initiation (33). Another mechanism that is responsible for tumor initiation triggered by chronic inflammation is the excessive generation of free radicals and consequent DNA damages. Free radicals can induce a lot of alterations, including gene mutations and post-transcriptional modifications of cancer related genes, such as p53. Activated neutrophils and macrophages are responsible for ROS or RNS generation (34). A variety of pro-inflammatory cytokines and chemokines, such as TNF, IL-1, IL-6, IL-17, IL-12 and IL-8, are associated with link between chronic inflammation and carcinogenesis. Recent studies demonstrated that IKK-β [inhibitor of NF-κB (IκB) kinase-β] driven NF-κB activation pathway plays a pivotal role in tumor promotion and tumor progression (35). Activation of the classic IKK-β/NF-κB pathway causes increased transcription of genes, including mediators of inflammation (COX-2, iNOS, TNF and IL-6), proteases, and inhibitors of apoptosis (BCL-X<sub>L</sub>, cIAPs, GADD45β, BFL1 and SOD2) (36). We confirmed the engagement of these molecules in colitis-associated carcinogenesis and the cancer preventing mechanism of infliximab might be through either ameliorating inflammatory mediators or inducing apoptosis. Since a lot of proinflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 are secreted by activated monocyte or macrophages and these inflammatory cytokines driven responses are significant pathogenic factors in IBD, Infliximab exerted its anti-inflammatory effects by down-regulation of proinflammatory cytokines via binding to transmembrane TNF receptor, a process known as reverse signaling (37-38).

In addition to cytokines, several growth and angiogenic factors as well as matrix degrading proteases, such as MMP-2, MMP-3 and MMP-9, are generally involved in tumorigenesis, invasion and metastasis. MMPs can be released in response to pro-inflammatory cytokines such as TNF-α and IL-1β and play an important role in the process of tissue remodeling and destruction, especially MMP-9 is the most abundantly expressed protease in inflamed tissues of colon. Also, MMPs, including MMP-2 and MMP-9, are expressed in human and animal cancers (16, 39). Increased levels of COX-2 mRNA and proteins are found in premalignant lesions as well as malignant tissues (40). Regarding colorectal cancer, increased expression of COX-2 was reported in 85% and 50% of human colorectal cancers and adenomas, respectively. Infliximab imposed significant inhibition of these tumorigenic actions on colitic cancer. ACF are now frequently used as effective surrogate biomarkers colorectal
cancers, but the preneoplastic or precancerous nature of ACF in rodents and humans still remains inconclusive (41). Instead of these obscuring implications of ACF, early appearing BCAC have been described in en face preparations of colonic mucosa and are suggested to be premalignant in much higher fidelity than ACF. Histological observation showed that BCAC exhibit cellular dysplasia, higher cellular proliferation, and more likely to progress to malignant transformation, by which BCAC are acknowledged as an intermediate biomarker for colon carcinogenesis (42). Therefore, significant decreases in BCAC with infliximab as shown in Fig. 4d might be the significant evidence supporting cancer preventing action of infliximab.

5-Aminosalicylic acid (5-ASA), a first-line agent for the remission induction and maintenance of mild to moderate ulcerative colitis, has been identified as a candidate chemopreventive agent. Though some meta-analyses showed that 5-ASA use can lower the risk of CRC, there remain other reports showing no protective effect against colitis-associated CRC performed in a rather large case-control study (43-44). Ikeda et al (45) revealed that 5-ASA given in the remission phase of colitis significantly suppressed the development of colitis-associated cancer in a mouse model, which indicates the clinical importance of adopting chemopreventive strategies even in patients of remission state. However, the fact that we couldn’t observe any benefit of group treated with late intervention of infliximab and 5-ASA can impose hindrance to proliferation and mutagenesis, but not so higher levels of achievement lead to conclusion that the potency seems to be enriched to achieve higher goal of colitic cancer prevention. In conclusion, even though the more clear applicability of “top-down” approach for chemopreventive purpose should be decided with well-designed prospective clinical trials, our results drawn from animal study offered the perspective of colitic cancer prevention with “top-down” strategy in high risk IBD patients presenting with extensive and easily recurred clinical course.

