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Oxidants and Antioxidants Synergistically Activate Immuno-Responses During Tumorgenesis

Kui Yuan[1#], Weilu Liu[2#], Wen Guo[3*], Lili Zhang[4], Yawei Liu[5], Ming Zhao[5*]

[1]*Department of Gastroenterology, Shenzhen Hospital, Southern Medical University*

[2]*Department of Orthopaedics, Nanfang Hospital, Southern Medical University*

[3]*Department of Gastroenterology, Nanfang Hospital, Southern Medical University*

[4]*School of Nursing, Southern Medical University*

[5]*Department of Pathophysiology, School of Basic Medical Sciences, Southern Medical University*

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***Corresponding author:** *Ming Zhao, Department of Pathophysiology, Southern Medical University, Guangzhou 510515, P.R. China, Tel: +862-061-648-393; Fax: +862-061-648-299; E-mail: 15602239057@163.com*

Wen Guo, Department of Gastroenterology, Nanfang Hospital, Guangzhou 510515, P.R. China, Tel: +862-061-641-543; Fax: +862-087-280-770; E-mail: gwdoc@163.com

#Authors contribute equally in this work

Abstract

The human immune system possesses powerful surveillance functions to find and eliminate tumor cells. Several steps are involved, such as uptake and presentation of tumor antigens by dendritic cells (DCs) in order to activate T cells, trafficking and infiltration of the tumor with T cells and, finally, recognition and killing of tumor cells. Tumor cells release a number of soluble mediators that may disrupt these steps, thereby creating tolerance or immune escape. We previously published data on a rat animal model showing 1, 2-dimethylhydrazine (DMH) to inhibit DCs activation and enhance T regulatory cells (Treg) expression, ultimately inducing colon carcinogenesis. By feeding rats with mustard seeds (MS), the animals' immune system shifted from a Th2 to a Th1 pattern, Tregs were inhibited and DCs activation enhanced. In the present study, we tried to isolate the functional fractions of MS and, to our surprise, we found that the oxidant and antioxidant fractions of MS showed synergistic effect on the activation of DCs and CD8+ effector T cells. Neither of the fractions alone, however, had any inhibitory effect on DMH-induced colon carcinogenesis. Differently from the antioxidant fraction, the oxidant fraction activated DCs ($CD11c^+CD80^+$, $CD11c^+CD86^+$), but not the CD8+ effector T cells. By adding Vitamin E, an acknowledged antioxidant, the oxidant fraction then became able to activate both spleenic DCs and effector T cells ($CD8^+CD28^+$). Taken together, these data suggest that the MS oxidant fraction might play an important role in activation of DCs, while also showing that oxidative signals may induce depletion of effector T cells. Addition of the antioxidant MS fraction, or Vitamin E, might restore the survival of effector T cells and eventually help eradicate tumor cells. Thus, MS oxidants, which normally are regarded as having negative effects, may actually function synergistically with antioxidants to prevent chemical-induced carcinogenesis.

Keywords: Oxidants; Antioxidants; Immuno-responses; Mustard seeds; Colorectal cancer; Chemoprevention; T cells; Dendritic cells

Abbreviations: AITC: Allyl Isothiocyanate; APCs: Antigen-Presenting Cells; CTLs: Cytotoxic T Lymphocytes; DCs: Dendritic Cells; DMH: 1, 2-Dimethylhydrazine; EGCG: Epigallocatechin Gallate; LPO: Lipid Peroxides ; MDA: Malondialdehyde; MHC: Major Histocompatibility Complex; MS: Mustard Seeds; MSE1: Ether Extract of Mustard Seeds; MSE2: Ethanolic Extract of Mustard Seeds; PBS: Phosphate-Buffered Saline; RBC: Red Blood Cell; ROS: Reactive Oxygen Species; TCRs: T Cell Receptors; Treg : T Regulatory Cells; VitE : Vitamin E

Introduction

The immune response against tumors is provided by a multitude of cells, although T cells are considered the major mediators of anti-tumor immunity [1-6]. The exact type of T cell response required for optimal anti-tumor immunity is not entirely clear, but a potent CD8+ effector T cell response is certainly necessary. Additionally, a response directed by either $CD4^+$ T helper 1 (Th1) cells or Th17 cells appears to promote the $CD8^+$ effector T cell responses [7-9]. T cell response is initiated by specific recognition of cognate peptides presented by major histocompatibility complex (MHC) proteins on antigen-presenting cells (APCs) through T cell receptors (TCRs), which are referred to as the first signal for T cell activation. However, the ultimate magnitude and quality of T cell response is determined by a balance between co-stimulatory and co-inhibitory signals (collectively called co-signals) that are transduced into T cells and is referred to as the second signal [10,11]. Following TCR engagement by cognate peptide-MHC complexes, co-signaling receptors are often mobilized and colocalized with TCRs, forming immunological synapses between APCs and T cells. This synaptic interface is the place where cross-talks between co-signaling ligands and receptors synergize or antagonize with TCR signaling, rendering T cells activated or inhibited [12], it is also the place where oxidant radicals are considered transported from APCs to T cells, which may influence the activation and survival of effector

 ***Corresponding author email:** 15602239057@163.com ; gwdoc@163.com*

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T cells. Holmdahl and his colleagues reported that a decreased capacity to produce ROS, due to a natural Ncf1 polymorphism, was found to be a major factor behind severe arthritis and increased T cell-dependent autoimmunity [13]. Peroxides are immunologic transmitters secreted by antigen-presenting cells that down-regulate the responses by autoreactive T cells. Ncf1 polymorphism results in the release of less ROS from APCs with ensuing activation of autoreactive $CD4^+$ T cells and resulting severe arthritis [14,15].

The B7 protein family is a group of surface glycoproteins that share structural features with immunoglobulins (Ig). Their extracellular domains bear homology to the IgV and IgC domains of Igs. A hallmark of the B7 family molecules is their capability to co-stimulate or co-inhibit T cell responses in the presence of peptide/MHC complex-mediated TCR signaling [12]. B7 family members primarily bind to members of the CD28 protein family, including CD28, CTLA-4, PD-1, ICOS and BTLA that transmit co-signals to T cells [16]. The B7 co-stimulatory ligands are important for full activation of naïve T cells in the lymphoid organs, in which APCs, particularly dendritic cells, are the primary cellular source for providing ligands, including B7.1 (CD80) and B7.2 (CD86). In contrast, B7 co-inhibitory ligands are crucial for the termination of over activated T cell responses, maintenance of self-tolerance, and protection of tissues from damage induced by invading pathogens. In addition, co-inhibitory ligands expressed in pathological compartments, such as tumor microenvironments, actively inhibit the effector functions of cytotoxic T lymphocytes (CTLs) and the generation of regulatory T cells [10,11]; thus, playing the role of important immune checkpoint proteins that are involved in the immune resistance to cancer cells.

