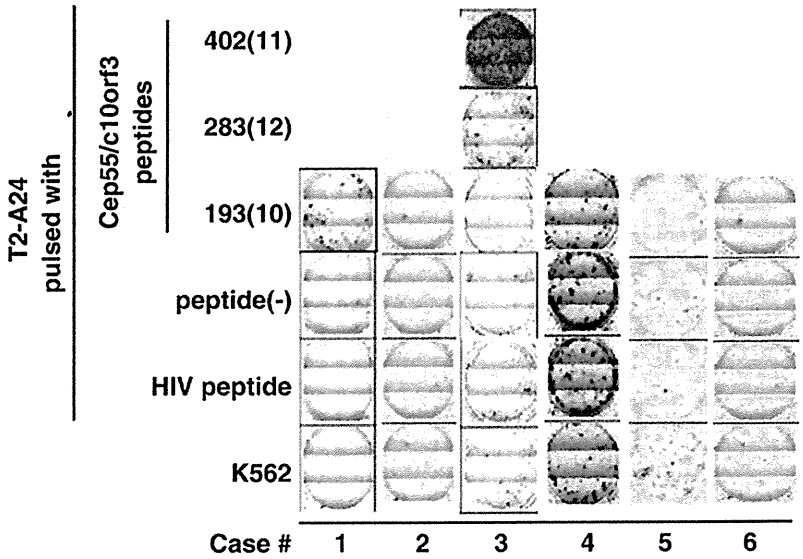

Fig. 2. ELISpot assay for Cep55/c10orf3 peptides in colorectal carcinoma patients. The Cep55/c10orf3 peptides specific CTLs were induced from HLA-A24 (+) colorectal carcinoma patients' PBMCs. The PBMCs were stimulated with the Cpe55/c10orf3 peptide cocktail for 3 times, and the peptide-specific reactivities were evaluated with the IFN-gamma ELISPOT assay using Cep55/c10orf3 peptide pulsed T2-A24 cells. The HIV derived peptide was used as a negative control. K562 is used as a negative control.

after 2days of post-infection the HCT15 cells were selected by a medium containing puromycin (5 μg/ml). The expression of *B2M* mRNA was confirmed by RT-PCR.

Results and discussion

We described that Cep55/c10orf3 was expressed in 25% (6/25) colorectal carcinoma cases previously (Inoda et al., 2009). The protein expression frequencies were relative low compared with breast carcinoma. In this study, to evaluate the protein expression in more detail, we improved the method of immunohistochemical staining. With this protocol, the stained specimens showed lower background, and more suitable for monoclonal antibody #11-55. With this protocol, we re-stained 70 colorectal carcinoma tissue specimens. As shown in Fig. 1A and Table 2, 44 of 70 colon cancer tissues showed positive staining for monoclonal antibody (#11-55), while normal colon epithelial cells showed negative for #11-55. The positivities for #11-55 in well differentiated adenocarcinoma was 47% (47% of weak positive and 0% of strong positive); in moderately differentiated adenocarcinoma, 66% (57% of weak positive and 9% of strong positive); and in poorly differentiated adenocarcinoma, 86% (29% of weak positive and 57% strong positive). Thus, we hypothesized that Cep55/c10orf3 might also be the immunological target of CTLs in CRC patients. In this study, we evaluated 6 CRC patients for immunological assays (Table 3). Fig. 1B shows the representative Cep55/c10orf3 protein staining patterns used for the CTL assays. In the colorectal

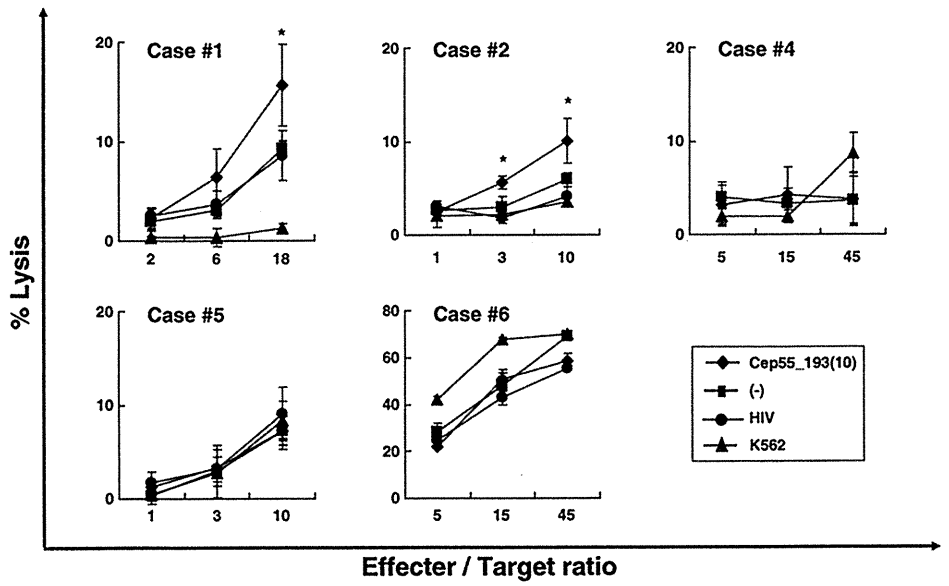

Fig. 3. Cytotoxic activity for Cep55/c10orf3 peptide in colorectal carcinoma patients. The cytotoxicity of the CTLs from colorectal carcinoma patients' PBMCs against K562 cells and T2-A24 cells pulsed with the Cep55/c10orf3_193(10) peptide, HIV peptide, or no peptide were analyzed using the ^{51}Cr release assay at various effector/target (E/T) ratios. Asterisks denote statistically significant differences from negative control peptide and no peptide pulsed T2-A24 cells. Each point represents the mean ± SD. Asterisks indicate statistically significant differences from the highest negative control the Cep55/c10orf3_193(10) peptide pulsed T2-A24 and negative controls by the Student's *t*-test (*, $p<0.05$).

carcinoma of patient #2, the primary colon carcinoma lesion did not show any Cep55/c10orf3 protein expression. In contrast, the regional lymph node metastasis lesion showed Cep55/c10orf3 protein expression. Because Cep55/c10orf3 is essential for the G2/M phase of the cell cycle (Fabbro et al., 2005), the protein is presumably expressed in mitotic cells. In our previous study, we could detect Cep55/c10orf3 mRNA expression in all of colorectal carcinoma cell lines (Inoda et al., 2009), suggesting that the proliferating colorectal carcinoma cells express Cep55/c10orf3 mRNA and protein.

To evaluate the feasibility of colorectal carcinoma immunotherapy using Cep55/c10orf3 peptides, we generated CTLs specific for Cep55/c10orf3 peptides in 6 colorectal carcinoma patients. To determine which Cep55/c10orf3 peptide is immunogenic in the PBMCs of colorectal carcinoma patients, we stimulated CD8-positive T cells of HLA-A24-positive colorectal carcinoma patients using an 11 Cep55/c10orf3 peptide cocktail. As shown in Fig. 2, case #1 showed Cep55/c10orf3_193(10) specific reactivity with the ELISPOT assay, which is the only immunogenic peptide for HLA-A24-positive breast carcinoma patients, as described before (Inoda et al., 2009). On the other hand, case #3 showed vigorous reactivity for T2-A24 cells pulsed with Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) compared to control peptides, which showed also high HLA-A24 binding affinity before (Inoda et al., 2009). Interestingly, these peptides are 11- and 12-mer, which are longer than the standard HLA-A24-restricted peptides (Kondo et al., 1995), and these peptides were not immunogenic for breast carcinoma patients. These observations suggest that not only the Cep55/c10orf3_193(10) peptide but also the Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) peptides can be presented by HLA-A24-positive colorectal carcinoma cells and caused the clonal expansion of the CTL precursor (CTLp) *in vivo*.

To assess the cytotoxic activity of the CTLs, we performed the ^{51}Cr release assay. Unfortunately, the CTLs of case #3 were used up in the ELISPOT assay, so we could not perform the ^{51}Cr release assay. As shown in Fig. 3, case #1 and case #2 showed specific reactivity for T2-A24 cells pulsed with the Cep55/c10orf3_193(10) peptide compared to the control peptides. The CTL of case #1 showed specific reactivity for the Sw480 colorectal carcinoma cell line, which is Cep55/c10orf3 positive and HLA-A24 positive, suggesting this CTL recognized the endogenously expressed Cep55/c10orf3_193(10) peptide (data not shown). To analyze further, we characterized Cep55/c10orf3_193(10) CTL clone #41, which was previously isolated from the PBMCs of HLA-A24-positive breast carcinoma patients (Inoda et al., 2009). As shown in Fig. 4B, Cep55/c10orf3_193(10) specific CTL clone #41 showed a specific lytic activity for HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinoma cell lines, including Sw480 and HT29. The beta 2 microglobulin (*B2M*) gene of the HCT15 cell line is known to be mutated, and the B2M protein is downregulated, which causes the low expression of the HLA-A24 molecule on the cell surface. We therefore established the wild type *B2M* gene stably transduced subline (HCT15-B2M). HCT15-B2M

