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The Interplay Between Innate Immunity (TLR-4) and sCD40L in the Context of an Animal Model of Colitis-associated Cancer

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Abstract. *Background/Aim: Several studies have found elevated soluble CD40 Ligand (sCD40L) in the serum of patients with malignancies as well as those with inflammatory bowel disease (IBD). Our goal was to determine the possible causal role of sCD40L in colitis-associated colorectal cancer (CAC) by using the well-established azoxymethane/dextran sulfate sodium (AOM/DSS) protocol. Materials and methods: Twelve wild type (WT) and twelve TLR4 knock out (KO) female C57BL6 mice were divided into 4 experimental groups. Six WT and six TLR4 KO mice were treated with a single intraperitoneal dose (10 mg/kg of body weight) of AOM followed by three 7-day cycles of oral 2.5% DSS. The other two groups included 6 WT and 6 TLR4 KO mice that received only water and served as the control groups. The mice were sacrificed after 84 days. Results: All mice in the AOM/DSS WT group developed CAC while all mice from the AOM/DSS TLR4 KO group were protected from CAC. We measured the serum and pathologic tissue levels of sCD40L with quantitative sandwich enzyme-linked immunoassay (ELISA) and found that serum sCD40L was significantly higher in wild-type mice that developed CAC compared to their healthy counterparts (wild-type and TLR-4 KO controls). In comparison, serum sCD40L levels were comparable between TLR-4 KO mice, which are protected from developing CAC, and their healthy counterparts (wild-type and TLR-4 KO controls). Of note, tissue levels of sCD40L were not affected by the development of CAC. Conclusion: Our findings point to the presence of an axis between TLR-4 and sCD40L, which may lead to decreased immunosurveillance and the subsequent development of colitis-associated cancer.*

The CD40 molecule is a co-stimulatory protein receptor and a member of the TNF family of receptors. It is expressed by various antigen-presenting cells, such as B-lymphocytes, activated macrophages, endothelial cells, and platelets (1, 2). Its soluble form, namely soluble CD40L (sCD40L), is a 18-KDa trimer that is released by activated T-lymphocytes and platelets (3-5). Cognasse *et al*. and Assinger *et al*. have shown that the release of sCD40L is mediated by TLR-4 (6, 7).

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The sCD40L molecule has been implicated in several malignancies. For example, Caggiari *et al*. found elevated sCD40L in the serum of patients with undifferentiated nasopharyngeal carcinoma, Roselli *et al*. found elevated sCD40L in the serum of patients with lung cancer, and a pioneering study by a NIH group found elevated sCD40L in the serum of patients with breast, prostate, and colon cancer (8-10). Most importantly, Danese *et al*. showed that elevated serum levels of sCD40L in IBD patients reflect enhanced surface expression and release of CD40L (11). However, to date, no experimental study has examined the potential correlation between elevated levels of sCD40L and IBD-related colon cancer (12, 13).

Although we do not know whether a correlation exists between elevated levels of sCD40L and IBD-related cancer, we do know that TLR-4 mediates the release of sCD40L, resulting in increased levels in the serum of patients with several malignancies and IBD. In addition, we know that mice that do not express the TLR-4 gene (*i.e*. TLR-4 KO mice) are protected from IBD-related cancer (14). As such, our hypotheses are that a) soluble CD40L will be decreased in TLR-4 KO mice, and b) soluble CD40L will be increased in wild-type mice that develop CAC. To test this hypothesis, we employed the novel AOM/DSS mouse model of CAC carcinogenesis in wild type and TLR-4 KO mice. The AOM/DSS protocol reliably induces colitis-associated cancer. Specifically, the use of DSS following administration of the mutagen azoxymethane (AOM) shortens the process of tumor formation from several months to as little as 8-10 weeks (15-17).

Materials and Methods

Experimental conditions, animals and experimental protocol. All experiments described herein were approved by ELPEN Laboratories and the veterinary authorities of the East Attica Region in accordance with the European Directive 63/2010 (national legislation PD 56/2013) and the principles of the Helsinki Declaration.