In this paper, in order to better understand the biochemistry behind the MS activity, we tried to check for crudely purified fractions of MS, using ether- and ethanol-soluble fractions of mustard seeds, on activation of DCs, CD80 and CD86 expression, and their positive activation ligand CD28 on $CD8^+$ T cells.

MS has been used as a spice and herbal medicine since the Tang dynasty (618-907) [17]. Glucosinolates and their breakdown products, such as allyl isothiocyanate (AITC), sinigrin and sinapine are major components of MS and possibly responsible for inducing a reduced risk of cancer [18-21]. A previous study of ours indicated that MS reduced the incidence of DMH-induced colorectal cancer [22]. The proportion of spleenic B- and dendritic cells was amplified in the MS group. The expression of MHCI, as well as that of MHCII, was increased in dendritic cells and the percentages of $CD8^+CD28^+$ and $CD4^+CD28^+$ cells were increased in the MS group, while the $CD4^+CD25^+Foxp3^+$ subset was depressed. Plasma analyses showed that DMH-exposure induced amplified amounts of IL-4, IL-5, IL-10 and TGF-beta, while MS feeding counteracted this effect but enhanced IL-2, IL12p70, IL21, TNF-alpha, and IFN-gamma. On the SW480 colon adenocarcinoma cell-line, the cytotoxicity of spleenic T-cells from MS-fed animals was significantly increased. In DMH-exposed rats, the expression of perforin in the spleenic T-cells was dramatically decreased, while MS abolished this depression. These data indicated that dietary MS suppresses DMH-induced immuno-imbalance as well as colon carcinogenesis in rats. When we explored the activities of ether- and ethanol-extracted MS fractions with respect to how they activate DCs and effector T cells, and eventually execute tumor cells, we found that an ether-extracted MS fraction is an oxidant, rather than an antioxidant and activates DCs, although not T cells. The ethanol-extracted MS fraction, possessing anti-oxidant activity, did not activate DCs but might enhanced the survival of T cells that were activated by DCs when the latter present antigens to T cells, and also supply free radicals that might hurt T cells. Thus, both oxidant and antioxidant fractions of MS appear to play synergistic roles in activating the immune system against tumorgenesis.

Materials and Methods

Animals and Diets

Four weeks old male Kunming mice with an average body weight of 20 g were obtained from the Southern Medical University animal center. All animals were cared for according to the Institutional Animal Care Guidelines. The Committee for Animal Care and Use of the Southern Medical University, Guangzhou, China, approved the study. The mice were maintained under controlled conditions of temperature (24 ± 2 °C), humidity (50 ± 10%), 12 h light/12 h dark cycles and were acclimated to their new diet and environment for 2 weeks before experiments. Each cage housed three mice. Tap water was provided ad libitum. Animals were fed standard chow or standard chow containing 7.5% crushed fresh MS [21].

MS Extraction

MS fractions were prepared as previously reported [23]. Briefly, MS was first extracted in ether and then in ethanol. 300 g mashed MS were extracted in 1000 ml ether and the soluble fraction collected, filtrated, air dried by evaporation at room temperature (25 °C) and named MSE1, which was an oily material. The solid MS remains were then again extracted, but now in 1000 ml ethanol at 25 °C. The substances dissolved in ethanol were then precipitated at 4 °C over night; the sediment collected by centrifugation and named MSE2. The MSE2 was a solid substance that was dissolved in PBS to 1.5% at room temperature.

Experiment I: Influence of mustard seeds and its fractions on DMH-induced colorectal cancer in mice

Experimental design: The mice were randomly divided into five groups:

Group I (n = 15): Mice were fed standard chow diet and received i.p. injections of normal saline (0.2 ml) once a week for 20 weeks. This group served as an untreated control group.

Group II (n = 15): Mice were fed standard chow diet and received i.p. injections of DMH (Sigma-Aldrich) at a dose of 20 mg/kg once weekly for 20 weeks.

Group III (n = 15): Mice were fed chow diet containing 7.5% MS.

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They received injections with the same dose of DMH as in group II once a week for 20 weeks.

Group IV (n = 15): Mice were fed standard chow diet and received i.p. injections of 50 µl MSE1 once a week, as well as the same DMH injections as the group II animals for altogether 20 weeks.

Group V (n = 15): Mice were fed standard chow diet and received i.p. injections of 100 µl MSE2, which was prepared at a concentration of 1.5% in PBS, once a week for 20 weeks as well as the same DMH injections as group II.

Mice were sacrificed by ether inhalation 12 weeks after their last injection of DMH or saline. Serum was collected for analysis of malondialdehyde (MDA) and Th1/Th2 cytokines. Spleens were removed and assayed for proportions of lymphocytes. Colons were removed and cut open longitudinally for further microscopical examination of selected areas.

Experiment II: Influence of MSE1 on dentritic cells in mice with DMH-induced colon tumors

Experimental design: The mice were randomly divided into groups as in Experiment I. The animals were sacrificed after 6 weeks of injections with DMH or saline. Spleens were removed, prepared and assayed for dendritic cells.

Experiment III: Influence of MSE1 with added VitE (vitamin E) on dentritic cells and CD8+ T cells in the spleens of DMH-exposed mice

Experimental design: The mice were randomly divided into the following groups:

Group I (n = 15): Mice were fed standard chow diet and received i.p. injections of normal saline (0.2 ml) once a week for 6 weeks.

Group II (n = 15): Mice were fed standard chow diet and received i.p. injections of DMH at a dose of 20 mg/kg once a week for 6 weeks.

Group III (n = 15): Mice were fed standard chow diet and received i.p. injections of 50 µl MSE1 and 20 mg/kg DMH once a week for 6 weeks.

Group IV (n = 15): Mice were fed standard chow diet and received 50 µl MSE1, 100 mg/kg VitE and 20 mg/kg DMH once a week for 6 weeks.