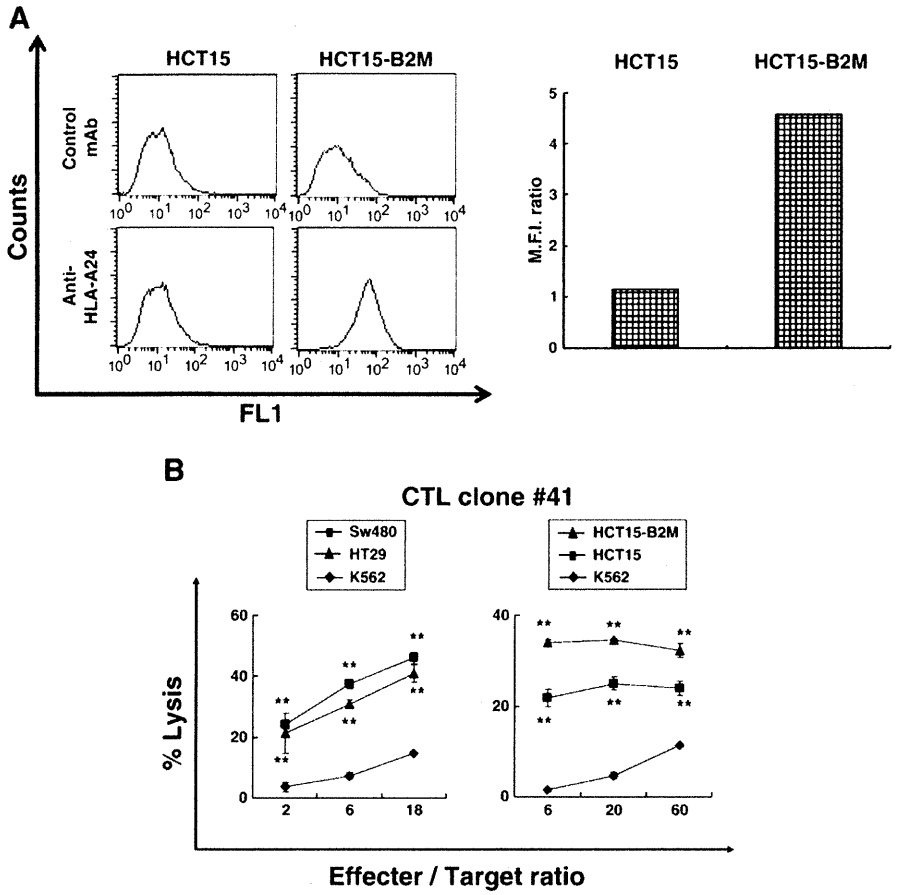

Fig. 4. Cytotoxicity for HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinomas. A. Expression of HLA-A24 on HCT15 and HCT15-B2M cells. The expression of HLA-A24 on the HCT15 and HCT15-B2M cells were evaluated by the FACS analysis. The HCT15 and HCT15-B2M cells were stained by the control antibody and anti-HLA-A24 antibody and detected by FACS Caliber. The right bar graph indicates the ratio of mean fluorescent intensity (M.F.I.), which is calculated as follows: M.F.I. ratio = (M.F.I. with the anti-HLA-A24 antibody)/(M.F.I. with control antibody) B. Cep55/c10orf3_193(10) specific CTL clone (#41) was assessed for the cytotoxicity for HLA-A24-positive and Cep55/c10orf3-positive colorectal carcinoma cell lines (Sw480, HT29 and HCT15) with the ^{51}Cr release assay. HCT15-B2M is a beta-2 microglobulin gene transduced cell line. HLA-A24 (−) and Cep55/c10orf3 (+) K562 were used as negative controls. Each point represents the mean ± SD. Asterisks indicate statistically significant differences from the K562 negative control cell by the Student's *t*-test (**, $p<0.01$).

showed the enhanced HLA-A24 expression with the FACS analysis (Fig. 4A). CTL clone #41 recognized HCT15 cells compared with K562 cells, and the lytic activity was enhanced with the ectopic expression of the *B2M* gene (Fig. 4B). These observations suggest that the Cep55/c10orf3_193(10) peptide is endogenously expressed in a number of colorectal carcinoma cell lines and that the Cep55/c10orf3_193(10) peptide is suitable for colorectal carcinoma immunotherapy.

Cep55/c10orf3 is expressed in a wide variety of cancer cells (Inoda et al., 2009; Sakai et al., 2006), which makes it a reasonable target for cancer immunotherapy. Cep55/c10orf3_193(10) is a high immunogenic peptide in breast carcinoma patients. However, the immunoreactivity for Cep55/c10orf3_193(10) in colorectal carcinoma is less frequent than that of breast carcinoma. Very interestingly, the Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) peptides are only immunogenic for colorectal carcinoma patient case #3. This may result from the difference in the peptide presentation profiles *in vivo* between colorectal carcinoma and breast carcinoma. In the previous study, we have shown that only the Cep55/c10orf3_193(10) peptide is suitable for breast carcinoma immunotherapy. However, the results of the current study indicate that not only Cep55/c10orf3_193(10) but also Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) may be useful for colorectal carcinoma immunotherapy.

The centrosome is the principal microtubule organizing center of the mammalian cell, consisting of a pair of barrel-shaped microtubule assemblies that are non-identical and are referred to as the mother and daughter centrioles. Defects in the number, structure or function of centrosomes can generate monopolar or multipolar mitotic spindles and cytokinesis defects, resulting in aneuploidy and chromosome instability, which are common characteristics of tumor cells. Thus, abnormal centrosome constituents may be exploited as therapeutic targets for malignantly transformed or dysplastic cells (Wang et al., 2004). In addition to Cep55/c10orf3 other centrosome residing molecules have been reported to be the target of CTLs, such as survivin (Hirohashi et al., 2002) and Aurora-A kinase (Ochi et al., 2009). These centrosome antigens are essential for G2/M phase transition and are expressed universally in malignant cells (Hirohashi et al., 2009). Thus targeting centrosome antigens is a reasonable approach for cancer immunotherapy, and more centrosome related antigens might be identified in the future.

In summary, we were able to generate CTLs specific for Cep55/c10orf3_193(10), Cep55/c10orf3_402(11) and Cep55/c10orf3_283(12) in HLA-A24-positive colorectal carcinoma patients. This suggests that these peptides may be useful for HLA-A24-positive colorectal carcinoma immunotherapy.

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Stem Cells, Tissue Engineering, and Hematopoetic Elements

Cytotoxic T Lymphocytes Efficiently Recognize Human Colon Cancer Stem-Like Cells

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Cancer stem-like cells (CSCs) and tumor-initiating cells (TICs) are a small population of cancer cells that share three properties: tumor initiating ability, self-renewal, and differentiation. These properties suggest that CSCs/TICs are essential for tumor maintenance, recurrence, and distant metastasis. Here, we show that cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigen CEP55 can efficiently recognize colon CSCs/TICs both *in vitro* and *in vivo*. Using Hoechst 33342 dye staining, we isolated CSCs/TICs as side population (SP) cells from colon cancer cell lines SW480, HT29, and HCT15. The SP cells expressed high levels of the stem cell markers SOX2, POU5F1, LGR5, and ALDH1A1 and showed resistance to chemotherapeutic agents such as irinotecan or etoposide.To evaluate the susceptibility of SP cells to CTLs, we used CTL clone 41, which is specific for the CEP55-derived antigenic peptide Cep55/c10orf3_193 (10) (VYVKGLLAKI). The SP cells expressed HLA class I and CEP55 at the same level as the main population cells. The SP cells were susceptible to CTL clone 41 at the same level as main population cells. Furthermore, adoptive transfer of CTL clone 41 inhibited tumor growth of SW480 SP cells *in vivo*. These observations suggest that Cep55/c10orf3_193(10) peptide-based cancer vaccine therapy or adoptive cell transfer of the CTL clone is a possible approach for targeting chemotherapy-resistant colon CSCs/TICs. *(Am J Pathol 2011, 178:1805–1813; DOI: 10.1016/j.ajpath.2011.01.004)*

Colon cancer is one of the most common malignancies worldwide. With recent progress in treatment, the prognosis has improved to some extent. In advanced disease, however, the prognosis remains unfavorable, because of recurrence, distant metastasis, and resistance to treatment. Thus, novel treatment modalities are needed.

Cancers contain morphologically heterogeneous populations. This fact has led to the cancer stem cell theory,[1] the idea that cancers are composed of several types of cells, and that only a small population of cancer cells that can regenerate cancer tissues, much as normal tissue can be regenerated only by a small population of stem-like cells. Recently, cancer stem-like cells and tumor-initiating cells (CSCs/TICs) have been isolated from various types of malignancies, including colon cancer.[2–6] In colon cancer, CSCs/TICs can reinitiate tumors that resemble mother colon cancer tissues morphologically when transplanted into immunodeficient mice.[3] Furthermore, these CSCs/TICs have higher tumorigenic potential than do non-CSCs/TICs. Previous reports have shown that CSCs/TICs are resistant to a variety of treatments, including chemotherapy and radiotherapy, with varied mechanisms of resistance, including high expression of drug transporters, relative cell cycle quiescence, high levels of DNA repair machinery, and resistance to apoptosis.[7] These reports[3–6] support the hypothesis that malignant cancers comprise heterogeneous populations that organize in a hierarchical differentiation model. The CSCs/TICs are located at the top of this hierarchy, and targeting CSCs/TICs is essential to achieve efficient effects for treatment of malignant diseases. Recently, some trials targeting CSCs/TICs have been reported for hema-

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topoietic malignancies.[8] Hedgehog signaling is essential for maintenance of myeloid leukemia stem cells, and inhibition of hedgehog signaling by cyclopamine is effective for imatinib-resistant myeloid leukemia.[9] To date, however, no such CSC/TIC targeting approach has been reported for colon cancer.