TLR-4 KO mice (8 weeks old) were purchased from Oriental Bio Service, Inc. (Kyoto, Japan). All knockout mice were backcrossed to C57BL/6 mice for at least 8 generations. C57BL/6 wild-type mice were purchased from the Hellenic Pasteur Institute. All animals were maintained at the ELPEN Animal Facility, in compliance with Institutional Animal Care Guidelines and EU regulations (directive 63/2010). Animals were housed in plastic cages (6 mice/cage) with free access to drinking water and a pelleted basal diet, under controlled conditions of humidity (50±10%), light (12/12 h light/dark cycle), and temperature (23±2˚C). The experimental animals were quarantined for the first 7 days and then randomly placed into experimental and control groups. The colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO, USA), while the pro-inflammatory DSS was purchased from ICN Biochemicals, Inc (Aurora, OH, USA).

A total of 24 mice were divided into 4 groups (2 experimental groups and 2 control groups), with 6 in each group. Specifically, groups A (n=6) and B (n=6) were the control groups (wild type C57BL6 and TLR-4 KO, respectively). Groups C (n=6) and D (n=6) were treated with a single intraperitoneal injection of AOM (10 mg/kg). Starting one week after AOM administration, the animals received 2.5% DSS in the drinking water for 7 days, followed by a 2-week rest period without DSS. They subsequently received another 7-day cycle of 2.5% DSS followed by a second 2-week rest period and underwent a final 7-day cycle of 2.5% DSS. Control groups (A and B) received only water. Following the sacrifice of the animals at 84 days, the colon was flushed with saline and excised.

Pathology. Colon length from the ileocecal junction to the anal verge was measured, and the colon was cut open longitudinally along its main axis and washed with saline. The specimen was then macroscopically inspected, sectioned, and fixed in 10% buffered formalin for two weeks. The resulting paraffin-embedded sections underwent hematoxylin & eosin (H&E) staining and were examined for the presence of histological alterations, such as mucosal ulceration, dysplasia, aberrant crypt foci, and/or carcinoma.

Sample preparation and enzyme-linked immunosorbent assay. In this study, we applied the quantitative sandwich enzyme immunoassay technique. Serum samples were centrifuged at approximately 1000 × *g* for 15 min, and the serum was subsequently removed. Colonic samples were homogenized and the resulting suspension was subjected to either ultrasonication or to two freeze-thaw cycles to further break the cell membranes. The homogenates were then centrifugated for 15 minutes at 1500 × *g* and the supernatant was removed. Following homogenization, sCD40L was quantified by a quantitative sandwich enzyme immunoassay assay. The procedure was performed according to the manufacturer's instructions (Elisa kit Mouse soluble Cluster of differentiation 40 ligand: MBS732869, Mybiosource San Diego, CA, USA). The results were expressed as nanograms per milligram of serum or tissue.

Statistics. Normality of the data was tested using the Skewness-Kurtosis test. Continuous variables were described by both median and mean, and compared using the Wilcoxon-Mann-Whitney test. A *p*-Value - ≤0.05 was considered statistically significant. Statistical analysis was performed using Stata/MP version 13.1 (StataCorp, College Station, TX, USA).

Results

Clinical course and pathology. All mice in the control groups (A and B) had an uneventful clinical course and normal colon in the pathologic specimens. Group C (wild type) mice developed loose stools with gross evidence of blood during DSS administration and experienced significant weight loss the week following DSS administration. Mice clinically recovered during the water-only rest period. Following sacrifice of the mice, histological examination revealed colitis of varying severity with the presence of adenocarcinoma (3-5 tumors/mouse). Characteristic findings included mucosal ulceration, infiltration with mononuclear and polymorphonuclear leukocytes in the lamina propria and submucosa, hyperplastic epithelia and ulcers in the process of healing *via* re-epithelialization, and adenocarcinoma (Figure 2). Multiple adenomas were also noted.