Mice were sacrificed after 6 weeks and their spleens were removed for analyzes of dentritic and CD8+ T cells.

Experiment IV: Influence of different combination of mustard seeds extraction on DMH-induced colorectal cancer in mice

Experimental design: The mice were randomly divided into the following groups:

Group I (n = 9): Mice were fed standard chow diet and received i.p. injections of phosphate buffer saline (0.2 ml) once a week for 20 weeks. This group served as an untreated control group.

Group II (n = 9): Mice were fed standard chow diet and received i.p. injections of DMH (Sigma-Aldrich) at a dose of 20 mg/kg once weekly for 20 weeks.

Group III (n = 9): Mice were fed standard chow diet and received i.p. injections of 50 µl MSE1, 100 µl MSE2 once a week, as well as the same DMH injections as the group II animals for altogether 20 weeks.

Group IV (n = 9): Mice were fed standard chow diet and received i.p. injections of 50 µl MSE1, 100 mg/kg VitE once a week, as well as the same DMH injections as the group II animals for altogether 20 weeks.

Mice were sacrificed by continued given other medicine for 12 weeks after their last injection of DMH or saline. Colons were removed and then cut open longitudinally for further microscopical examination of selected areas. The area of tumor on the surface of colons were measured to assess the situation of colorectal cancer.

Tumor Assay

The colon tumors that resulted in Experiment I were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Sections (4-µm-thick) were cut and stained with hematoxylin and eosin for histological examination. Sections of each tumor were examined under a light microscope (Olympus, Japan) by a pathologist blinded to the experimental design. If tumor cells invaded the submucosa, the tumor was diagnosed as an adenocarcinoma. When tumor cells did not invade the submucosa, the tumors were diagnosed as adenomas (low- or high-grade intraepithelial neoplasias).

Preparation of Mice Splenocytes for Flow Cytometry

Single cell suspensions of mice splenocytes were prepared by grinding one half of each spleen with the plunger of a disposable syringe. The homogenate was then passed through a 100-mesh screen. Erythrocytes were removed by suspending cells in red blood cell (RBC) lysis buffer (155 µM NH_4Cl, 10 µM $KHCO_3$ and 100 µM EDTA) for 5 min followed by three washes in phosphate-buffered saline (PBS) and resuspending of the remaining splenocytes at 1×107 cells/ml. Experiment I splenocytes were first surface-labeled with anti-mouse CD4-FITC, CD8-PE/Cy5, CD25-PE and CD28-PE/Cy7 (Miltenyi Biotec) for 15 min in a dark refrigerator (4°C). After fixation and permeabilization with a FoxP3 staining buffer set, cells were washed and intracellularly stained with APC-labeled anti-mouse Foxp3 for 30 min in the dark at 4°C according to the instruction offered by the manufacturer. Experiment II and III splenocytes were labeled with anti-mouse CD11c-APC, CD80-FITC, CD86-FITC, CD8-PerCP and CD28-PE/Cy7 for 15 min in a dark refrigerator (4°C) before ensuing flow cytometry. Cells were run on a Becton Dickinson FACSVerse and data analyzed using the CellQuest software.

Assay for Malondialdehyde (MDA)

MDA is a degradation product of lipid peroxides (LPO). Blood samples from Experiment I were collected from the hearts and

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centrifuged for 10 min at 3000 × g The obtained serum was assayed for possible thiobarbituric acid-reactive substances using a colorimetric Diagnostic Reagent Kit (Nanjing Jiancheng Bioengineering, Nanjing city, P.R. China) according to the manufacturer's instructions.

Assay for Cytokines

Plasma cytokines (IFN-γ, IL-12, IL-4, IL-5, IL-6, IL-10 and IL-17) from the different groups of mice of Experiment I were analyzed using the Milliplex map kit (Millipore Corporation, USA). The assay was performed according to the manufacturer's protocol. Briefly, plasma was diluted 1:5 with PBS to maximize sensitivity at baseline levels. The diluted plasma (25 µl) was incubated with an antibody-immobilized mix of beads for 16 h at 4 °C. The beads were then washed twice and incubated with detection antibodies for 1 h at room temperature. Streptavidin–Phycoerythrin was then added and the mixture was incubated for an additional 30 min period at room temperature. The beads were again washed twice and finally analyzed on a Luminex-100 cytometer (Millipore Corporation, USA) using Start Station software (Version 2.0; Applied Cytometry Systems, Dinnington, UK). Cytokine concentrations were calculated from standard curves. The minimum significant value of the assay was 3.2 pg/ml.

Results

MSE1 and MSE2 do not inhibit DMH-induced formation of colon tumors, while MS does (Experiment I)

Table 1 depicts the effects of the two MS fractions and MS itself on colonic tumor formation in DMH-exposed mice. All DMH exposed mice developed tumors (100%) including adenocarcinomas (86.67%) and adenomas (13.33%). MS significantly inhibited tumor formation. By 40% it decreased the total tumor incidence and by nearly 50% the adenocarcinoma incidence, whereas neither MSE1 nor MSE2 had any significant effect on tumor incidences. Sample images of colon tumors are shown in figure 1. The macroscopic appearance of several solitary (upper illustration) and multiple (lower illustration) colonic tumors is shown in figure 1A, whereas figure 1B shows microscopic views of colon section stained with hematoxylin and eosin.

MS, but not MSE1 and MSE2, decreased $CD4^+CD25^+Foxp3^+$ Treg cells. (Experiment I)

The immunologic action of MS and its two extracts in preventing colon carcinogenesis was investigated by analyzing the percentages of spleen $CD4^+CD25^+Foxp3^+$ Treg cells (Figure 2). The DMH alone group showed increased numbers of Treg cells compared to the saline group (from 0.12% to 0.26%), indicating that tumor-infiltrating Treg cells are detrimental to the tumor-bearing host. Treg cells of mice exposed to MS + DMH decreased to 0.16%, which is close to that of the saline group. Both MS fractions + DMH resulted in similar values for Treg cells as were found for the DMH alone group. Obviously, they had no effect.