In the present study, we evaluated the efficiency of CTL-based immunotherapy targeting colon CSCs/TICs. Using Hoechst 33342 dye, we isolated colon CSCs/TICs as side population (SP) cells from six colon cancer cell lines. The SP cells derived from SW480, HT29, and HCT15 showed higher tumorigenicity than did main population (MP) cells. On the other hand, SP cells from KM12LM, Lovo, and Colo320 did not show any increase in tumorigenicity, compared with MP cells. This suggests that SW480, HT29, and HCT15 SP cells (but not KM12LM, Lovo, and Colo320 SP cells) were enriched with CSCs/TICs. In RT-PCR analysis the SW480, HT29, and HCT15 SP cells showed a stem cell-like gene expression signature, including SOX2, POU5F1, LGR5, and ALDH1A1. Furthermore, these SP cells also showed resistance to chemotherapeutic agents, including irinotecan and etoposide. These observations support the idea that these SP cells had stem cell-like features. To assess the immunogenicity of SP cells, we evaluated the expression of HLA class I and of CEP55, which is a tumor-rejection antigen of breast and colon cancer.[10,11] The SP cells expressed HLA class I (and also HLA-A24) at the same level as MP cells. The SP cells also expressed CEP55 messenger RNA (mRNA) at the same level as MP cells in RT-PCR. To confirm the susceptibility of SP cells to cytotoxic T lymphocytes (CTLs), we used CTL clone 41, which recognizes CEP55 in an HLA-A24-restricted manner.[10] CTL clone 41 killed SW480, HT29, and HCT15 SP cells at the same level as it killed MP cells and presorted cells. These observations suggest that colon CSCs/TICs are also sensitive to CTLs, as non-CSC/TIC populations are. Furthermore, adoptive transfer of CTL clone 41 inhibited the tumor growth of SW480 SP cells in immunodeficient mice. These observations suggest that CTL-based colon cancer immunotherapy is efficient for colon CSCs/TICs. To our knowledge, the present study provides the first direct evidence that colon CSCs/TICs are susceptible to CTLs and thus opens possibilities for future applications in immunotherapy using CSC/TIC-specific vaccines.

Materials and Methods

Cell Lines

Colon adenocarcinoma cell lines SW480 (HLA-A*0201/2402), HCT15 (HLA-A*0201/2402), HT29 (HLA-A1/24), Lovo, and Colo320 were kind gifts of Dr. K. Imai (Sapporo, Japan), and KM12LM was a kind gift of Dr. K. Itoh (Kurume, Japan). All cell lines except K562 were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). K562 was cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum. HCT15-B2M, a stable transfectant of HCT15 cells with B2M (β2 microglobulin) cDNA, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 μg/mL puromycin (Sigma-Aldrich).[11]

Side Population Analysis

Side population analysis was performed as described previously, with some modifications.[12] Trypsinized cultured cells were washed with PBS and were resuspended at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. After 10 minutes preincubation, the cells were labeled with Hoechst 33342 dye (Lonza, Walkersville, MD) for 90 minutes at concentrations of 3.75 μg/mL for Colo320, 5 μg/mL for SW480 and Lovo, 7.5 μg/mL for HT29 and KM12LM, and 10 μg/mL for HCT15, with or without verapamil (Sigma-Aldrich), which is an inhibitor of ABC transporters, at concentrations of 50 μmol/L for SW480, HCT15, and Colo320, 75 μmol/L for Lovo, and 100 μmol/L for HT29. Cells were counterstained with 1 μg/mL propidium iodide to label dead cells. Next, 1×10^6 viable cells were analyzed and sorted using a BD FACSAria II fluorescence-activated cell sorting system (BD Biosciences, Franklin Lakes, NJ). The Hoechst dye was excited at 355 nm, and its fluorescence was measured at two wavelengths using optical filters 405 DF20 [450/20 nm bandpass filter O (Hoechst Blue)] and 635LP [635 nm longpass edge filter (Hoechst Red)]. Propidium iodide labeling was measured through a 630/BP30 filter for discrimination of dead cells.

Xenograft Model

The SP cells, MP cells, and presorted cells from colon cancer cell lines were mixed 1:1 by volume with Matrigel (BD Biosciences) and were injected subcutaneously into the backs of female 4- to 8-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size in cubic millimeters was assessed weekly with calipers and was calculated as Tumor Size = (Longest Diameter $\times$ Shortest Diameter^2)/2.

RT-PCR Analysis of SP and MP Cells

RT-PCR analysis was performed as described previously.[10] Total RNAs were isolated from both SP cells and MP cells using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen). The PCR amplification was performed in 20 μL of PCR mixture containing 1 μL of cDNA mixture, 0.5 μL of Taq DNA polymerase (Qiagen) and 4 pmol of primers. The PCR mixture was initially incubated at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The following primer pairs were used for RT-PCR analysis (forward and reverse, respectively): 5′-CATGATG-

GAGACGGAGCTGA-3′ and 5′-ACCCCGCTCGCCATGCTATT-3′ for SOX2, with an expected PCR product size of 410 bp; 5′-TGGAGAAGGAGAAGCTGGAGCAAAA-3′ and 5′-GGCAGATGGTCGTTTGGCTGAATA-3′ for POU5F1, with an expected PCR product size of 163 bp; 5′-CTCTTCCTCAAACCGTCTGC-3′ and 5′-GATCGGAGGCTAAGCAACTG-3′ for LGR5, with an expected PCR product size of 181 bp; 5′-TGTTAGCTGATGCCGACTTG-3′ and 5′-TTCTTAGCCCGCTCAACACT-3′ for ALDH1A1, with an expected PCR product size of 154 bp; 5′-TGAGTTTGCCATCACAGAGC-3′ and 5′-TTGCTTGCTGGTGCATTAAC-3′ for CEP55, with an expected PCR product size of 521 bp; and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with an expected product size of 452 bp. GAPDH was used as an internal control.

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primers and probes were designed by the manufacturer (TaqMan gene expression assays; Applied Biosystems). Thermal cycling was performed using 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Each experiment was done in triplicate, with normalization to the *GAPDH* gene as an internal control.

Flow Cytometric Analysis and Monoclonal Antibodies

Cells were incubated with mouse monoclonal antibodies at saturation concentration for 30 minutes on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with fluorescein isothiocyanate for 30 minutes. Samples were analyzed using a BD FACSCalibur flow cytometry system (Becton Dickinson, Mountain View, CA). Anti-pan HLA class I (W6/32) and anti-HLA-A24 monoclonal antibodies (C7709A2.6 hybridoma, a kind gift from Dr. P.G. Coulie, Brussels, Belgium) were prepared from hybridomas.

Survival Studies for Etoposide and Irinotecan

We isolated SP and MP cells of SW480 and HCT15 and seeded them into 96-well culture plates at 1×10^4 cells per well for each population of cells. The cells in both populations were treated with etoposide (1 and 5 μg/mL) or irinotecan (40 and 400 μg/mL for SW480, 10 and 100 μg/mL for HCT15). After 72 hours of exposure to the chemotherapeutic agents, viability of the cells was determined using the SOD assay kit WST-1, which was performed according to the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan; Rockville, MD).

Cytotoxicity Assay for SP Cells with CTL Clone 41

We had previously established CTL clone 41, which recognizes an HLA-A24 restricted antigenic peptide (VYVKGLLAKI) termed Cep55/c10orf3_193(10), from an HLA-A24-positive breast cancer patient's peripheral blood mononuclear cells.[8] The lytic activity of CTL clone 41 for SP cells, MP cells, and presorted cells was evaluated by ^{51}Cr release assay. Briefly, SP cells, MP cells and presorted cells were labeled with 100 μCi of ^{51}Cr for 1 hour at 37°C, washed four times with PBS, and resuspended in AIM-V medium (Invitrogen). The ^{51}Cr-labeled target cells (2000 cells/well) were then incubated with various numbers of effector cells for 6 hours at 37°C in 96-well culture plates. Radioactivity of the culture supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated as follows: % Specific Lysis = (Experimental Release − Spontaneous Release) × 100/(Maximum Release − Spontaneous Release). Target cells were treated with 100 units/mL interferon-γ for 48 hours before the assay.

Winn Assay

SW480 SP cells were mixed with CTL clone 41 at a ratio of 1 SP cell to 10 CTL cells. The resulting mixture (200 μL with 1×10^6 CTL clone 41 and 1×10^5 SP cells) was injected subcutaneously into the backs of NOD/SCID mice. A control group of five mice was injected with SP cells alone. Tumor size was assessed weekly.

CTL Adoptive Transfer

NOD/SCID mice were inoculated subcutaneously on the back with 1×10^3 SW480 SP cells. Three weeks later, when the tumor started to be palpable, 5×10^4 Cep55/c10orf3_193(10)-specific CTL clone cells or PBS was injected intravenously. The same adoptive transfer procedure was performed 4 weeks after inoculation with SP cells. Tumor size was assessed weekly.

Statistical Analysis

In the xenograft model, survival studies using chemotherapeutic agents, cytotoxicity assay, Winn assay, and adoptive transfer model, the data were analyzed using the Mann-Whitney *U*-test, with $P < 0.05$ conferring statistical significance.

Results

Isolation of Colon CSCs/TICs as SP Cells

Several methods to isolate colon cancer CSCs/TICs has been reported, including cell surface markers such as CD44 or PROM1 (CD133), SP cells, and the Aldefluor assay.[3–6,13] In the present study, we isolated colon CSCs/TICs using SP cell analysis. Several colon cancer cell lines were dyed with Hoechst 33342 and then analyzed with a BD FACSAria II flow cytometer as

