both CD4 and CD8 T cell responses, and supposedly in enhancing the anti-tumor activity of TAMs as well.

Although our previous study supported the notion that the number of TAMs at the invasive front of CRC tissues is associated with good prognosis of the patients with LN metastasis, recently the roles of TAMs on immunity and angiogenesis in malignancies have been variously discussed. For example, in breast cancer, prostate cancer and renal cell carcinoma, the dense invasion of TAMs in tumor tissues is a sign of worse prognosis; conversely, in stomach cancer, CRC and malignant melanoma, it indicates a favorable prognosis (11); hence more intense studies should be accumulated to elucidate its whole feature (12,13). To elucidate the immunological function of TAMs in those malignancies, not only to correlate the number of CD14+ TAMs in cancer tissues with clinical characteristics, but also to elucidate the roles of cell surface antigens on TAMs which we herein discuss, will be important. In this report we studied the cell surface antigens (CD86, CD80, HLA-DR, CD1a, CD40 and CD83) in the CD14+ TAMs with flow cytometry and correlated the results with prognosis of CRC patients to find out prognostic indicators for the OS of those patients.

METHODOLOGY

Patients and tissue samples

Tissue samples from 31 CRC patients undergoing curative or palliative resections of primary tumors at Tohoku University Hospital or Sendai Postal Services Agency Hospital were included in this study. The clinicopathological characteristics of the patients are summarized in Table 1. Tumor depth, histological types, stages and other factors were defined according to the rules for the Japanese society for cancer of the colon and rectum (6th edition) (14). We collected all samples after obtaining informed consent and the tissue samples for flow cytometry were taken immediately after surgical removal.

Isolation of mononuclear cells

To avoid any contamination of either necrotic or ulcerative lesions, we selectively sampled the tissues from invasive fronts under visual control and the specimens were histopathologically diagnosed with hematoxylin and eosin staining (data not shown). Both the cancerous and corresponding normal mucosal tissue specimens were washed by Hanks' balanced salt solution (HBSS; Invitrogen, Carlsbad, CA, USA) containing 1mM dithiothreitol (Wako Pure Chemical, Osaka, Japan) for 30 minutes, then washed by HBSS containing 1mM EDTA for 60 minutes three times. Finally, they were washed by PBS containing 10% fetal bovine serum (FBS; Trace Biosciences, Castle Hill, NSW, Australia) for 30 minutes, four times. After washing, the samples were minced with a scalpel and treated with HBSS containing 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Wako Pure Chemical), 0.3g/mL collagenase (Wako Pure Chemical), 50μg/mL deoxyribonuclease (Worthington Biochemical, Lakewood, NJ, USA), 12.5μg/mL penicillin-streptomycin (Invitrogen) and 0.6μg/mL amphotericin B (SIGMA, St. Louis, MO, USA) followed by stirring at 37ºC for 6-8 hours until complete dissolution was achieved.

The enzymatically disaggregated cell suspensions were filtered through a nylon mesh screen to remove cell clumps. After filtering, the cell suspensions were washed by PBS containing 1% FBS and the mononuclear cells were isolated by Ficoll-Paque PLUS (Amersham Pharmacia Biotech, Uppsala, Sweden).

Flow cytometric analysis (Figure 1)

The cells were pelleted and suspended in PBS containing 0.2% bovine serum albumin (Wako Pure Chemical) and 0.02% sodium azide (Wako Pure Chemical). After blocking with 10% normal mouse serum, the cells were stained with the following fluorophore-conjugated mouse monoclonal antibodies (mAbs) against human cell surface antigens of the CD markers at 4ºC for 30 minutes: phycoerythrin (PE)-CD14 (clone MφP9; Becton Dickinson, San Jose, CA, USA), fluorescein isothiocyanate (FITC)-CD14 (clone MφP9; Becton Dickinson), PE-CD80 (clone T22; BD Pharmingen, San Diego, CA, USA), PE-CD86 (clone MAB104; Beckman Coulter, Fullerton, CA, USA), FITC-HLA-DR(clone 243; Becton Dickinson), PE-CD1a (clone BL6; Beckman Coulter, Fullerton, CA, USA), FITC-CD40 (clone 5C3; BD Pharmingen), and FITC-CD83 (clone HB15a; Beckman Coulter).