MSE1 and MSE2 do not activate $CD8^+$ T cells, while MS does (Experiment I)

$CD8^+$ T cells functionally play a role as cytotoxic T lymphocytes (CTLs). CD28 molecules are highly expressed on the surface of naive T cells and initiate T cell activation. Mice in the DMH group had a 100% tumor incidence with a low ratio of CD8+ T cells (5.10%) and $CD8^+CD28^+$ cells (3.38%) as compared to the control (17.64% and 12.66%, respectively) and the MS group (11.23% and 7.31%, respectively). Mice exposed to DMH and simultaneously fed diets with MS had a lower total tumor incidence and an increased ratio of $CD8^+$ T (11.23%) and $CD8^+CD28^+$ cells (7.31%), indicating that MS played a role in preventing carcinogenesis by increasing CTLs. Neither of the MS fractions caused any significant increase of $CD8^+$ T or $CD8^+CD28^+$, indicating that they alone do not activate effector T cells (Figure 3).

MSE1 induces a Th1 reaction, while MSE2 induces type Th2 and Th17 reactions (Experiment I)

Our previous studies showed that DMH induced a Th2 pattern of immune responses while MS reversed it to a Th1 pattern. We next tried to explore the effects of the two MS fractions on formation of Th1, Th2 and Th17 type cytokines. As shown in figure 5, MSE1 significantly enhanced the Th1-type cytokines, including IFN-γ and IL-12, while MSE2 significantly induced Th2 and Th17 reactions, including formation of IL-4, IL-5, IL-6, IL-10 and IL-17, compared to DMH (Figure 4).

MSE1 is an oxidant, while MSE2 is an antioxidant (Experiment I)

The biochemical difference between MSE1 and MSE2 became better understood when we investigated their redox capability. By checking lipid peroxidation products (Figure 5), we found MSE1 to increase the level of MDA as did also DMH, while MSE2 had the opposite activity. MS behaved as MSE2, indicating that they both act mainly as antioxidants.

MSE1, but not MSE2, activate dendritic cells (Experiment II)

Dendritic cells (DCs) are sensors of immune responses; their main function is to process antigen and present it to T cells. CD11c is the most widely used specific marker of mouse DCs. Cell-surface receptors, such as CD80 and CD86 act as co-stimulating-ligands in T-cell activation and inducers of primary T cell responses. Figure 6 shows that MSE1 significantly enhances the DCs population and activates DCs (CD11c+CD80+ and CD11c+CD86+), while MSE2 does not.

MSE1 $^+$ VitE enhances the dendritic cell population and the activation of $CD8^+$ T cells in spleens (Experiment III)

Since experiment I showed that MSE1 acts as an oxidant, while MSE2 performs as an antioxidant and since experiment II showed that MSE1 activates dendritic but not $CD8^+$ T cells (Figures 3 and 6). We proposed that adding an antioxidant together with MSE1 might help dendritic cells to survive and activate CD8+ effector T cells. Figure 7 shows that MSE1 + VitE enhanced the numbers of dendritic and activated CD8+ T cells.

Both MSE1 + MSE2, or MSE1 + VitE decrease DMH-induced colon

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Table 1: Effects of MS and itsfractionson colonic tumor formation DMH-induced mice[a]

Groups	Adenoma incidence	Adenocarcinoma incidence	Total, tumor incidence
Saline	—	—	—
DMH	2/15 (13.33%)	13/15 (86.67%)	15/15 (100%)
DMH + 7.5%MS	2/15 (13.33%)	7/15 (46.67%) [b]	9/15 (60%) [b]
DMH + MSE1	3/15 (20%)	10/15 (66.67%)	13/15 (86.67%)
DMH + MSE2	1/15 (6.67%)	11/15 (73.33%)	12/15 80%)

Note: [a]Analyses of incidence were performed using the χ^2 test, n=15 colons/group.
[b]Value is significantly different from the DMH group, $p<0.05$.