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Figure Legends

Fig. 1. Repeated colitis-induced tumorigenesis as animal model for colitic cancer. (a & c) Overview of experimental protocol of repeated DSS-induced colitic cancer model in mice (a shows top-down strategy and c shows step-up strategy). (a) Control mice were fed a normal diet and given ordinary sterilized water (Group 1). Colitis was induced by 15 cycles (totally 255 days) of 0.7% of DSS in drinking waters, with each cycle consisting of 7 days of 0.7% DSS, followed by 10 days of ordinary sterilized water (Group 2). The Group 3 administered with infliximab at 1st, 3rd, and 7th weeks on the protocol same as Group 2. The gross and microscopic picture of colitic cancers. Colon tumor was observed in 9/12 of mice of Group 2 on the gross inspection (Left), of which pathology of each representational tumor in each mice developed in Group 2 was shown (Right, x100 magnification). (b) The incidence of colon tumor in colitic cancer model according to group. Statistically significantly decreases in tumor incidence were noted in group with early intervention of infliximab (Group 3). (c) Overview of experimental protocol of repeated DSS-induced colitic cancer model in mice (infl iximab administered based on “step up” strategy). Control mice were fed a normal diet and given ordinary sterilized water (Group 1). Group 4; colitis was induced by 15 cycles (totally 255 days) of 0.7% of DSS in the drinking water, with each cycle consisting of 7 days of 0.7% DSS, followed by 10 days of ordinary sterilized water as same with Group 2. However, Group 5 was
different with Group 3 in the intervention point of biologics that infliximab was administered at later stage of the 25th, 27th, and 31st weeks compared to the 1st, 3rd, and 7th weeks in Group 3. (d) The incidence of colon tumor in colitic cancer model according to group. There was no significant difference in tumor incidence between Group 4 and Group 5, suggesting that later intervention of infliximab did not was not effective in cancer prevention as noted in Group 3.

**Fig. 2.** Changes of TNF-α, cytokine essentially engaged in colitic cancer according to group (a) Repetitive bouts of colitis (Group 2) was significantly associated with elevated serum levels of TNF-α and tissue levels of TNF-α, and TNF-α mRNA expressions of non-tumorous colon tissue (b). These elevated expression and levels of TNF-α in Group 2 was significantly attenuated in Group 3. (c) Mast cells identified with tryptase immunostaining. The mean numbers of tryptase positive mast cells were significantly elevated in colon tumor of Group 2, whereas the numbers of mast cells were statistically significantly decreased in Group 3, suggesting that infliximab was very effective in inactivating mast cells, one of main sources of TNF-α. Arrows indicate the mast cells showing positive staining with tryptase antibody. (d) Multi-probe RPA system for inflammatory cytokines [mCK-2 (Cat. #45002P) and mCK-5 (Cat. #45026P), respectively, PharMingen, San Diego, CA]. Compared to Group 2, the expression levels of IL-12, IL-1β, MIF, MIP-1, and IL-10 were considerably decreased in Group 3.

**Fig. 3.** Apoptosis index according to group. (a) TUNEL findings in tumors of Group 2 and Group 3. The TUNEL positive cells were scantly detected in tumor tissues of Group 2, whereas TUNEL positive cells were apparently increased in tumor tissues in addition to nearby non-tumor tissues in Group 3. (b) Apoptotic index (AI) according to group. Apoptotic index was significantly increased in Group 3 compared to Group 1 or Group 2. ($p<0.001$), suggesting that infliximab induced apoptosis in the tumor tissues. Apoptotic index was calculated as the mean number of positive cells under x100 magnified filed of microscopic examination. (c) Multi-probe RNase protection assay system for apoptosis [mAPO-3 (Cat. #45355P)]. The most executive molecules engaged in apoptosis were increased in Group 3 compared to Group 2.
Fig. 4. MMPs and BCAC according to group (a-b) Inhibitory actions of infliximab on MMP-2, MMP-9, and MT1-MMP (a) mRNA expression of each MMPs according to group (pooled samples) measured with RT-PCR. MMP-3, MMP-9 and MMP-13 were apparently increased in Group 2 compare to Group 1. However, the expression and activity of MMP-9 and MMP-11 were significantly decreased in Group 3 compared to Group 2 (p<0.05). (b) Zymography for MMP activities Gelatinolytic activity was measured by zymography using colon homogenates. Genolytic activity of MMP-2, MMP-9, and MMP-11 was significantly decreased in Group 3 compared to Group 2. Since the molecular size of MMP-3, MMP-10, and MMP-20 was around 45 kDa, it was not discerned with zymography experiment. (c) β-catenin accumulated crypt (BCAC) is generally regarded as better biomarker for malignant transformation than ACF (aberrant crypt foci), which is stained strong with β-catenin in membrane and nucleus. (d) The mean numbers of BCAC were significantly decreased in Group 3 than Group 2 (p<0.01), suggesting that infliximab was quite effective in suppressing BCAC formation.