The specificity of the staining was confirmed using equal concentrations of isotype-matched control mAbs. After washing, the cells were suspended in fluorescence-activated cell sorting (FACS) buffer (Becton Dickinson) and stored on ice until analysis. The cells were analyzed by double-staining flow cytometry using FACS Calibur with a Cell-Quest software program (Becton Dickinson) within 12 hours after staining. At least 10,000 cells per sample were analyzed and their data were expressed as dot plots.

Statistical analysis

The statistical analyses were performed using the SPSS II computer software program (SPSS Inc., Chicago, IL, USA). The correlation between the numbers of positive cells for each of the cell surface antigens and clinicopathological characteristics was analyzed using Mann-Whitney U test (two categories) and Kruskal-Wallis test (three or more categories) (**Table 1** and **Figure 1**).

With the median number of CD14+ cells positive for each of the cell surface antigens (CD80, CD86, HLA-DR, CD1a, CD40 and CD83) among the 31 CRC cases as a borderline, the 31 cases were divided into two subgroups: "high group (n=16)" in which the number of positive cells was equal to or higher than the median, and "low group (n=15)" in which the number of positive cells was lower than the median. Univariate analysis and multivariate analysis were performed using the Cox proportional hazards model for each of the factors listed in Tables 2 and 3. The OS curves were drawn following the Kaplan-Meier method (**Figure 2**) and the differences between the two survival curves were tested by the log-rank test; a p-value of less than 0.05 was considered to be statistically significant.

RESULTS

Clinicopathological characteristics

Clinicopathological characteristics of the 31 CRC patients in this study (18 males and 13 females; mean age: 65.5 years) are summarized in Table 1. This study did not include patients with other malignant diseases or with neoadjuvant chemotherapy.

A flow cytometric analysis of CD14+ cells

The positivity and negativity of CD14 in mononuclear cells using a PE-CD14 mAb were highly consistent with those using FITC-CD14 mAb (data not

Fig. 1

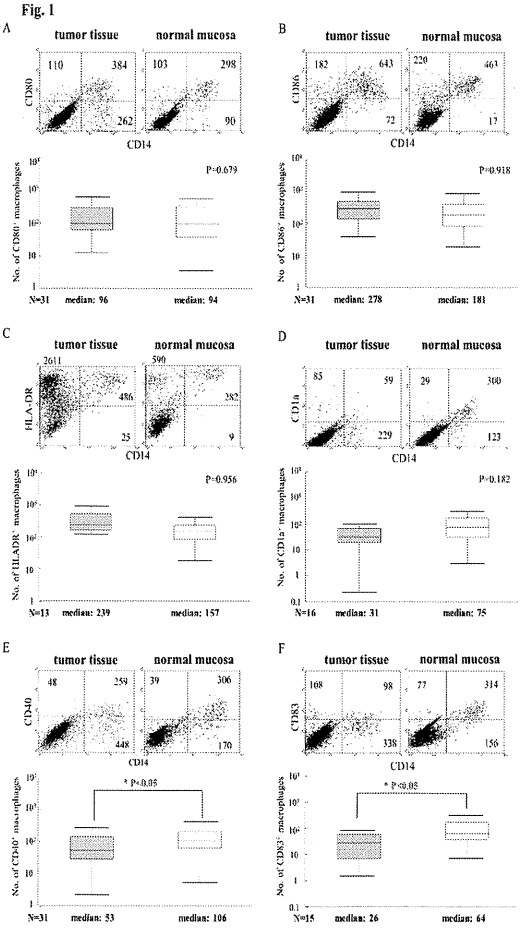

FIGURE 1. Representative dot plots of flow cytometric analysis of CD14, CD80, CD86, HLA-DR, CD1a, CD40 and CD83 in CRC tissues and their corresponding tissues. The numbers of positive cells for each of the cell surface antigens are summarized in box whisker plots. The upper and lower endpoints of the whiskers represent the 10th and 90th percentiles, respectively. The upper and lower edges of the boxes represent the 25th and 75th percentiles, respectively. The line inside the box represents the median. *p<0.05, Mann-Whitney U test. **(A)** A representative dot plot of flow cytometric analysis for CD14 and CD80. The cell counts of CD80+ macrophages in CRC and their corresponding normal tissues are shown with box whisker plots (n=31). **(B)** CD14 and CD86 (n=31). **(C)** CD14 and HLA-DR (n=13). **(D)** CD14 and CD1a (n=16). **(E)** CD14 and CD40 (n=31). **(F)** CD14 and CD83 (n=15).