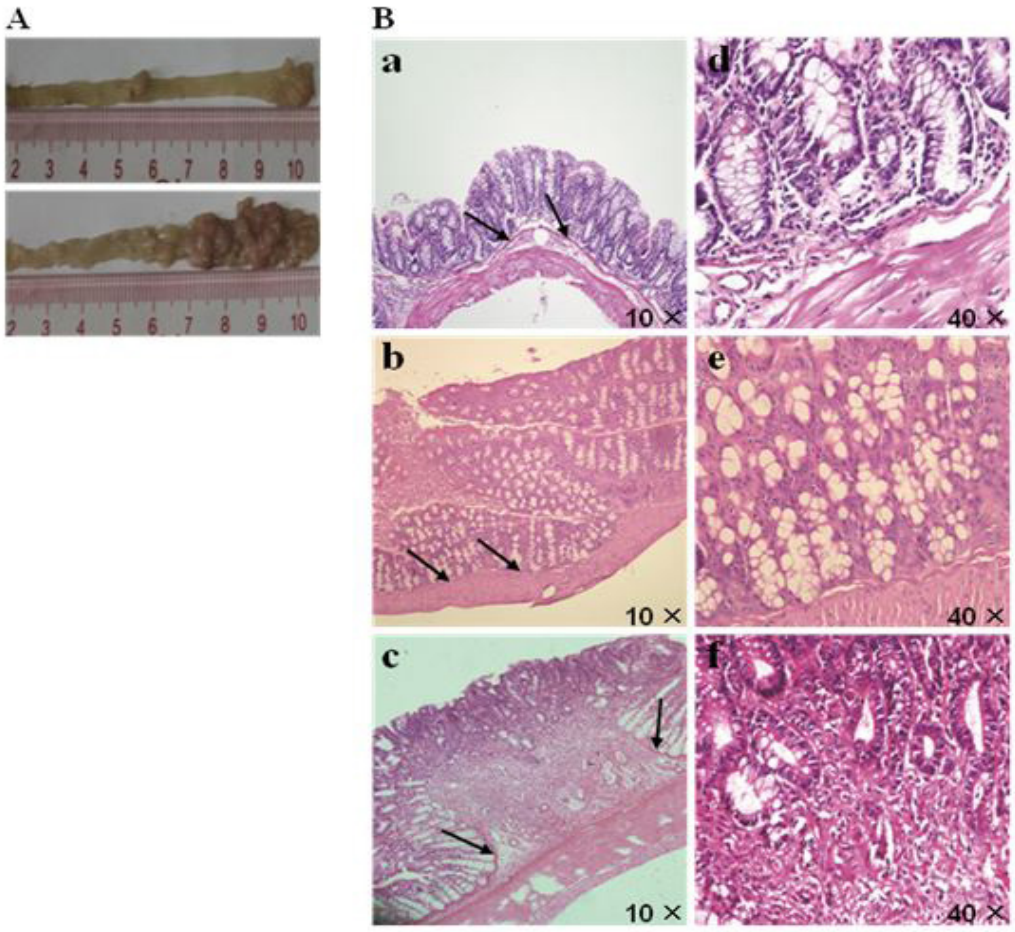

Figure 1: Sample images of colonic tumors
A: Macroscopic view of colonic tumors in a longitudinally cut colon. The tumors are solitary (upper) and multiple (lower). B: Microscopic view of a colon section stained with hematoxylin and eosin. Arrows show muscularis mucosa, **Figure 1Ba** (10×) and d (40×) showthe normal colonic type epithelium, characterized by small round nuclei and abundant goblet cells. **Figure 1Bb** (10×) and e (40×) show an adenoma of colon mucosa with moderate grade of dysplasia, loss of polarity and decreased mucine excretion, however, the muscularis mucosa is intact. **Figure 1Bc** (10×) and f(40×) show adenocarcinomas that penetrate through the muscularis mucosa into the submucosa. The muscularis mucosa is apparently destroyed by the invading tumor cells, showing disordered arrangement and enlarged hyperchromatic nuclei.

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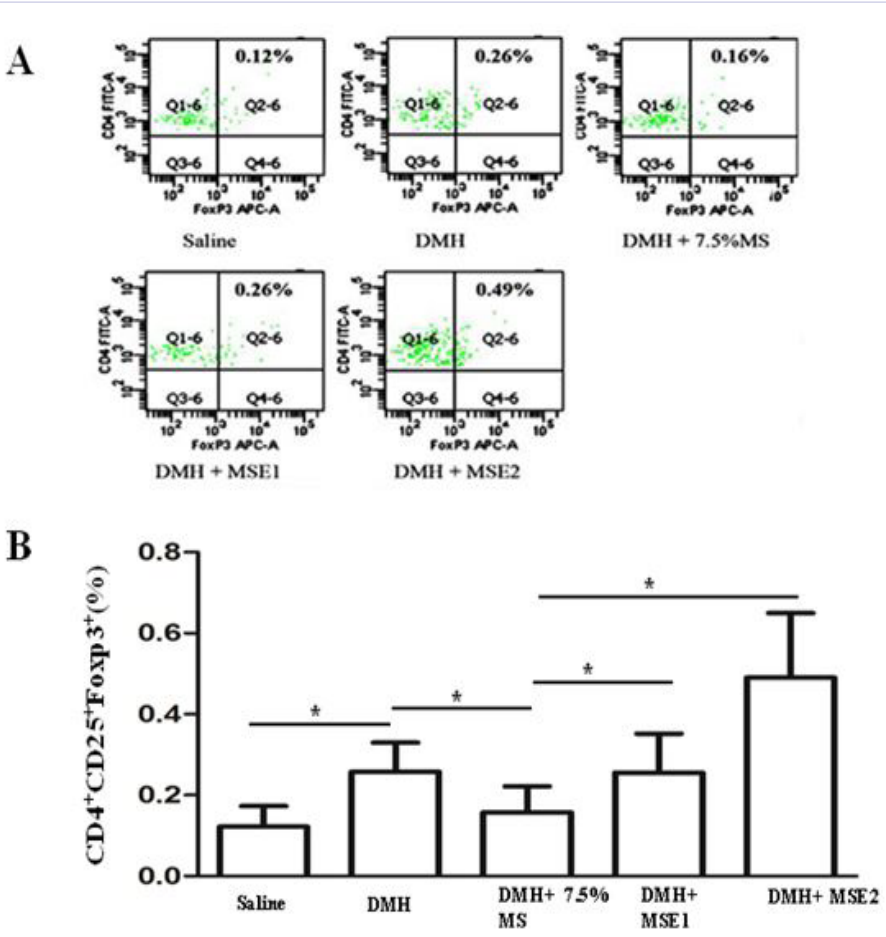

Figure 2: While MS decreases spleen Treg cells, MSE1 and MSE2 do not
A:Representive FACS samples of $CD4^+CD25^+Foxp3^+$Treg cells from experiment I. Numbers in the upper right area of the dot-plot indicate the percentage of Treg cells. B: The percentages of $CD4^+CD25^+Foxp3^+$ Treg cells in the five-group mice summarized. Compared with the saline group, a significant increase of Treg cells were observed in the DMH group. Both MS fractions also increased Treg cells, whereas there was a decrease in the MS group (**P<0.05*).

cancer area in Kunming mice model (Experiment IV).

Thus, components in MSE1 are good enough to activate Dc cells, and anti-oxidant components are good enough to survive CD8+ T cells. Whether they are good enough to suppress tumor-genesis? Although MS itself are good for inhibition of DMH-induced colon cancer incidents, we did not see similar effect from both MSE1 + MSE2, or MSE1 + VitE combinations (probably we need to do more optimal ratio of these combinations), but comparing the tumor size, we found both groups of MS extracts combination and MSE1 with VitE have good effect on inhibition of cancer growth (Figure 8).