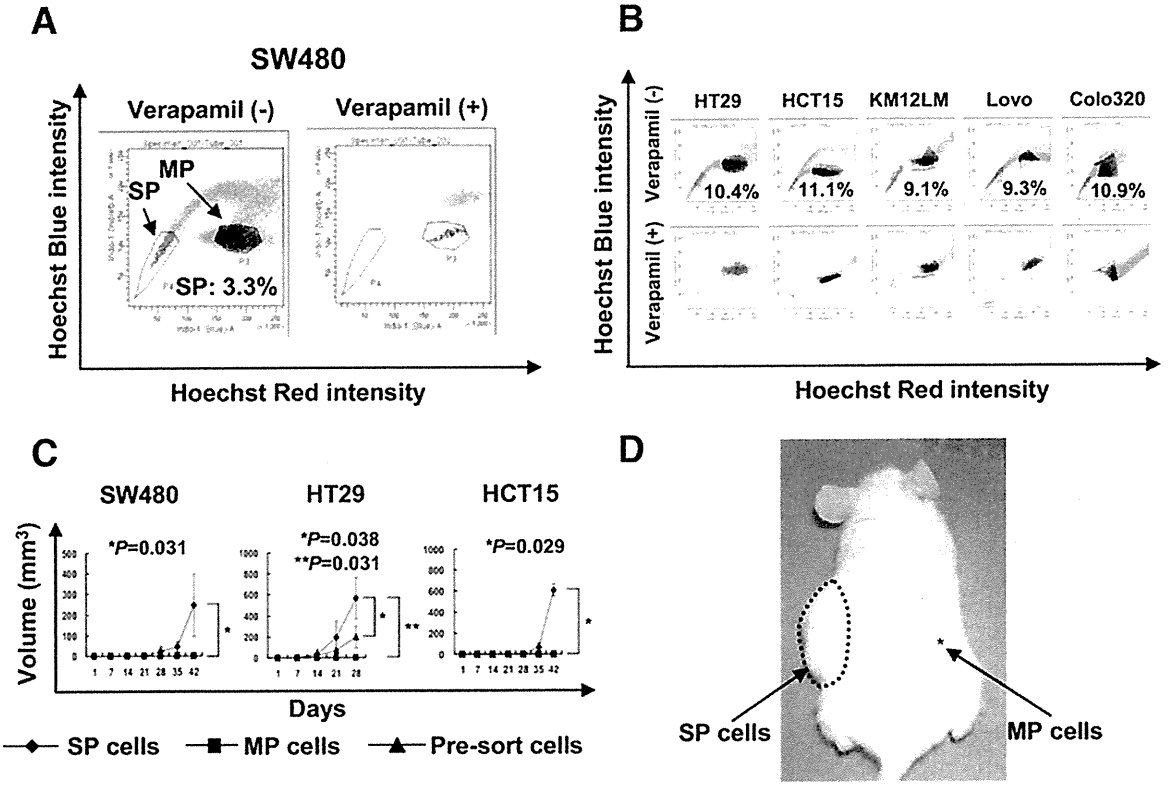

Figure 1. Isolation of colon CSCs/TICs from colon cancer cell lines and tumor growth of the SP cells. **A:** Colon cancer cell line SW480 was stained with Hoechst 33342 dye as described under *Materials and Methods*, with or without verapamil. Stained cells were analyzed using a BD FACSAria II fluorescence-activated cell sorting system. Frequency of SP cells was 3.3%. **B:** Colon cancer cell lines (HT29, HCT15, KM12LM, Lovo, and Colo320) were stained with Hoechst 33342 dye with or without verapamil. Stained cells were analyzed using a BD FACSAria II system. Frequencies of SP cells ranged from 9.1% for KM12LM cells to 11.1% for HCT15 cells. **C:** SP cells, MP cells, and presorted cells of colon cancer cell lines SW480, HT29, and HCT15 were inoculated subcutaneously into the backs of NOD/SCID mice (1×10^3 cells injected). Data are reported as means ± SD. *P* values indicate differences between cell types according to a Mann-Whitney *U*-test. **D:** Representative tumor growth in NOD/SCID mice at the SP cell injection site (1×10^3 cells injected). SP cells and MP cells were inoculated subcutaneously into the left and right side of the back, respectively.

described under *Materials and Methods*. Side population cells could be detected in all six colon cancer cell lines analyzed (ie, SW480, HT29, HCT15, Colo320, Lovo, and KM12LM) (Figure 1, A and B). The frequency of SP cells ranged from 3.3% for SW480 to 11.1% for HCT15 cells. All these SP cells were specifically inhibited by verapamil, as has been shown previously,[14] suggesting that these SP cells were specific for ABC transporter expression. Because previous studies showed that some colon cancer SP cells were not enriched with a CSC/TIC population,[15] it was essential to confirm the presence of CSCs/TICs in SP cells for further analysis. We inoculated these SP cells subcutaneously into the back of immunodeficient NOD/SCID mice using serial dilution. The SP cells derived from SW480, HCT15, and HT29 showed higher tumor initiating ability, compared with MP cells (Table 1). Furthermore, SW480, HT29, and HCT15 SP cells showed faster tumor growth, compared with MP cells (Figure 1, C and D), suggesting the presence of CSCs/TICs in these SP cells. In contrast, the SP cells derived from Colo320, Lovo, and KM12LM did not show any difference in tumorigenicity or tumor growth, compared with MP cells. We therefore restricted further analysis to the SW480, HT29, and HCT15 SP cells as colon cancer CSCs/TICs.

RT-PCR Analysis of Colon Cancer SP Cells

To examine the molecular properties of SP cells, we performed RT-PCR analysis. SOX2 and POU5F1 are representative markers for embryonal stem cells and CSCs/TICs.[16] The SP cells derived from SW480, HT29, and HCT15 showed higher expression of both SOX2 and POU5F1, compared with MP cells (Figure 2A). ALDH1A1, a colon CSC/TIC marker,[6] was expressed at a higher level in SP cells of HCT15 than in MP cells, but SP cells of SW480 and HT29 did not show any difference in comparison with MP cells. SW480 and HT29 SP cells also showed higher expression of LGR5, which is known as a normal colon stem cell marker.[17] To confirm the expression of stem cell markers, we also performed real-time PCR. The SW480 SP cells expressed 90 times higher SOX2, 7 times higher POU5F1, 153 times higher LGR5, and 6.1 times higher ALDH1A1, compared with MP cells (Figure 2B). These findings indicate that these SP cells had molecular properties similar to those of embryonal stem cells.

Resistance to Chemotherapeutic Reagents

Although SP cells derived from liver cancer cell line HuH7 have showed resistance to chemotherapy,[13] we know of no conclusive previous studies of such resistance in co-

Table 1. Tumor Initiating Ability of Colon Cancer SP Cells

Cell line (% SP cells)	Tumor initiating ability* $1 \times 10^{4\dagger}$	$1 \times 10^{3\dagger}$	$1 \times 10^{2\dagger}$
SW480 (3.3)			
SP cells	4/4	4/6	4/4
MP cells	2/4	3/5	0/4
HT29 (10.4)			
SP cells	3/3	2/3	3/3
MP cells	3/3	0/3	0/3
HCT15 (11.1)			
SP cells	3/3	3/4	3/3
MP cells	1/3	1/4	0/3
Colo320 (10.9)			
SP cells	2/2	1/2	1/2
MP cells	2/2	2/2	1/2
Lovo (9.3)			
SP cells	0/1	1/1	0/1
MP cells	1/1	0/1	0/1
KM12LM (9.1)			
SP cells	1/2	2/2	1/1
MP cells	1/2	2/2	1/1

MP, main population; SP, side population.

*Tumor initiating ability is expressed as the ratio of tumor-initiation to injection.

†The tumor initiation abilities were evaluated at day 42 after injection of the indicated number of cells.

lon SP cells. We performed a cell survival study of colon cancer SP cells using the chemotherapeutic agents irinotecan and etoposide. The SW480 and HCT15 SP cells were more resistant to both irinotecan and etoposide than were MP cells (Figure 3, A and B). This finding is consistent with findings for CSCs/TICs derived from other organs.[22,24]

Expression of HLA and Tumor-Associated Antigens in SP Cells

Because CTLs recognize tumor-associated antigen (TAA)-derived antigenic peptides presented by HLA class I molecules, expression of HLA class I molecules is essential for activation of CTLs. Several types of malignancies have been reported to lose the expression of HLA class I molecules through various mechanisms and so escape CTL attack.[18] We therefore evaluated the expression of HLA class I molecules and TAA. We assessed the differences of HLA class I and HLA-A24 expression between SP cells and MP cells by flow cytometry. Because ELISA study has revealed that HCT15 cells lack B2M because of gene mutations of *B2M*,[19] we transduced wild-type B2M cDNA into HCT15 cells and so established HCT15-B2M cells. The SW480, HT29, and HCT15-B2M SP cells showed HLA class I and HLA-A24 expression at the same level as MP cells (Figure 4, A and B). Furthermore, we assessed the expression of one of the colon cancer TAAs, CEP55, by both RT-PCR and real-time PCR (Figure 2, A and B). Both SP cells and MP cells derived from SW480, HT29, and HCT15-B2M expressed CEP55 mRNA at the same level. These data raised the possibility that SP cells are also sensitive to CTLs specific for the CEP55-derived antigenic peptide. Because both SP cells and MP cells expressed CEP55 mRNA at the same level, this appeared to be an ideal target for comparing the susceptibilities of SP cells and MP cells to CTLs.

Susceptibility of CSCs/TICs to CTLs, in Vitro *and* in Vivo

We had previously established CTL clone 41, which is specific for the cancer-related, antigen-derived, HLA-A24-restricted peptide Cep55/c10orf3_193(10).[10] CTL clone 41 recognized CEP55-positive and HLA-A24-positive cancer cells, as described previously.[10,11] In the present study, we used a ^{51}Cr release assay to examine whether CTL clone 41 can recognize SP cells. All SP cells derived from SW480, HT29, and HCT15-B2M showed susceptibility to CTL clone 41 at the same level as the MP cells and the presorted bulk cell lines (Figure 4C). This indicates that the colon CSCs/TICs were sensitive to CTLs.