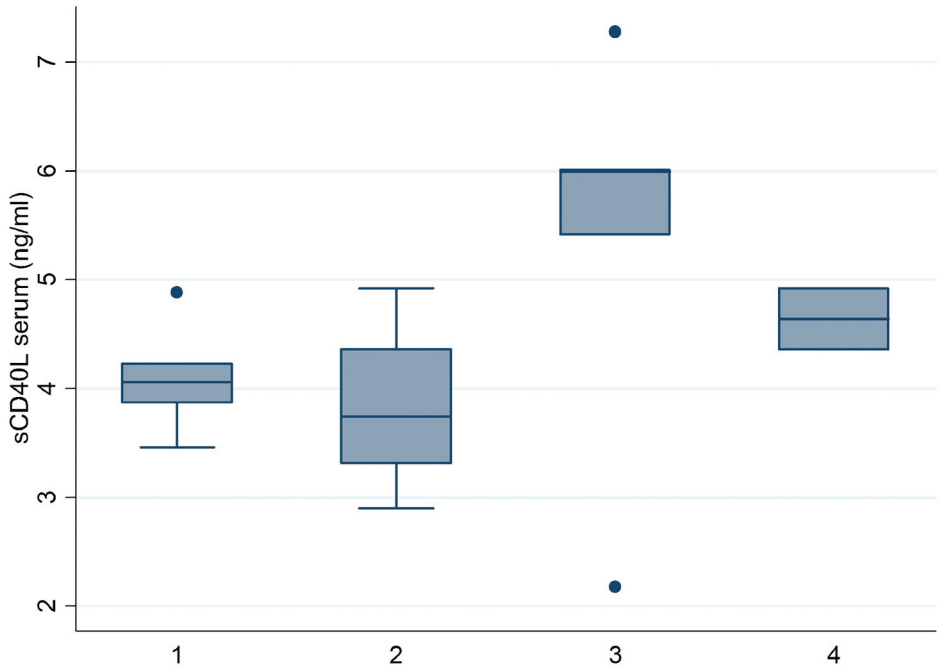

Figure 1. *Serum sCD40L in different groups [group 1: wild-type control (N=6), group 2: TLR-4KO control (N=6), group 3: wild-type AOM/DSS (N=6) and group 4: TLR-4KO AOM/DSS (N=2)]. The dots represent the outliers (values outside the interquartile range).*

Group D (TLR4 KO) mice similarly developed loose stools with gross evidence of blood during DSS administration, and also experienced significant weight loss the week following DSS administration. Mice clinically recovered during the water-only rest period, with the exception of four mice that succumbed after the first and second dose of DSS. Chronic colitis was the characteristic finding on histological examination of the specimens. Of note, adenocarcinomas were not observed.

Regarding the four mice of group D that succumbed, during the first week of the experiment and 5 days following the administration of AOM, one mouse died from group D. Signs of peritonitis were found on autopsy. After the first cycle of DSS, another mouse died from Group D, with remarkable weight loss (from 23.64 to 18.46 gr) and excessive rectal bleeding. After the second dose of DSS, we noticed another two deaths associated with similar symptoms (weight loss and rectal bleeding).

ELISA results in serum. The sCD40L molecule serum levels in groups C and D were compared to those in groups A and B (Table I and Figure 1). The mice in group C had significantly higher median and mean levels of sCD40L compared to the mice in both groups A and B (Table II). The mice in group D had comparable median and mean levels of sCD40L compared to those in both groups A and B (Table II).