Fig. 5. In vitro documentations showing anti-inflammatory and apoptosis inducing actions of infliximab Using Raw 264.7 macrophage cells and Jurkat T cells, LPS was stimulated for 4hrs in case of Raw 264.7 cells and 16 hrs in case of Jurkat T cells based on preliminary study showing highest stimulation of inflammatory cytokines. (a) RT-PCR results for inflammatory cytokines including TNF-α, IL-8, iNOS, COX-2, and GAPDH and quantitation results with real-time PCR in Raw264.7 cells and Jurkat T cells. Infliximab (1μg) suppressed LPS-induced TNF-α, iNOS, and COX-2 expression in Jurkat T cells, whereas only suppressed TNF-α expressions was noted in Raw264.7 macrophage cells with infliximab treatment. (b) RT-PCR results for inflammatory cytokines including TNF-α, IL-8, iNOS, COX-2, and GAPDH and real-time PCR for quantitating mRNA. As the dose of infliximab is increased, these suppressive effects on LPS-stimulated inflammatory cytokines were more prominent in Jurkat T cells. These suppressive effects of infliximab were also prominently noted in Raw 264.7 cells, when the dose of infliximab was increased up to 10 μg. 1. control; 2. LPS 1 mg; 3. LPS 1 mg + infliximab 1μg (24 hrs); 4. LPS 1 mg + infliximab 5 μg (24 hrs); 5. LPS 1 mg + infliximab 10 μg (24 hrs). (c) Western blotting for apoptosis executors and their densitometric analyses. Cytochrome c release was main execution of apoptosis in Jurkat T cells treated with infliximab, whereas FAS, caspase-3, TRADD were significantly downregulated.
operated in Raw 264.7 cells after infliximab treatment.

Fig. 6. **Connection between chronic inflammation and carcinogenesis and their blockings through top-down strategy.** Repeated bouts of inflammation resulted in tumorigenesis without any intervention of carcinogen supporting the evidence of crosslink between inflammation and carcinogenesis. The early administration of infliximab (“top-down” strategy) was very effective in the prevention of colitic cancer, suggesting the early and appropriate intervention of efficient anti-inflammatory and anti-mutagenic strategy can lead to ultimate way of chemoprevention in longstanding inflammatory bowel disease. Infliximab, even though its primary indication is to induce strong anti-inflammatory action refractory to other modality, could impose significant levels of anti-mutagenic actions, apoptosis execution, attenuate TNF-α levels, and exert the hindrance of β-catenin accumulation in colitic cancer model far beyond the designed action of blocking TNF-α.
Group 1 Normal control

Group 2 Repeated cycles of colitis induction

Group 3 Repeated cycles of colitis induction + infliximab injection (↑ 1st, 3rd, 7th week iv, 4mg/kg)

0.7% DSS in sterilized drinking water for 7 days followed by sterilized tap water for 10 days

117 days/cycle

Sacrifice (37 weeks)
### Tumor incidence & multiplicity according to group

<table>
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<th>Groups</th>
<th>Treatments</th>
<th>Tumor Incidence (%)</th>
<th>Tumor multiplicity</th>
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<tr>
<td>Group 1</td>
<td>None</td>
<td>0/10 (0.0)</td>
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<tr>
<td>Group 2</td>
<td>Repeated DSS</td>
<td>9/12 (75.0)</td>
<td>1.57±0.55</td>
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<td>Group 3</td>
<td>Repeated DSS + Infliximab (1st, 3rd, 7th)</td>
<td>2/12 (16.7)*</td>
<td>1.00</td>
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* p<0.001 (vs Group 2)
Group 1: Normal control

Group 4: Repeated cycles of colitis induction (15 cycles)

Group 5: Repeated cycles of colitis induction + infliximab injection (25th, 27th, 31st week iv, 4mg/kg)