To analyze the cytotoxic activity of the CTL clone *in vivo*, we performed a Winn assay. SW480 SP cells with or without CTL clone 41 were injected into the backs of NOD/SCID mice subcutaneously. CTL clone 41 significantly inhibited the tumorigenicity of SW480 SP cells (Figure 4D). Because CTLs were injected at the same time and place as the SP cells in the Winn assay, we could not rule out the possibility that SP cells were killed *in vitro*. We

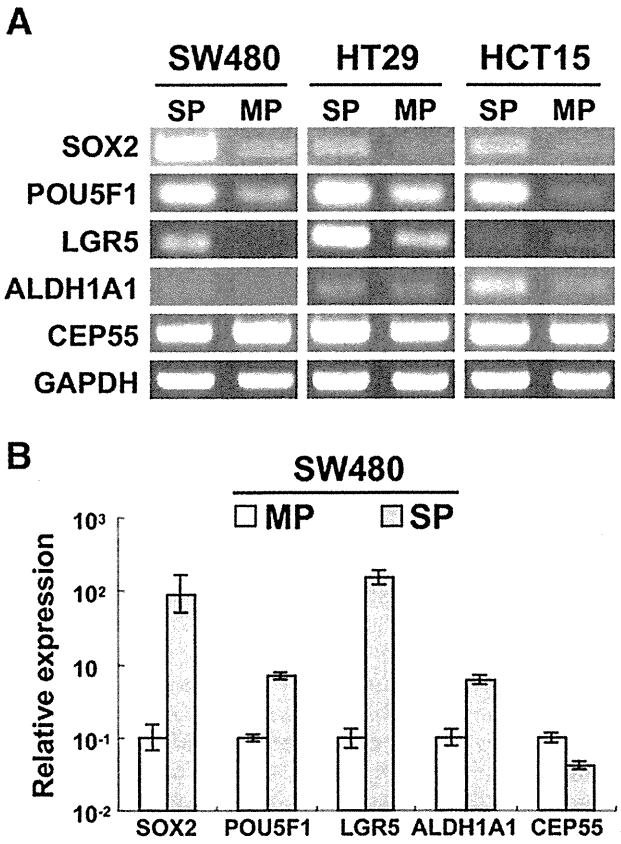

Figure 2. Expression of stem cell markers in SP and MP cells. **A:** mRNAs purified from SW480, HT29, and HCT15 SP and MP cells were analyzed by RT-PCR. **B:** mRNA purified from SW480 SP cells and MP cells were analyzed with real-time PCR. mRNA expression level is relative to MP cells. Data are reported as means ± SD.

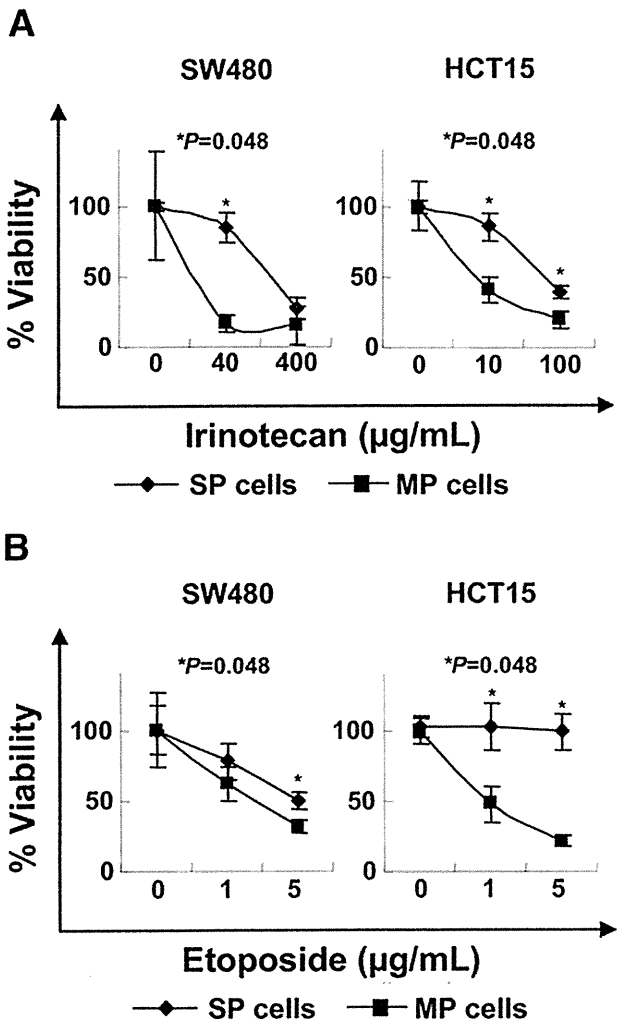

Figure 3. Sensitivity to chemotherapeutic agents. SP cells and MP cells derived from SW480 and HCT15 were incubated in the presence of irinotecan (CPT-11) (**A**) or etoposide (VP-16) (**B**) for 3 days. After incubation, the cell viabilities were measured by WST-1 assay. Data are reported as means ± SD. Differences between SP cells and MP cells were examined for statistical significance using the Mann-Whitney *U*-test.

therefore used an adoptive transfer model, as described under *Materials and Methods*. SW480 SP cells were inoculated into the back of NOD/SCID mice subcutaneously. Three weeks later, after confirmation of palpable tumors, CTLs were injected intravenously. Tumors of CTL-injected mice were significantly inhibited in growth, compared with tumors of control mice (Figure 4E). These data indicate that CTLs could recognize CSCs/TICs both *in vitro* and *in vivo*.

Discussion

In the present study, we successfully isolated colon cancer CSCs/TICs as SP cells, using Hoechst 33342 staining. Side population cells were first described by Goodell et al,[12] and CSCs/TICs of several types of malignancies were successfully isolated as SP cells in subsequent studies.[14,20–24] Haraguchi et al[13] isolated SP cells from gastrointestinal cancer cell lines; they reported the gene expression profiles and resistance to chemotherapeutic agents of SP cells derived from liver cancer cell line Huh7, but did not determine their tumorigenicity. Burkert et al[15] found that SP cells derived from gastrointestinal cancers cell lines HT29, HGT101, Caco2, and HRA19a1.1 were not enriched with a CSC/TIC population. In the present study, we were able to isolate SP cells from all six colon cancer cell lines studied (SW480, HT29, HCT15, KM12LM, Lovo, and Colo320). However, in only three of the six cell lines did the SP cells show higher tumorigenicity than MP cells, suggesting that these SP cells were enriched with CSC/TIC populations. Thus, SP cells might not be the definitive phenotype of CSCs/TICs, and confirmation of tumorigenicity in immunodeficient mice is essential for validation of SP cells as a source of CSCs/TICs. In the present study, the SP cells derived from SW480, HCT15, and HT29 cells were confirmed to be enriched with CSCs/TICs. Furthermore, these SP cells expressed stem cell markers, including SOX2, POU5F1 and LGR5, at higher levels than MP cells, suggesting correspondence with CSCs/TICs. Thus, these SP cells would be a useful tool for analysis of colon CSCs/TICs.

In the present study, we evaluated the immunogenicity of colon CSCs/TICs. Colon cancer CSCs/TICs expressed HLA class I molecules, and also CEP55, which is one of the TAAs. Furthermore, colon CSCs/TICs expressed several other TAA-encoding genes (data not shown), including BIRC5 (encoding apoptosis inhibitor survivin), BIRC7 (encoding livin), WT1, CTAG1B (alias *NY-ESO-1*), and MAGEA4. As a novel finding, colon cancer CSCs/TICs were sensitive to CTLs both *in vitro* and *in vivo*. Recently, Todaro et al[25] showed that colon CSCs/TICs were sensitive to γδT cells. Because both CTLs and γδT cells kill target cells through secretion of perforin (encoded by the *PRF1* gene) and granzyme B (encoded by *GZMB*), these observations strongly suggest that CSCs/TICs are sensitive to PRF1- and GZMB-dependent apoptosis. Todaro et al[26] had earlier reported that PROM1-positive ($CD133^+$) colon cancer CSCs/TICs secrete IL-4 in an autocrine manner and upregulate the antiapoptotic proteins CFLAR (c-FLIP), BCL2L1 (Bcl-xL), and PEA15 (PED), thereby gaining resistance to chemotherapeutic agents. Saigusa et al[27] reported that distant recurrence of rectal cancer after chemotherapy was related to the expression of CSC/TIC markers such as PROM1 (CD133), POU5F1 (Oct3/4), and SOX2. These reports support the idea that colon CSCs/TICs are resistant to apoptotic cell death. The fact that immunocytes induce apoptosis in their target cells raises the question of whether colon CSCs/TICs are also sensitive to immunotherapy.

In the present study, and in that of Todaro et al,[25] colon CSCs/TICs were sensitive to perforin- and granzyme B-dependent apoptosis. Thus, both CTLs and γδT cells can be useful tools for colon CSC/TIC targeting therapy. However, because γδT cells do not recognize target cells in an antigen-specific manner, immunotherapy using γδT cells should also recognize the non-CSC/TIC population. Because the number of γδT cells is restricted *in vivo*, it may be in doubt whether γδT cell can recognize colon