shown); hence either one of the antibodies was chosen for the double-staining flow cytometry in combination with another antibody: CD80, CD86, HLA-DR, CD1a, CD40, or CD83. In Figure 1, representative dot plots of double-staining flow cytometry in the paired cancer and corresponding normal tissues are shown. In Figure 1, the number of cells doubly positive for CD14 and another cell surface antigen (CD80, CD86, HLA-DR, CD1a, CD40 or CD83) in 10,000 cells among 31 cases were compared between the paired tissues and the results were shown with box-whisker plots. Among the TAMs which were CD14-positive, the numbers of CD80+ cells, CD86+ cells and HLA-DR+ cells in cancer tissues were higher than those in corresponding normal tissues (**Figure 1A**, **B** and **C**, respectively); on the other hand, the number of CD1a+ cells in cancer tissues was lower than that in corresponding normal tissues although those results were not statistically significant (**Figure 1D**). The numbers of CD40+ cells and CD83+ cells in cancer tissues were

Fig. 2

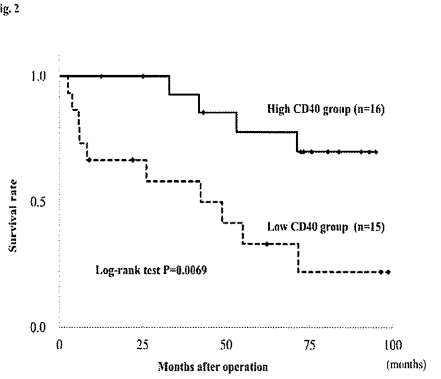

FIGURE 2. Survival analysis. Kaplan-Meier estimates of the overall survival of 31 CRC cases. The 31 CRC cases were classified into two subgroups according to the number of CD40+ TAMs infiltrated in the CRC tissues: "high CD40 group (n=16)" and "low CD40 group (n=15)".

significantly lower than those in corresponding normal tissues (**Figure 1E** and **F**, respectively) ($p<0.05$). Since CD40 as a co-stimulatory molecule was considered to have important roles for cancer immunity and the number of CD40+ TAMs in cancer tissues was significantly lower than that in corresponding normal tissues, we further analyzed the 31 cases for the numbers of CD40+ TAMs in cancer tissues associated with each of their clinicopathological characteristics listed in Table 1. The mean number of the CD40+ TAMs in cases without lymphatic invasion (n=9) was significantly higher than that with lymphatic invasion (n=22) (302±108 vs. 113±23; $p=0.044$). Again, the mean number of the CD40+ TAMs in cases without distant metastasis (n=27) was significantly higher than that with distant metastasis (n=4) (188±42 vs. 34±7.8; $p=0.034$). On the other hand, the mean number of the CD40+ TAMs isolated from normal tissues did not show any correlation with the clinicopathological characteristics listed in **Table 1** (data not shown).

Survival analysis

To find out prognostic indicators of OS among the 31 CRC patients, we performed the univariate analysis. Among them, stage ($p=0.001$), lymph node metastasis ($p=0.032$), distant metastasis ($p=0$) and the number of CD1a+ cells (immature DCs) ($p=0.041$) were shown to be the prognostic indicators (**Table 2**). In addition to the above indicators, the number of CD40+ TAMs was also shown to be a prognostic indicator ($p=0.016$). We further performed the multivariate analysis (**Table 3**) and the number of CD40+ TAMs ($p=0.033$), lymph node metastasis ($p=0.007$) and the distant metastasis ($p=0.004$) were shown to be independent prognostic indicators of OS. Survival data were available for all of the 31 patients in this study, with an observation period ranging from 2.5 to 99 months (median 53 months). Thirty one patients were classified into high CD40 group (n=16) and low CD40 group (n=15), as explained in the methodology section. With the Kaplan-Meier method, the estimated 5-year OS rate in the high CD40 group and that in the low CD40 group were 70.1% and 22.2%, respectively, the difference being statistically significant ($p=0.0069$) (**Figure 2**). In the same way, the OS rates associated with expression of cell surface antigens (CD80, CD86, HLA-

DR, CD1a and CD83) in TAMs were also evaluated and there was no significant difference (data not shown).