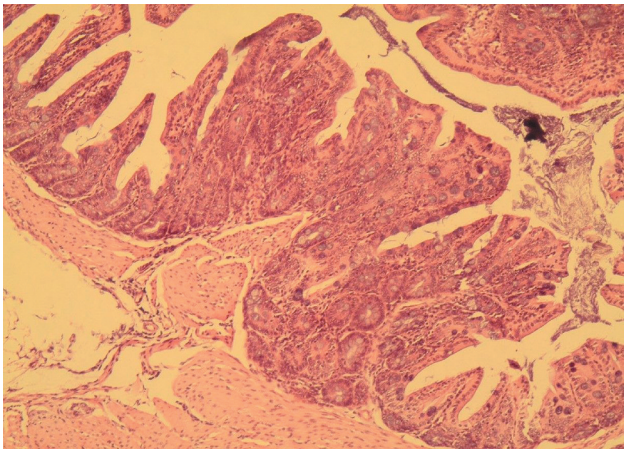

Figure 2. *Colon carcinoma induced post AOM/DSS treatment for 12 weeks in C57BL/6 mice (H-E, original magnification ×200).*

ELISA results in tissue. The sCD40L molecule tissue levels in groups C and D were compared to those in groups A and B (Table I). The mice in group C had comparable median

Table I. *Serum and tissue sCD40L in different groups.*

	sCD40L serum (ng/ml)					Tissue sCD40L (ng/ml)		
	Mean (SD)		Median (IQR)		Range	Mean, SD	Median, IQR	Range
Total (n=20)	4.5 (1.2)	4.3 (3.7-5.2)	2.1-7.3	3.3 (2.3)	2.1 (1.7-4.5)	1.6-8.8		
Group 1 (n=6)	4.1 (0.47)	4.1 (3.9-4.2)	3.5-4.9	4.9 (3.1)	4.6 (2.3-7.6)	1.7-8.8		
Group 2 (n=6)	3.8 (0.77)	3.7 (3.3-4.4)	2.9-4.9	1.8 (0.7)	1.8 (1.7-1.9)	1.6-2.0		
Group 3 (n=6)	5.5 (1.7)	6.0 (5.4-6.0)	2.2-7.3	3.4 (2.0)	2.9 (1.7-5.3)	1.6-6.2		
Group 4 (n=2)	4.6 (0.4)	4.6 (4.4-4.9)	4.4-4.0	2.1 (0.4)	2.1 (1.8-2.4)	1.8-2.4		

and mean levels of sCD40L compared to the mice in groups A and B (Table III). The mice in group D had comparable median and mean levels of sCD40L compared to those in groups A and B (Table III).

Discussion

To our knowledge, this is the first experimental study to investigate the correlation between sCD40L levels and CAC. We employed the AOM/DSS protocol, which is a powerful tool for investigating the pathogenesis of CAC (18). Specifically, according to Tanaka *et al*., a single dose of AOM followed by 2% DSS results in 100% incidence of colonic adenocarcinoma within four weeks (19). Most importantly, for our hypothesis, TLR-4 KO mice are protected from the development of CAC (14).

In agreement with our hypothesis, we found that serum sCD40L is significantly higher in wild-type mice that developed CAC compared to their healthy counterparts (wild-type and TLR-4 KO controls, Figure 1). In comparison, serum sCD40L levels were comparable between TLR-4 KO mice, which are protected from developing CAC, and their healthy counterparts (wild-type and TLR-4 KO controls). Of note, tissue levels of sCD40L were not affected by the development of CAC. This study also confirms previous reports that secretion of serum sCD40L, at least in animal models of CAC, is nullified in TLR-4KO mice, which highlights the critical role of innate immunity in the secretion of sCD40L (15, 20).

These findings raise the question of correlation versus causality with regard to serum sCD40L levels and the development of CAC. Unfortunately, we did not measure serum sCD40L levels during the earlier stages of tumorigenesis to examine a possible association between the two (*i.e*. lower serum sCD40L levels in the earlier stages of CAC versus higher sCD40L levels in late stages), which may be more consistent with a causal relationship. Nonetheless, findings in humans may support a causal relationship. For example, Danese *et al*. not only reported elevated levels of sCD40L in the circulation of IBD patients, but also found that the levels of sCD40L were higher with more extensive anatomical involvement (11). Since greater anatomic involvement is a risk factor for the development of colon cancer, this indicates that sCD40L may be directly involved in the pathogenesis of CAC (21).