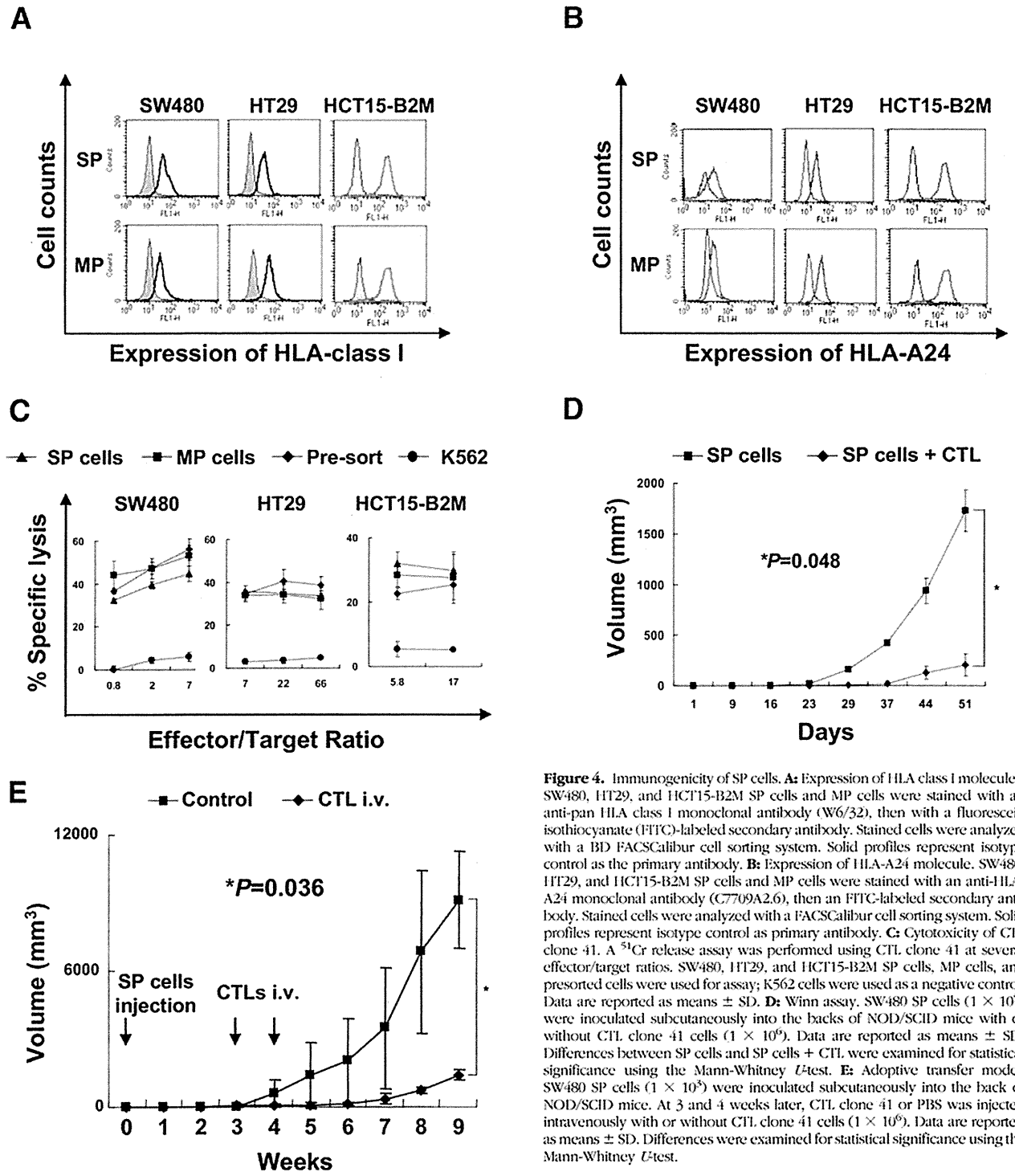

Figure 4. Immunogenicity of SP cells. **A:** Expression of HLA class I molecules. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-pan HLA class I monoclonal antibody (W6/32), then with a fluorescein isothiocyanate (FITC)-labeled secondary antibody. Stained cells were analyzed with a BD FACSCalibur cell sorting system. Solid profiles represent isotype control as the primary antibody. **B:** Expression of HLA-A24 molecule. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-HLA-A24 monoclonal antibody (C7709A2.6), then an FITC-labeled secondary antibody. Stained cells were analyzed with a FACSCalibur cell sorting system. Solid profiles represent isotype control as primary antibody. **C:** Cytotoxicity of CTL clone 41. A ^{51}Cr release assay was performed using CTL clone 41 at several effector/target ratios. SW480, HT29, and HCT15-B2M SP cells, MP cells, and presorted cells were used for assay; K562 cells were used as a negative control. Data are reported as means ± SD. **D:** Winn assay. SW480 SP cells (1×10^5) were inoculated subcutaneously into the backs of NOD/SCID mice with or without CTL clone 41 cells (1×10^6). Data are reported as means ± SD. Differences between SP cells and SP cells + CTL were examined for statistical significance using the Mann-Whitney *U*-test. **E:** Adoptive transfer model. SW480 SP cells (1×10^5) were inoculated subcutaneously into the back of NOD/SCID mice. At 3 and 4 weeks later, CTL clone 41 or PBS was injected intravenously with or without CTL clone 41 cells (1×10^6). Data are reported as means ± SD. Differences were examined for statistical significance using the Mann-Whitney *U*-test.

cancer CSCs/TICs *in vivo* efficiently. Recently, based on a large cohort study, Ogino et al[28] reported that lymphocytic reaction to tumor was associated with longer survival of colorectal cancer patients. They did not analyze the subtypes of infiltrating lymphocytes; however, the findings from this large-scale study strongly support the notion that immune reaction to tumor cells is important for control of the disease.

Wei et al[29] reported recently that glioma-derived CSCs/TICs suppressed T-cell proliferation and activation, and induced T-cell apoptosis through expression of co-stimulatory inhibitory molecule CD274 (B7-H1) and soluble LGALS3 (galectin-3); glioma CSCs/TICs enhance the induction of regulatory T cells. We also observed that SW480 SP cells express higher mRNA of the immunosuppressive cytokine IL-10 than MP cells (data not

shown). Thus, colon CSCs/TICs may have immunosuppressive potential and so inhibit CTL induction. However, colon CSCs/TICs are efficiently killed by CTLs, and colon CSCs/TICs have no influence on the effector phase of CTLs. Thus, adoptive cell transfer of CSC/TIC-specific CTL clones, T-cell-receptor–induced T cells, or peptide vaccination accompanied by an anti-IL-10 monoclonal antibody might be an effective approach for eliminating colon CSCs/TICs.

In the present study, we observed that both colon CSCs/TICs and non-CSCs/TICs were sensitive to CEP55-specific CTLs at the same level. This finding seems reasonable, given that CSCs/TICs express CEP55 mRNA at the same level. Huge numbers of TAAs have already been reported,[30,31] and the next challenge is to identify which TAAs would be the most suitable targets for cancer immunotherapy. According to the manner of expression in CSCs/TICs and non-CSCs/TICs, TAAs can be classified into three categories: i) CSC/TIC-specific antigens, such as SOX2 and ALDH1A1; ii) non-CSC/TIC-specific antigens; and iii) shared antigens, such as CEP55.[32] The frequencies of colon CSCs/TICs are 1% to 10%, and in the present study these cells had 10- to 100-fold higher tumorigenicity than non-CSCs/TICs. It is likely, therefore, that 1% to 10% of colon CSC/TIC populations have almost the same tumorigenic potential as 90% to 99% of the non-CSC/TIC population. To achieve a complete cure of the disease, shared antigens seem to a be reasonable candidate strategy. *In vivo*, however, CTL numbers are limited. Given that 1 L of peripheral blood contains approximately 5×10^9 lymphocytes, there are approximately 5×10^8 CD8 T cells in 1 L of peripheral blood and approximately 3×10^9 CD8 cells in the total volume of peripheral blood in a human adult. If the CTL precursor frequency reaches 0.1% of CD8 T cells in a patient receiving peptide vaccination therapy, then the total peptide-specific CTLs can be calculated as 3×10^6 cells in whole blood. This is not an inconsiderable number. Visible tumors as large as 1 cm diameter contain 1×10^9 tumor cells, and the estimated effector/target ratio (E/T) *in vivo* is 0.003. This ratio may be too low to expect an anti-tumor effect *in vivo*. However, if we focus only on CSCs/TICs, then the effector/target ratio will be improved. For targeting CSCs/TICs with 1% frequency, the effector/target ratio is correspondingly improved (E/T = 0.3). Thus, focusing only on the CSC/TIC population with CSC/TIC-specific antigens seems to be a better approach for advanced cancer cases. For prevention of disease recurrence after treatment, the target cells are likely to be limited, so shared antigens might be a reasonable choice for cancer immunotherapy.

Recently, some research groups have reported that monoclonal antibodies for insulin-like growth factor-1 receptor (IGF-1R), δ-like 4 ligand (DLL4), and CD47 efficiently eliminate colon cancer and leukemia CSCs/TICs.[33–36] These approaches are also fascinating, and a reasonable option for elimination of CSCs/TICs. An antibody is a relatively stable protein, but the half-life in peripheral blood is approximately 2 to 3 weeks, and therefore serial administration is needed to maintain the effects of the antibody. On the other hand, antigenic peptide vaccination can induce specific CTLs as memory cells *in vivo*, such that the specific immunity will last for several years. Thus, peptide vaccination therapy may also be useful for prevention of post-treatment cancer recurrence.

In conclusion, we report here the novel finding that colon cancer CSCs/TICs are as sensitive to CTLs as are non-CSCs/TICs, and that CEP55, a tumor-associated antigen, is a suitable antigen for targeting colon cancer CSCs/TICs.

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Immunopathology and Infectious Diseases

Arachidonate 5-Lipoxygenase Establishes Adaptive Humoral Immunity by Controlling Primary B Cells and Their Cognate T-Cell Help

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In this study, we report the unique role of arachidonate 5-lipoxygenase (Alox5) in the regulation of specific humoral immune responses. We previously reported an L22 monoclonal antibody with which human primary resting B cells in the mantle zones of lymphoid follicles are well-defined. Proteomics analyses enabled identification of an L22 antigen as Alox5, which was highly expressed by naive and memory B cells surrounding germinal centers. Cellular growth of mantle cell lymphoma cells also seemed to depend on Alox5. *Alox5*$^{-/-}$ mice exhibited weak antibody responses specific to foreign antigens at the initial and recall phases. This was probably attributable to the low number of follicular and memory B cells and the functional loss of interleukin-21–mediated responses of follicular B cells. Moreover, *Alox5*$^{-/-}$ mice could not fully foster the development of follicular B helper T (Tfh) cells even after immunization with foreign antigens. Further experiments indicated that Alox5 affected mortality in experimentally induced enterocolitis in germ-prone circumstances, indicating that Alox5 would endow immunologic milieu. Our results illustrate the novel role of Alox5 in adaptive humoral immunity by managing primary B cells and Tfh cells *in vivo*. *(Am J Pathol 2011, 178: 222–232; DOI: 10.1016/j.ajpath.2010.11.033)*

Regulation of specific humoral responses directs a center of adaptive immunity to prevent infection, autoimmune disease, and cancer.[1,2] Mechanisms underlying antibody production wholly depend on the cellular function of B cells. After the somatic mutation of genes encoding B-cell receptor (BCR) and its editing in the bone marrow, B cells are distributed to the peripheral lymphoid tissues, where they reside as primary resting B cells.[3,4] On stimulation of the surface BCRs and with cognate help from CD4$^+$ T cells, primary B cells differentiate into germinal center B cells, where somatic hypermutations and class switch recombination occur, resulting in further differentiation into plasma cells or memory B cells. It is considered that for the sake of efficient production of specific antibodies, primary resting B cells around germinal centers keep BCR repertories as a major anatomical source of lymphoid follicles.[5] By the function of antiapoptotic factors, such as Bcl-2, it is thought that primary B cells would survive and eventually maintain BCR repertoires in individuals.[6] However, the mechanism underlying primary B-cell regulation has not yet been elucidated.