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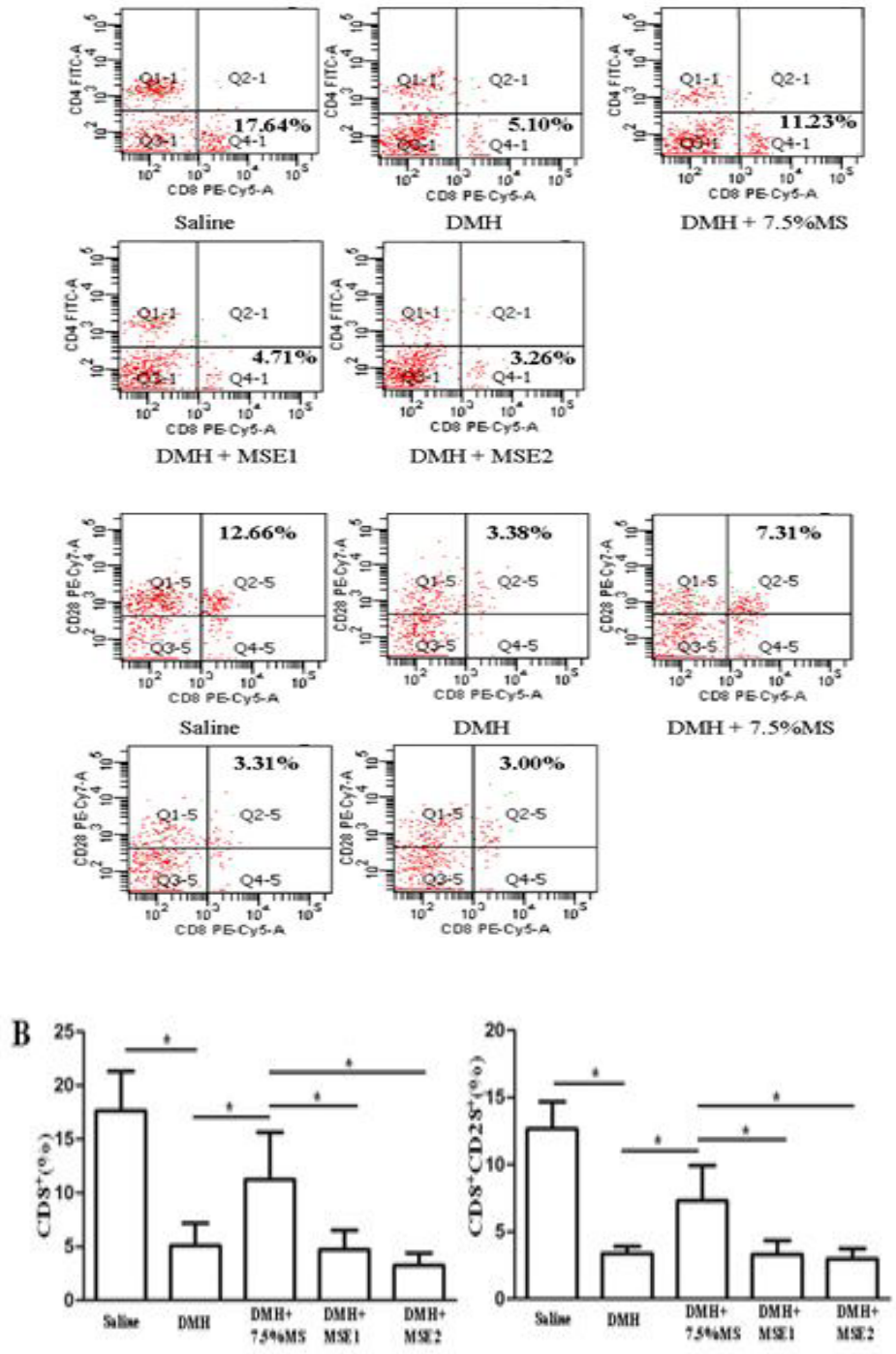

Figure 3: DMH depresses spleen CD8+T cells and CD8+CD28+cells, which is counter-acted by MS but not by its fractions
A: Representative FACS samples of $CD8^+$T cells and $CD8^+CD28^+$cells. The five graphs to the left represent $CD8^+$T cells with ratios given in the lower right quadrant while the five graphs to the right show ratios of $CD8^+CD28^+$ cells in the upper right quadrant. B: The percentages of $CD8^+$T cells (left) and $CD8^+CD28^+$ cells (right) in the five groups of mice are shown summarized. Compared with the saline group, a significant decrease occurs in the DMH group, while the MSE1 and MSE2 + DMH show no significant increase of $CD8^+$T and $CD8^+CD28^+$cells.

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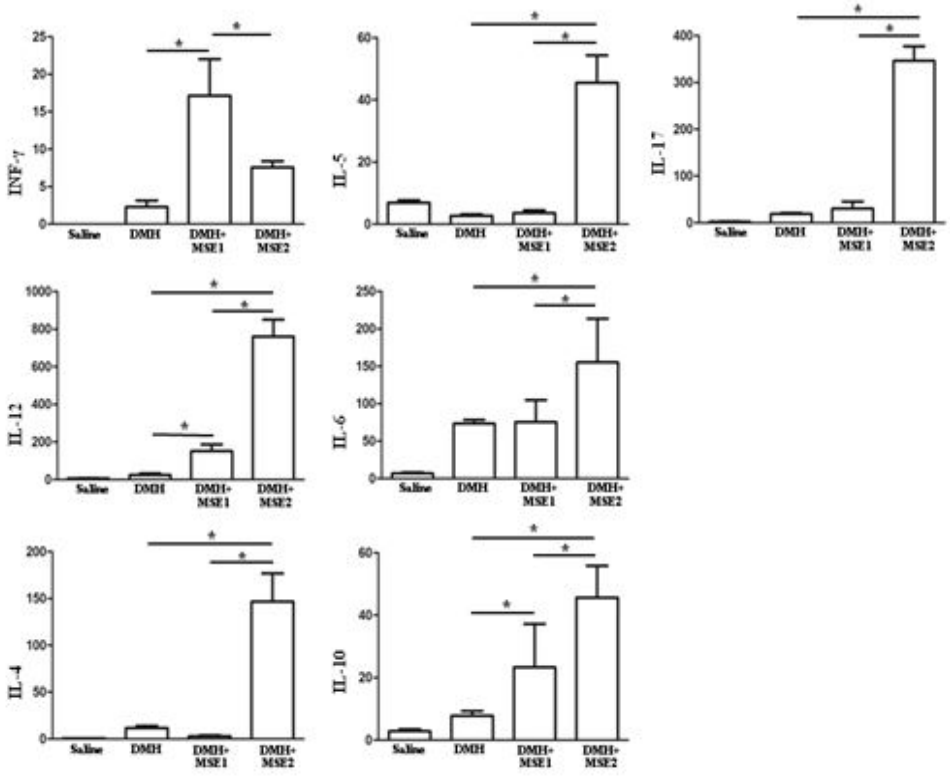

Figure 4: MSE1 induces Th1 reaction, while MSE2 induces Th2 and Th17 type reactions
MSE1 significantly enhanced a Th1 reaction following exposure to DMH, while Th2 and Th17 type cytokines were significantly enhanced in the DMH + MSE2 group. In opposition to MS, neither of the MS fractions prevented carcinogenesis, suggesting that the MSE1 and MSE2 fractions might have a synergetic effect on regulating immunoreactions.