O.7% DSS in drinking water for 7 days followed by ordinary tap water for 10 days

17 days/cycle

Sacrifice (37 weeks)
<table>
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<th>Groups</th>
<th>Treatments</th>
<th>Tumor Incidence (%)</th>
<th>Tumor multiplicity ±</th>
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<tr>
<td>Group 1</td>
<td>None</td>
<td>0/10 (0.0)</td>
<td>-</td>
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<tr>
<td>Group 4</td>
<td>Repeated DSS (control to group 5)</td>
<td>8/10 (80.0)</td>
<td>1.85 ± 0.31</td>
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<td>Group 5</td>
<td>Repeated DSS + Infliximab (25&lt;sup&gt;th&lt;/sup&gt;, 27&lt;sup&gt;th&lt;/sup&gt;, 31&lt;sup&gt;th&lt;/sup&gt;)</td>
<td>7/10 (70.0)</td>
<td>1.60 ± 0.42</td>
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</table>
(b)

<table>
<thead>
<tr>
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<th>Group 2</th>
<th>Group 3</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
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</table>

**TNF-α**

**GAPDH**

* *p<0.05 (vs Group 2)*

![Graph showing TNF-α (non-tumorous mucosa)/GAPDH levels for different groups. Group 1 has the highest level, followed by Group 2, and then Group 3, with Group 3 showing a statistically significant difference compared to Group 2.]
Mean number of tryptase (+) mast cells (M±SD)

- Group 1
- Group 2
- Group 3

* p<0.001 (vs Group 2)
Kim YJ et al, Cancer Prevention Research Fig. 2
**Group 2**

**Group 3**

(b) Apoptotic index (M±SD)

* p<0.001 (vs Group 2)

---

Kim YJ et al, Cancer Prevention Research Fig. 3
Kim YJ et al, Cancer Prevention Research Fig. 3
Kim YJ et al, Cancer Prevention Research Fig. 4
1.2 * p<0.05 (vs Group 2)

0.8

D

H ratio

0.4

MMP-9/GAPDH ratio

0.0

0.2

0.4

0.6

0.8

1.0

1.2

0.0

0.2

0.4

0.6

0.8

1.0

1.2

MMP-11/GAPDH ratio

1

2

3

Group

* p<0.05 (vs Group 2)

1

2

3

Group

* p<0.05 (vs Group 2)
(b) Kim YJ et al, Cancer Prevention Research Fig. 4
Mean Number of BCAC (M±SD)

<table>
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<th>Group</th>
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<tr>
<td>Group 1</td>
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</tr>
<tr>
<td>Group 2</td>
<td>9.8 ± 2.3</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

* p<0.01 (vs Group 2)
Kim YJ et al, Cancer Prevention Research Fig. 5

(a) Raw264.7

Control
LPS (4hrs)
LPS (4hrs) + infliximab (24hrs)
Infliximab (24hrs)

GAPDH
COX-2
iNOS
IL-8
TNF-α
* p<0.05 (vs LPS 16hr)

** TNF-α mRNA

** IL-8 mRNA

** iNOS mRNA

** COX-2 mRNA
Kim YJ et al, Cancer Prevention Research Fig. 5
Kim YJ et al, Cancer Prevention Research Fig. 5
Kim YJ et al, Cancer Prevention Research Fig. 5
Kim YJ et al, Cancer Prevention Research Fig. 5
Fig. 5: Graphs showing the expression levels of different mRNAs (COX-2, TNF-α, iNOS, IL-8) across different samples (1 to 5). The graphs indicate a significant increase in expression levels with a *p<0.05 (vs #2) comparison. The y-axis represents the mRNA expression levels, and the x-axis represents different samples.

Kim YJ et al, Cancer Prevention Research
Cytochrome c
Caspase-3
Caspase-9
Fas
TRADD
PARP
GAPDH

Raw264.7

Jurkat T
Infliximab administered in later stage of colitis
“no cancer preventive outcome”

IBD
ulcerative colitis
Crohn’s disease

Mild, Moderate, Severe colitis

“Inflammation - dysplasia - carcinoma”

Colitis associated cancer

Infliximab administered in early stage of colitis
“cancer preventive outcome”
through
1) attenuated levels of TNF-α
2) induced apoptosis of inflammatory cells
3) decreased inflammatory mediators

Kim YJ et al, Cancer Prevention Research Fig. 6
Cancer Prevention Research

Prevention of colitis-associated carcinogenesis with infliximab
Yoon Jae Kim, Kyung Sook Hong, Jun Won Chung, et al.


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