DISCUSSION

Macrophages differentiate into DCs under various types of stimulation such as cytokines (2) and they exert different roles in each type of malignancies, but the mechanism has yet to be elucidated (15,16). In our previous study (9), we reported that in CRC patients with LN metastasis, the 5-year OS rate in high CD14 group was better than that in low CD14 group; however, the difference was not evident in patients without LN metastasis, the reason of which was yet to be clarified. In addition, it is still difficult to uniformly discuss the roles of TAMs on immunity and angiogenesis in a series of malignancies.

CD14 has been used as a marker of tissue macrophages (7,8), CD1a as a marker of immature DCs (10), and CD83 as a marker of mature DCs (10). On the other hand, both CD80 and CD86 are molecules expressed on APCs and they provide important co-stimulatory signals necessary for T cell activation and survival (6). All of the CD14, CD1a, CD83, CD80, and CD86 were analyzed and discussed in our previous study. In our current study, we analyzed two additional markers: HLA-DR and CD40. HLA-DR is one of the MHC class II molecules and binds the TCR on CD4+ helper T cells to present exogenous antigen peptides (17). CD40 is a co-stimulatory protein found on APCs and is required for their activation. The binding of CD40 ligand (CD40L) on helper T cells to CD40 on APCs activates APCs and induces a variety of downstream effects (18). In this report, we evaluated those cell surface antigens and co-stimulatory molecules with flow cytometry to clarify their roles in TAMs in CRC tissues.

For T cell activation, two pathways are concurrently required: i) the stimulation of TCR with antigen-MHC complex and ii) co-stimulatory signals from APCs towards the CD28 on T cells (19). It means that in T cell activation, both HLA-DR as MHC class II molecules and CD80/CD86 as CD28 ligands have to be presented on APCs. In our present study, the numbers of CD80+ TAMs, CD86+ TAMs and HLA-DR+ TAMs in CRC tissues were higher than those in corresponding normal tissues. These results suggest that TAMs can phagocytose tumor antigens and present the antigens along with MHC class II molecules on their cell surfaces, and they concurrently stimulate CD28 on T cells as co-stimulatory signals in CRC tissues. On the other hand, the numbers of CD1a+ TAMs and CD83+ TAMs in CRC tissues were lower than those in normal tissues, which meant that maturation of macrophages into DCs was inhibited in CRC tissues compared with normal tissues.

With the multivariate analysis, the number of CD40+ TAMs, as well as lymph node metastasis and distant metastasis, was shown to be an independent prognostic indicator of OS among 31 CRC patients. With the stimulatory and co-stimulatory signals from APCs, the activated T cells expressed CD40L on their cell surfaces (18) and the CD40L bound the CD40 on APCs, which reaction eventually stimulated the T cells and promoted the production of IL12 in APCs (18). With the production of IL12, T cells became to express IL12 receptors, and the IL12 receptors increasingly bound to IL12 and T cells producing more INF γ along with stimulation of "IL12-INF γ pathway" (20). Our results may suggest that activation of the "IL12-INFγ pathway" is inhibited in CRC tissues compared with normal tissues, due to a lower population of CD40+ TAMs. Cancer immunotherapy is expected as the fourth modality of cancer treatment, following surgical treatment, chemotherapy and radiotherapy (21,22). In our current study, we demonstrated that the numbers of CD40+ TAMs and CD83+ TAMs in CRC tissues were less than those in corresponding normal tissues and the results indicated that the OS of CRC patients with a higher number of CD40+ TAMs was better than that with a lower number of CD40+ TAMs. They suggest that the number of CD40+ TAMs may be utilized as a prognostic or predictive indicator in the clinical setting, especially when postoperative adjuvant chemotherapy is indicated among high-risk Stage II CRC patients (23).