Previously, we created an L-series of monoclonal antibodies (mAbs) for use in the identification of immune cells by means of immunohistochemical analysis or flow cytometry.[7–9] The L-series panel includes L26 mAbs, which specifically bind to the cytoplasmic domain of CD20. This mAb is widely used for immunopathologic examinations of lymphoid tissue to decipher B-cell–related disorders, including malignancies and immune-associated diseases.[7,10] L22 mAbs have exhibited a unique distribution profile: L22 antigen (Ag) is specifically expressed in the cytoplasm of primary resting B cells in the mantle zones of lymphoid follicles but not in activated B cells of germinal centers and in T cells.[8] Therefore, it is of great interest to investigate the nature of L22 Ag because we consider that its functional features may provide new fundamental insights into the regulatory mechanism of adaptive humoral immune responses.

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Herein, we first identified an L22 Ag as arachidonate 5-lipoxygenase (Alox5) and elucidated its unique role of coordinating specific antibody responses. Alox5 oxidizes arachidonic acid as a substrate initially to produce 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid, which is an intermediate for various leukotrienes of lipid mediators.[11] We observed that naive ($CD19^+CD23^+CD27^-CD38^-$) and memory ($CD19^+CD23^-CD27^+CD38^-$) phenotypes of human primary B cells in the mantle zones preferentially expressed Alox5.[12] Studies of *Alox5*$^{-/-}$ mice revealed that this enzyme was required for the humoral response against T-cell–dependent foreign antigens at the initial and recall phases. This was probably because of the loss of follicular B cells ($B220^+CD21^{int}CD23^+$) and memory B cells ($B220^+IgG1^+NP^+$) due to cell death in *Alox5*$^{-/-}$ mice.[13] Furthermore, mantle cell lymphoma (MCL) would depend on the growth activities of Alox5, implying a growth regulatory role of Alox5 in normal primary B cells and their derived tumor cells. Moreover, note that *Alox5*$^{-/-}$ mice showed fewer germinal center cells after immunization of the foreign antigens and developmental insufficiency of follicular B helper T (Tfh) cells ($CD3^+CD4^+CD25^-CXCR5^+ICOS^+$).[14,15] Experimental evidence has revealed that *Alox5*$^{-/-}$ and recombination activating gene $1^{-/-}$ (*Rag1*$^{-/-}$) chimera mice were fairly susceptible to the condition of enterocolitis, especially in conventional facilities; this reiterates the pivotal role of Alox5 in the regulation of specific antibody responses. In addition to the evidence indicating the possible role of Alox5 in MCL, these and further investigations may shed light on the association of lipid metabolism of arachidonic acid by Alox5 with the regulation of adaptive humoral immunity and the nature of MCLs.[16–18]

Materials and Methods

Tissues and Cell Lines

All tissues were obtained after receiving informed consent from the subjects and with the approval of the Institutional Review Board. Tonsillar tissues were obtained from patients undergoing tonsillectomy for the treatment of recurrent tonsillitis, and lymphoma tissues were obtained by means of biopsy. Portions of the tissues were stored at −80°C as frozen tissue sections. Human embryonic kidney (HEK) 293 and P1.4 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μg/ml of streptomycin, and 100 U/ml of penicillin. Cell lines derived from MCL, including G519, HBL2, JEKO1, MINO, and REC1, as well as Jurkat, Molt4, and Daudi cells, were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 2 mmol/L l-glutamine, 50 μg/ml of streptomycin, and 100 U/ml of penicillin. All cells were cultured at 37°C in a humidified atmosphere with 5% carbon dioxide.

Mice and Immunization

All the experiments were performed in accordance with the institutional guidelines for the care and use of animals. *Alox5*$^{-/-}$ mice[19] and C57BL/6-SV129S F1 hybrid mice used as a control were purchased from The Jackson Laboratory (Bar Harbor, ME). All these mice were maintained in specific pathogen-free or conventional animal facilities of Sapporo Medical University School of Medicine. Age- (6 to 12 weeks) and sex-matched mice from each group, which were simultaneously raised in the same environment at the facilities, were used for the experiments. Lin^-Scal^+ bone marrow stem cells of *Alox5*$^{+/+}$ or *Alox5*$^{-/-}$ mice were transferred into recombination activating gene 1–deficient mice (C57BL/6 background; The Jackson Laboratory) to generate *Rag1*$^{-/-}$ bone marrow chimera mice. Eight weeks after stem cell transplantation, levels of serum Igs of *Rag1*$^{-/-}$ chimera mice were examined before being used for experiments. Mice were immunized by means of i.p. administration of 200 μL of 5% sheep red blood cells (SRBCs) or 100 μg of (4-hydroxy-3-nitrophenylacetyl)36 coupled to chicken gamma globulin (NP36-CGG) (with alum at initial immunization).

Reagents

L22 (clone TB1-2C3) and TE4 mAbs were purified from mouse ascitic fluid and were used for immunoprecipitation and immunohistochemical analysis. The following antihuman mAbs were purchased from BD Biosciences (San Jose, CA): fluorescein isothiocyanate (FITC)–anti-CD3 (clone SK7), FITC–anti-CD4 (RPA-T4), FITC–anti-CD8 (clone RPA-T8), antigen-presenting cell (APC)–anti-CD19 (clone HIB19), phycoerythrin (PE)-Cy7–anti-CD25 (clone MA251), PE–anti-CD27 (clone MT271), FITC–anti-CD38 (clone HIT2), PE–anti-CD278 (ICOS; clone DX29), biotin–anti-CXCR5 (clone RF8B2), FITC–anti-IgD (clone IA6-2), FITC–anti-IgM (clone G20-127), FITC–anti-IgG (clone G18-145), and Ig isotype control mAbs. Rabbit antihuman-CD23 mAbs (clone SP23), antihuman-Bcl-2 polyclonal antibodies (pAbs), and anti-human-Alox5 pAbs were obtained from Nichirei Bioscience Inc. (Tokyo, Japan), Neomarkers Inc. (Fremont, CA), and Cayman Chemical Co (Ann Arbor, MI), respectively. The following antimouse mAbs were purchased from BD Biosciences, Affinity BioReagents Inc. (Golden, CO), eBioscience Inc. (San Diego, CA), and Genetex Inc. (Irvine, CA): biotin–anti-CD1 days (clone 1B1), anti-CD3 (hamster clone 500A2), APC–anti-CD3 (clone 145-2C11), FITC–anti-CD4 (clone L3T4), PE–anti-CD8 (clone 53-6.7), anti-CD19 mAb (clone 1D3), FITC–anti-CD21 (clone 7G6), PE–anti-CD23 (clone B3B4), PE-Cy7–anti-CD25 (clone PC61), FITC– or APC–anti-CD45R/B220 (clone RA3–6B2), PE–anti-CD278 (ICOS; clone 7E17G9), biotin–anti-CXCR5 (clone 2G8), FITC–anti-IgG1 (clone LO-MG 1–2), biotin–anti–major histocompatibility complex class II (clone M5/114.15.2), and isotype control mAbs. Horseradish peroxidase–conjugated rabbit pAbs against isotypes were purchased from Southern Biotechnology (Birmingham, AL). Mouse anti–enhanced green fluorescent protein (EGFP) mAbs (clone JL-8) and biotin-conjugated peanut agglutinin were obtained from Vector Laboratories (Burlingame, CA) and Invitrogen (Carlsbad, CA), respectively. NP-CGG, 4-hydroxy-3-nitrophenylacetyl coupled to bovine serum albumin (NP-BSA), and NP-BSA–biotin were purchased from Bioresearch Technologies Inc. (Novato, CA).

Immunohistochemical Analysis

Procedures for tissue sections were conducted as described previously.[20] In brief, tissue sections were stained with primary mAbs at 4°C overnight and then were reacted with secondary pAbs conjugated to Alexa Fluor (Molecular Probes Inc., Eugene, OR). Signals were detected using an immunofluorescence microscope (IX71; Olympus, Center Valley, PA) or a laser scanning confocal microscope (R2100AG2; Bio-Rad Laboratories, Hercules, CA).

Immunoprecipitation and Immunoblotting

Cells were treated in a lysis buffer solution consisting of 150 mmol/L sodium chloride, 50 mmol/L Tris-chloride (pH 7.5), 1 mmol/L EDTA, 0.3% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), and protease inhibitors (Roche Diagnostics GmbH) and then were subjected to immunoprecipitation or immunoblotting as described elsewhere.[21] After washing the beads, the specimens were separated by 5% to 20% gradient SDS–polyacrylamide gel electrophoresis and were stained using a silver staining MS kit (Wako Chemicals USA Inc., Richmond, VA) or blotted onto an Immobilon-P membrane (Millipore, Billerica, MA) for immunoblotting.[21]

Liquid Chromatography–Electrospray Ionization–Tandem Mass Spectrometry

Trypsin was used for in-gel digestion of the specimens, after which they were analyzed using tandem mass spectrometry (Gene World, Tokyo, Japan). The obtained peptides were further investigated through a Mascot search to predict an original protein.