Table II. *p-Values for comparisons of serum sCD40L in different groups.*

sCD40L serum (ng/ml)	Group 1	Group 2	Group 3	Group 4
Group 1	-	0.63	0.05	0.10
Group 2	-	-	0.05	0.17
Group3	-	-	-	0.18

Table III. *p-Values for comparisons of tissue sCD40L in different groups.*

Tissue sCD40L (ng/ml)	Group 1	Group 2	Group 3	Group 4
Group 1	-	0.03	0.20	0.32
Group 2	-	-	0.15	0.32
Group3	-	-	-	0.74

The mechanism behind this presumably causal relationship may relate to the immunosuppressive effect of this molecule. Specifically, a group has previously suggested that the observed differences between serum sCD40L levels in cancer patients and healthy donors implicates serum sCD40L as a factor promoting cancer development by suppressing immune activation and tumor immunosurveillance (9, 22). The exact mechanisms include enhancing the suppressive activity of myeloid-derived suppressor cells, expanding the Treg population, up-regulating PD-1 expression on CD4 T cells, increasing suppressive cytokine production, and inhibiting IL-12 production by monocytes (9).

If serum sCD40L is indeed a causal factor in CAC pathogenesis, the next step would be to test whether its pharmacologic suppression may halt tumorigenesis. Statins,

a well-known cardiovascular drug, can significantly decrease the levels of sCD40 (23). Interestingly, while unrelated to sCD40L research, there is a growing body of evidence that these well-tolerated compounds could form the basis of future chemopreventive strategies for colon cancer (24). Although the mechanism behind this effect is unknown, our study findings point to the decrease of serum sCD40 as a possible mechanism.

Interestingly, contrary to its serum counterpart, tissue sCD40 levels did not correlate with the development of CAC and were comparable across all groups. Of note, almost all studies that found a relationship between sCD40L and carcinogenesis refer to serum and not tissue sCD40L (8, 10, 11). Thus, although we cannot explain this finding, our results are aligned with those in other studies. This study is limited by the small number of mice in group D, as well as the fact that we did not test for a dose response relationship between sCD40L and the stage of carcinogenesis. Unfortunately, the AOM/DSS protocol does not allow for testing for different stages of carcinogenesis.

In conclusion, our findings indicate the presence of an axis between TLR-4 and sCD40L, which may lead to decreased immunosurveillance and the subsequent development of CAC. Importantly, only elevated levels of sCD40L in serum but not in pathologic tissue were associated with CAC. Future studies are needed to confirm these findings and investigate whether a dose response relationship exists between levels of serum sCD40 and stages of carcinogenesis in both animal models and patients with IBD.

Conflicts of Interest

None.

Authors' Contributions

Anastasios Angelou: conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, wrote the paper. Apostolos E. Papalois: conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, wrote the paper (equal contribution). Efstathios Antoniou: conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, wrote the paper. Jaeyun Wang: collected the data, contributed data or analysis tools. Neda Amini: collected the data, contributed data or analysis tools. Anastasia Pikouli: collected the data, contributed data or analysis tools. Nikolaos Andreatos: collected the data, contributed data or analysis tools. Stefan Buettner: collected the data, contributed data or analysis tools. Muhammad Munir: collected the data, contributed data or analysis tools. Georgios Theodoropoulos: corrected the manuscript, contributed data or analysis tools. Georgios C Zografos: corrected the manuscript, contributed data or analysis tools. Panagiotis Sarantis: contributed data or analysis tools. Alessandra Pulvirenti: collected the data, contributed data or analysis tools. Stamatios Theocharis: conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis. Emmanouil Pikoulis: conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, wrote the paper. Georgios Antonios Margonis: conceived and designed the analysis, collected the data, contributed data or analysis tools, performed the analysis, wrote the paper.

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