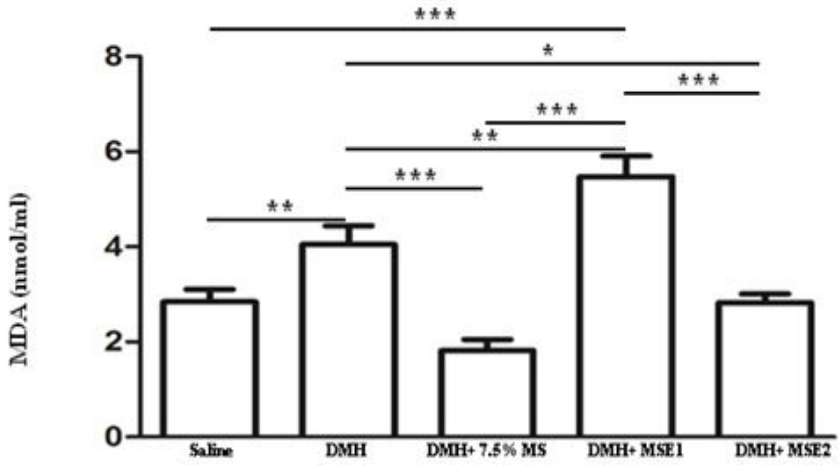

Figure 5: In opposition to MS and MSE2, MSE1 doesn't depress MDA blood levels following exposure to DMH
MSE1 significantly increased MDA levels following exposure to DMH, whereas a significant decrease was observed in the DMH + MS and DMH + MSE2 groups, suggesting that MSE1 acted as an oxidation activator, while MS and MSE2 are antioxidants.

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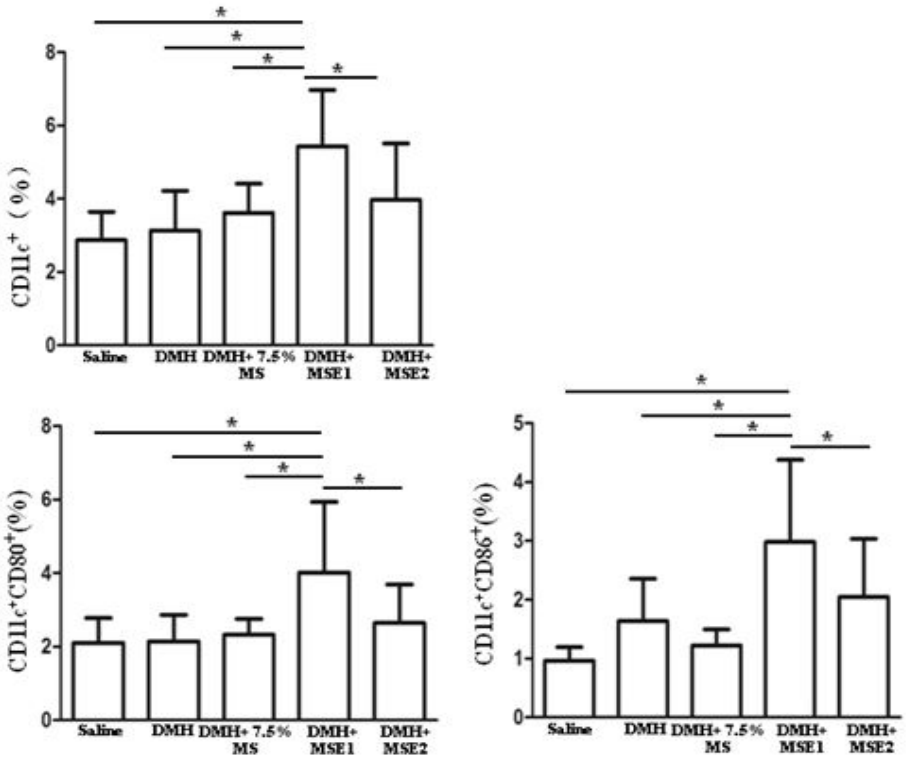

Figure 6: MSE1 enhances the spleen dendritic cells population
MSE1 significantly enhanced the population of dendritic cells including CD11c+CD80+ cells and CD11c+CD86+cells and thereby differed from MSE2, indicating that MSE1 affects the immune system by activating dendritic cells

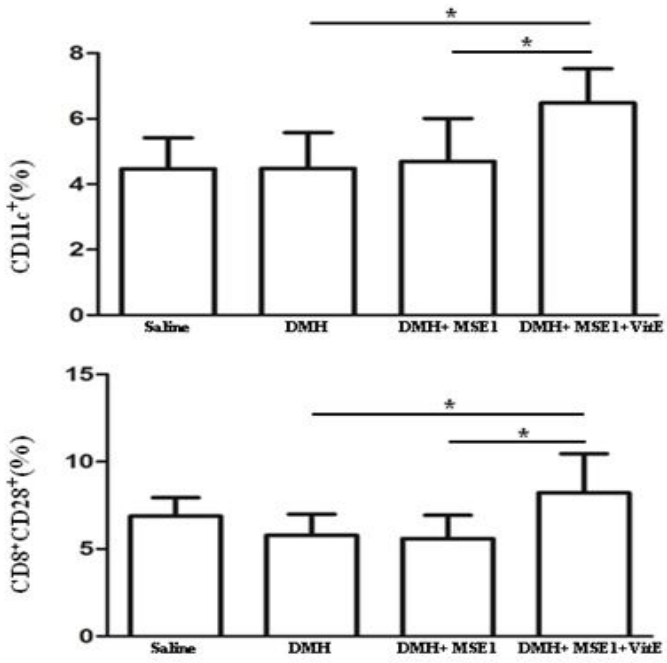

Figure 7: MSE1 +VitE enhances the spleen population of active dendritic and CD8+T cells
MSE1 +VitE significantly enhanced the dendritic and CD8+CD28+ T cell populations following exposure to DMH, thereby differing from MSE1 alone