TABLE 1. Clinicopathological characteristics of colorectal cancer patients and the mean number of CD40⁺ cells in TAMs (n=31)

	No. of patients	No. of CD40⁺ cells in TAMs* (mean±SE)	*p* value
Gender Male	18	198±60	0.712
Female	13	127±34	
Age (years, mean±SD)	65.5±9.5		
Tumor location Rt. side	5	104±53	0.134
Lt. side	10	102±38	
Rectum	16	229±65	
Depth SM/MP	6	346±141	
SS/A1	18	122±38	0.098
>SE/A2	7	134±50	
Histological type Well	7	187±138	
Mod	22	162±32	0.590
Por	2	161±105	
Stage I	4	401±215	
II	12	146±50	
III	10	164±37	0.128
IV	5	42±9.6	
Lymph node metastasis Negative	18	190±61	
Positive	13	137±32	0.468
Lymphatic invasion Negative	9	302±108	0.044
Positive	22	113±23	
Vascular invasion Negative	11	195±91	0.629
Positive	20	153±32	0.629

TAM: Tumor-Associated Macrophage; SM: Submucosa; MP: Muscularis Propria; SS: Subserosa; A1: cancer exceeds the muscular layer, but does not invade more deeply; SE/A2: cancer exceeds the muscular layer and invades more deeply, but does not invade to other organs; Well: well differentiated adenocarcinoma; Mod: moderately differentiated adenocarcinoma; Por: poorly differentiated adenocarcinoma. Depth, histological type, stage and other factors were defined according to the rules for the Japanese society for cancer of the colon and rectum (6th edition).
*The number in 10,000 of mononuclear cells by FCM.

TABLE 2. Univariate analysis by the Cox proportional hazards regression analysis for overall survival

Variable	Categories	HR	95% CI	*p* value
Stage	I vs. II vs. III vs. IV	3.2	1.616-6.334	0.001
Age	continuous value	1.008	0.995-1.064	0.768
Gender	Male vs. Female	2.458	0.903-6.695	0.078
Historical type	Well vs. Mod vs. Por	1.802	0.62-5.24	0.279
Depth	MP vs. SS vs. A1	1.278	0.652-2.501	0.475
Lymph node metastasis	Negative vs. Positive	2.885	1.094-7.606	0.032
Distant metastasis	Absent vs. Present	10.2	2.874-36.19	0
Lymphatic invasion	Negative vs. Positive	2.176	0.706-6.704	0.176
Vascular invasion	Negative vs. Positive	1.632	0.6-4.436	0.337
No. of $CD1a^+$ DCs	Low vs. High	0.191	0.039-0.933	0.041
No. of $CD83^+$ DCs	Low vs. High	0.463	0.115-1.861	0.278
No. of $CD14^+$ macrophages (TAMs)	Low vs. High	0.753	0.29-1.955	0.559
No. of $CD86^+$ cells in TAMs	Low vs. High	0.572	0.217-1.508	0.259
No. of $CD80^+$ cells in TAMs	Low vs. High	0.55	0.199-1.515	0.248
No. of $CD40^+$ cells in TAMs	Low vs. High	0.263	0.089-0.781	0.016
No. of HLA-DR^+ cells in TAMs	Low vs. High	0.622	0.103-3.765	0.605
No. of $CD1a^+$ cells in TAMs	Low vs. High	0.441	0.108-1.796	0.253
No. of $CD83^+$ cells in TAMs	Low vs. High	0.636	0.170-2.385	0.502

HR: Hazard Ratio; CI: Confidence Interval; DC: Dendritic Cell; TAM: Tumor-Associated Macrophage.

Currently the anti-CD40 monoclonal antibody (SGN-40; decetuzumab) has been clinically used for non-Hodgkin lymphoma patients (24). Our results suggest that CD40 plays an important role in tumor immunity of CRC patients as well and it may be possible to apply the antibody for CRC patients to enhance their tumor immunity. We believe that further analyses of CD40+ TAMs in CRC may lead to a novel cancer immunotherapy for CRC patients.

TABLE 3. Multivariate analysis by the Cox proportional hazards regression analysis for overall survival.

Variable	Categories	HR	95% CI	*p* value
Lymph node metastasis	Negative vs. Positive	5.574	1.604-19.37	0.007
Distant metastasis	Negative vs. Positive	10.297	2.092-50.65	0.004
No. of $CD40^+$ TAMs	Low vs High	0.253	0.071-0.897	0.033

HR: Hazard Ratio; CI: Confidence Interval; TAM: Tumor-Associated Macrophage.

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REFERENCES

1. **De Visser KE, Eichten A, Coussens LM:** Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 2006; 6:24-37.
2. **Hume DA:** Macrophages as APC and the dendritic cell myth. J Immunol 2008; 181:5829-5835.
3. **Gooden MJ, de Bock GH, Leffers N, et al.:** The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. Br J Cancer 2011; 105:93-103.
4. **Iwamoto M, Shinohara H, Miyamoto A, et al.:** Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. Int J Cancer 2003; 104:92-97.
5. **Du C, Wang Y:** The immunoregulatory mechanisms of carcinoma for its survival and development. J Exp Clin Cancer Res 2011; 30:12.
6. **Chen L:** Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. Nat Rev Immunol 2004; 4:336-347.
7. **Holness CL, Simmons DL:** Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood 1993; 81:1607-1613.
8. **Kielian TL, Blecha F:** CD14 and other recognition molecules for lipopolysaccharide: a review. Immunopharmacology 1995; 29:187-205.
9. **Kinouchi M, Miura K, Mizoi T, et al.:** Infiltration of CD14-positive macrophages at the invasive front indicates a favorable prognosis in colorectal cancer patients with lymph node metastasis. Hepato-gastroenterol 2011; 58:352-358.
10. **Kalinski P, Schuitemaker JH, Hilkens CM, et al.:** Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J Immunol 1998; 161:2804-2809.
11. **Lewis CE, Pollard JW:** Distinct role of macrophages in different tumor microenvironments. Cancer Res 2006; 66:605-612.
12. **Oosterling SJ, van der Bij GJ, Meijer GA, et al.:** Macrophages direct tumour histology and clinical outcome in a colon cancer model. J Pathol 2005; 207:147-155.
13. **Sickert D, Aust DE, Langer S, et al.:** Characterization of macrophage subpopulations and microvessel density in carcinomas of the gastrointestinal tract. Anticancer Res 2007; 27:1693-1700.
14. **Japanese Society for Cancer of the Colon and Rectum:** General rules for clinical and pathological studies on cancer of the colon, rectum and anus, 6th ed. Tokyo, Japan, Kanehara Publishers; 1998.
15. **Chen JJ, Lin YC, Yao PL, et al.:** Tumor-associated macrophages: The double-edged sword in cancer progression. J Clin Oncol 2005; 23:953-964.
16. **Forssell J, Oberg A, Henriksson ML, et al.:** High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. Clin Cancer Res 2007; 13:1472-1479.
17. **Brode S, Macary PA:** Cross-presentation: dendritic cells and macrophages bite off more than they can chew! Immunology 2004; 112:345-351.
18. **O'Sullivan B, Thomas R:** CD40 and dendritic cell function. Crit Rev Immunol 2003; 23:83-107.
19. **Harris NL, Ronchese F:** The role of B7 co-stimulation in T-cell immunity. Immunol Cell Biol 1999; 77:304-311.
20. **Frucht DM, Fukao T, Bogdan C, et al.:** IFN-gamma production by antigen-presenting cells: mechanisms emerge. Trends Immunol 2001; 22:556-560.
21. **Mellman I, Coukos G, Dranoff G:** Cancer immunotherapy comes of age. Nature 2011; 480:480-489.
22. **Topalian SL, Weiner GJ, Pardoll DM:** Cancer immunotherapy comes of age. J Clin Oncol 2011; 29:4828-4836.
23. **Engstrom PF, Arnoletti JP, Benson AB 3rd, et al.:** NCCN clinical practice guidelines in oncology: colon cancer. J Natl Compr Canc Netw 2009; 7:778-831.
24. **Lewis TS, McCormick RS, Emmerton K, et al.:** Distinct apoptotic signaling characteristics of the anti-CD40 monoclonal antibody dacetuzumab and rituximab produce enhanced antitumor activity in non-Hodgkin lymphoma. Clin Cancer Res 2011; 17:4672-4681.