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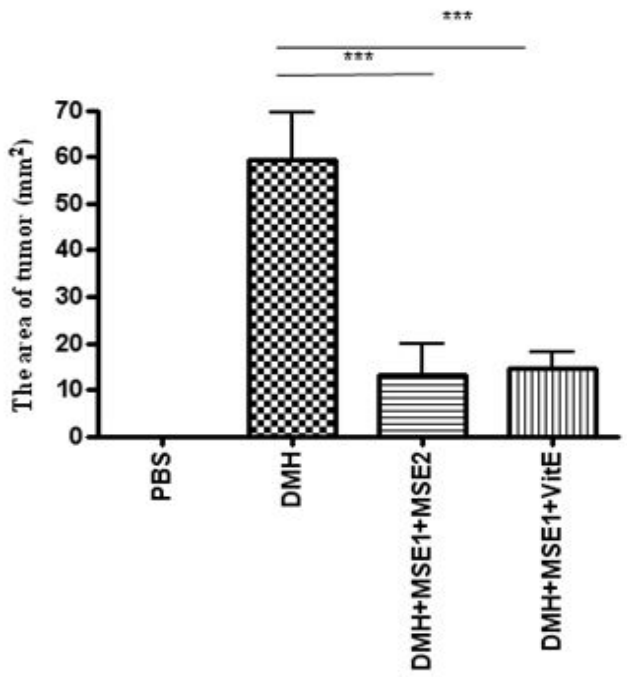

Figure 8: Both MSE1 + MSE2, and MSE1 + VitE combinations significantly decreases MDH-induced colon cancer area
In experiment IV, long-term feeding of MSE1 + MSE2 or MSE1 + VitE, we did not see the differences of DMH-induced tumor-genesis of incidents, but significantly decreased the tumors size

Discussion

Products from traditional medicinal herbs may act as potent anti-inflammatory, antioxidant and anticancer agents. Recent advances in genomics and metabolomics have enabled scientists to better understand the potential use of immuno-modulatory natural products for treatment or control of various malignancies. Plant products may block cancer initiation through several different mechanisms. These include prevention of attack of reactive oxygen species (ROS) on DNA, alteration of the metabolism of pre-carcinogens by phase-I drug metabolizing enzymes and excretion of reactive metabolites from the cell by a secondary line of defense that involves phase-II conjugating enzymes [such as glutathione-S-transferases (GST)] [24]. Paradoxically, some immunomodulatory plant products, such as epigallocatechin gallate (EGCG) of green tea and the polyphenol curcumin in turmeric and ascorbic acid of fruits act as both oxidants and antioxidants [25, 26]. Thus, oxidants and antioxidants derivatives obtained from natural products have been found to prevent tumorgenesis. Most agents were explored with respect to their effect on inflammation, cell cycle and apoptosis etc. Our data suggest that different fractions of natural products modulate different steps of the immune reactions. Oxidants, which commonly are regarded as inducers of inflammation and apoptosis, may actually activate dendritic cells in a positive way, while they probably damage T cells in close contact with the MHC-TCR complex. Therefore, antioxidants may be required for T cell survival.

Our previous publication indicated that MS reversed DMH-induced immune system Th2 profile to Th1 [22]. Next question is that which fraction of MS induces Th1 cytokines expression? Here we found that oxidant fraction did (inducing interferon-gamma expression), while anti-oxidant fraction actually induced IL-4, IL-5, IL-6, IL-10, etc., the Th2 profile of cytokines (Figure 4). This is another point of view to see oxidant in MS is necessary on preventing tumor-genesis. Although anti-oxidant fraction mainly induced Th2 profile of cytokines, but it also induced IL-17, so that anti-oxidant fracture may also have a tendency of a shift from Th2 to Th1. Thus, the difference in function of the two MS fractions on the expression of Th1 and Th2 cytokines suggest their synergistic role on immune regulation.

CD80 and CD86, which are expressed on DCs, bind to a cluster of members of the CD28 family, including CD28, CTLA-4, PD-1, ICOS and BTLA. Some of these ligands activate T cells (such as CD28), whilst others are negative regulators of T cells. We checked the effect of CD28 on CD8+ T cells, to see the activation of DC consequence is positive or negative activation of T cells. Activation signals may involve the expression of tumor-damaging enzymes, such as perforin in CD8+ T cells. We observed that mustard seeds reversed the DMH-induced down-regulation of perforin expression, while both the oxidant and antioxidant MS fractions were unable to replicate the effects of complete mustard seeds (data not shown), implicating that the expression of perforin by cytotoxic T cells may also be

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regulated by DCs activation.

Taken together, we found that the ether- and ethanol fractions of mustard seeds play different roles in activation of the immune system. When we found activation of DCs but not of CD8+ T cells, to be induced by the oxidant fraction MSE1, we hypothesized that the antioxidant effect needed to for T cells survival. We therefore exposed animals to the MSE1 + VitE, instead of the MSE2 fractions, and then successfully restored T cell activation. Therefore, we raise the theory that oxidants and antioxidants synergistically activate the immune system, possibly through immunological synapses between APCs and T cells. The activation of APCs needs oxygen radicals as second messengers, while the release of such radicals through MHC-TCR complexes may harm and damage T cells. Antioxidants seem to be required for secondary lymphocyte activation, which gives survival signals to activated T cells (Figure 9).

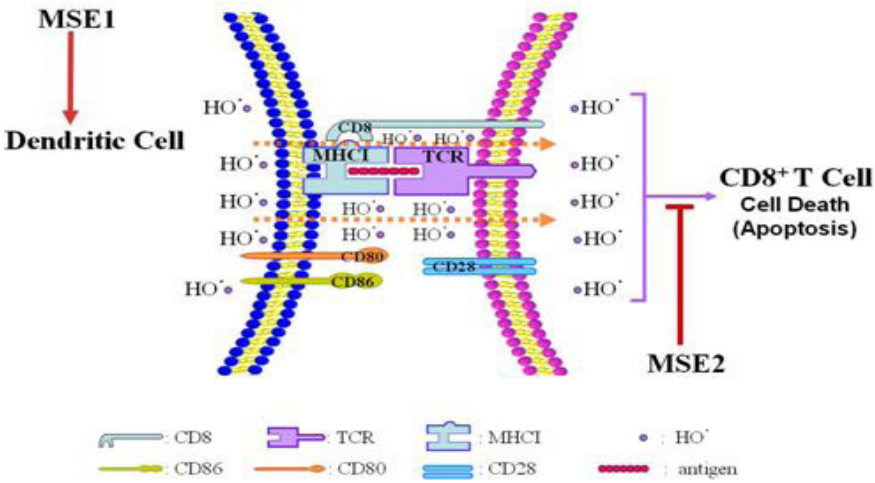

Figure 9: Schematic overview of the synergistic oxidant and anti-oxidant effects on APCs-induced activation of CD8+T cells

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Declarations

Ethical Approval: The experiments were approved by the Animal Ethics Committee of Southern Medical University.

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