Flow Cytometry and Cell Sorting

Lymphocytes were purified by means of centrifugation with Lymphoprep (human; Axis-Shield, Oslo, Norway) or Lympholyte-M (mouse; Cedarlane Laboratories Ltd, Burlington, NC) and then were stained using standard flow cytometry. After preparation of cell specimens, cells were analyzed using a FACSCanto II flow cytometer with DiVA software (BD Biosciences). A FACSAria II cell sorter was used to select and analyze follicular B cells and Tfh cells. AutoMACS (Miltenyi Biotec Inc., Auburn, CA) was also used for magnetically purifying lymphocytes from human tonsils or primitive hematopoietic stem cells ($Lin^{-}Scal^{+}$) from murine bone marrow. In each experiment, the purity of cells reached 95%. Apoptotic cells were stained by annexin V and were detected using an APC dye according to the manufacturer's protocol (BD Biosciences).

cDNA Transduction

EGFP-tagged expression plasmids carrying human Alox5, sorting nexin 5, sorting nexin 6, or autoimmune regulator were used in this study.[21,22] Plasmid DNAs were transfected into cells using LF2000 (Invitrogen).

Enzyme-Linked Immunosorbent Assay

A 96-well microtiter plate coated with SRBCs or NP-BSA was used to determine anti-SRBC and anti-NP Ig titers, as described previously.[13,23] Ig isotypes of mice were determined using isotype-specific enzyme-linked immunosorbent assay (Southern Biotechnology).

RT-PCR and Quantitative PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) and were reverse transcribed using oligo(dT) primers (Applied Biosystems, Foster City, CA). The RT-PCR primer pairs used in this study were designed as described previously and are summarized in Tables 1 and 2.[20] Real-time PCR was performed using an ABI-PRISM 7900 according to the manufacturer's protocol (Applied Biosystems). Target gene expression was calculated using ΔΔCT and comparative methods after normalization to 18S RNA or glyceraldehyde-3-phosphate dehydrogenase expression.

Induction of Enterocolitis

Experimental enterocolitis was induced in mice by supplementing drinking water with 1.5% dextran sodium sulfate (DSS; ICN Biomedicals, Irvine, CA), and the mice were monitored daily for survival as described previously.[24] For evaluating histologic findings, the middle parts of the colon were removed and fixed with 10% formalin containing PBS. Then, paraffin-embedded tissue sections were stained with H&E to examine histologic changes. The histologic score was examined as follows: epithelium (E), 0

Table 1. Mouse PCR Primer Pairs Used in This Study

Mouse primers	Forward primer	Genomic region	Reverse primer	Genomic region	Expected length (bp)
IL-6	5′-AGTTGCCTTCTTGGGACTGA-3′	Exon 2	5′-CAGAATTGCCATTGCACAA-3′	Exon 3	191
IFN-γ	5′-ACTGGCAAAAGGATGGTGAC-3′	Exon 2–3	5′-TGAGCTCATTGAATGCTTGG-3′	Exon 4	237
IL-4Rα	5′-CCTCACACTCCACACCAATG-3′	Exon 6	5′-AGCCTGGGTTCCTTGTAGGT-3′	Exon 7	168
IL-21R	5′-TGTCAATGTGACGGACCAGT-3′	Exon 4	5′-CACGTAGTTGGAGGGTTCGT-3′	Exon 5	163
IFN-γR1	5′-TCCTGCACCAACATTTCTGA-3′	Exon 3	5′-TACGAGGACGGAGAGCTGTT-3′	Exon 4	213
IFN-γR2	5′-GCTTCACCCTGTTCCTCAAA-3′	Exon 6	5′-AGCACATCATCTCGCTCCTT-3′	Exon 7	205
TLR4	5′-GCTTTCACCTCTGCCTTCAC-3′	Exon 3	5′-GAAACTGCCATGTTTGAGCA-3′	Exon 3	174
TLR7	5′-CCTGTTCTACTGGGGTCCAA-3′	Exon 3	5′-GCCTCAAGGCTCAGAAGATG-3′	Exon 3	167
TLR9	5′-GCTTTGGCCTTTCACTCTTG-3′	Exon 2	5′-AACTGCGCTCTGTGCCTTAT-3′	Exon 2	194
Alox5	5′-CTACGATGTCACCGTGGATG-3′	Exon 2	5′-GTGCTGCTTGAGGATGTGAA-3′	Exon 3	235

Table 2. Human PCR Primer Pairs Used in This Study

Human primers	Forward primer	Genomic region	Reverse primer	Genomic region	Expected length (bp)
BLT1	5′-TTGCTCACTGCTCCCTTTTT-3′	Exon 2	5′-AAAGGACAACACCCAGATGC-3′	Exon 2	237
BLT2	5′-GAGACTCTGACCGCTTTCGT-3′	Exon 1	5′-AAGGTTGACTGCGTGGTAGG-3′	Exon 1	183
CysLTR1	5′-TGATGACTTCCGCAATCAAG-3′	Exon 3	5′-AGCCAAATGCCTTTGTGAAC-3′	Exon 3	216
CysLTR2	5′-TCCACTTGACGACATGGAAA-3′	Exon 1	5′-GGCCTTTTCTGAGTGCAGAC-3′	Exon 1	165
MAF	5′-TGGAGTCGGAGAAGAACCAG-3′	Exon 1	5′-GCTTCCAAAATGTGGCGTAT-3′	Exon 2	228
BCL-6	5′-AACCTGAAAACCCACACTCG-3′	Exon 8	5′-TGACGGAAATGCAGGTTACA-3′	Exon 9	245
CXCR5	5′-CTCCCAAGAGAACCAAGCAG-3′	Exon 2	5′-CCAGCAGAGGAAGAAGATGC-3′	Exon 2	205
IL-21	5′-TTCTGCCAGCTCCAGAAGAT-3′	Exon 2	5′-TTGTGGAAGGTGGTTTCCTC-3′	Exon 3	153

= normal morphologic features; 1 = loss of goblet cells; 2 = loss of goblet cells in large areas; 3 = loss of crypts; and 4 = in large areas; infiltration (I), 0 = no infiltrate; 1 = infiltrate around the crypt basis; 2 = infiltrate reaching the lamina muscularis mucosae; 3 = extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; and 4 = infiltration of the lamina submucosa. The total histologic score was obtained by E + I.

Cell Growth Assay

Growth activities of cultured cells were investigated using a premix WST-1 cell proliferation assay system as per the manufacturer's instructions (Takara Bio Inc., Shiga, Japan).

Statistical Analysis

Results are expressed as mean and SE; the unpaired *t*-test was used to compare experimental groups unless otherwise stated, with $P < 0.05$ considered significant.

Results

L22 Ags in Primary B Cells Are Identical to Alox5

Mouse L22 mAbs were originally established by immunizing mice with human tonsillar lymphocytes per standard procedures.[8] Immunohistochemical analysis of tonsillar tissues demonstrated preferential distribution of L22 Ags in the cytoplasm of primary resting B cells in the mantle zones of germinal centers in lymphoid tissues (Figure 1, A and B).[25] We further examined the colocalization of L22 Ags with CD23, which was restricted to the IgM^+ or IgD^+ naive B-cell phenotype and were subsequently lost in germinal center and memory B cells.[5,26,27] Primary B cells of the mantle zones were found to contain a mixed population of $L22^+CD23^+$ and $L22^+CD23^-$ cells, indicating that primary B cells around germinal centers consisted of $CD23^+$ naive B cells and $CD23^-$ memory B cells, both of which presented L22 Ags (Figure 1C). Follicular dendritic cells of germinal centers also present CD23; however, L22 Ags were not expressed in $CD23^+$ follicular dendritic cells within germinal centers.[28] Collectively, L22 Ags were expressed by primary B cells with naive and memory phenotypes but not in follicular dendritic cells.

To identify the molecular nature of L22 Ags, we initially used L22 mAbs to perform immunoprecipitation on tonsillar lymphocytes and cell lines. After trials with lysis buffers containing different types of detergents and under different experimental conditions, a clear band was detected at approximately 78 kDa (Figure 1D). Such a band was also detected in Daudi B cells but not in Jurkat T cells and P1.4 thymic epithelial cells; these results concur with the tissue distribution of L22 Ags in human lymphoid tissues of the tonsils and thymus. Proteomics analysis of the protein band derived from tonsillar lymphocytes revealed the presence of at least four different peptides, all of which were completely matched to a core protein sequence of Alox5 (Figure 1E). Further immunoprecipitation and immunostaining experiments in which a plasmid DNA encoding EGFP-tagged Alox5 was introduced into HEK 293 cells indicated the binding specificity of L22 mAbs to Alox5 (Figure 1, F and G).

Primary B Cells Are Susceptible to Functional Loss of Alox5

To investigate the tissue distribution of L22 Ags, primary resting naive ($CD19^+CD27^-CD38^-$) and memory ($CD19^+CD27^+CD38^-$) B cells were sorted and analyzed (see Supplemental Figure S1 at *http://ajp.amjpathol.org*).[27,29] Quantitative PCR and immunoblot analyses demonstrated high Alox5 expression in naive and memory B cells, which were also noted to be with or without CD23, respectively (Figure 2, A and B). Meanwhile, germinal center B cells ($CD19^+CD27^-IgD^+$) expressed very low levels of Alox5; these results are in accordance with those shown in Figure 1, A–C. However, Alox5 was not detected in $CD4^+$ and $CD8^+$ T cells.

To further address the functional role of Alox5 in B cells, we cultured tonsillar B cells ($CD19^+$) with or without Alox5 inhibitors of MK886 or AA861 and studied the B-cell population therein. We observed that primary B cells easily perished in the presence of MK886 or AA861 (Figure 2C). Taking this result into account, we considered Alox5 to be a potential regulator of the maintenance of human naive and memory B cells.

By means of two different types of receptors, namely, BCRs and Toll-like receptors (TLRs), B cells sense various antigens.[2–6,30] When BCRs of tonsil-derived B cells were stimulated, the cells could secrete leukotrienes (data not shown).[11,31] This indicates that leukotrienes derived from naive and memory B cells, by affecting B cells themselves or surrounding immune cells in lymphoid tissues, might act as a prompt dispatch mode to the subsequent antibody response.

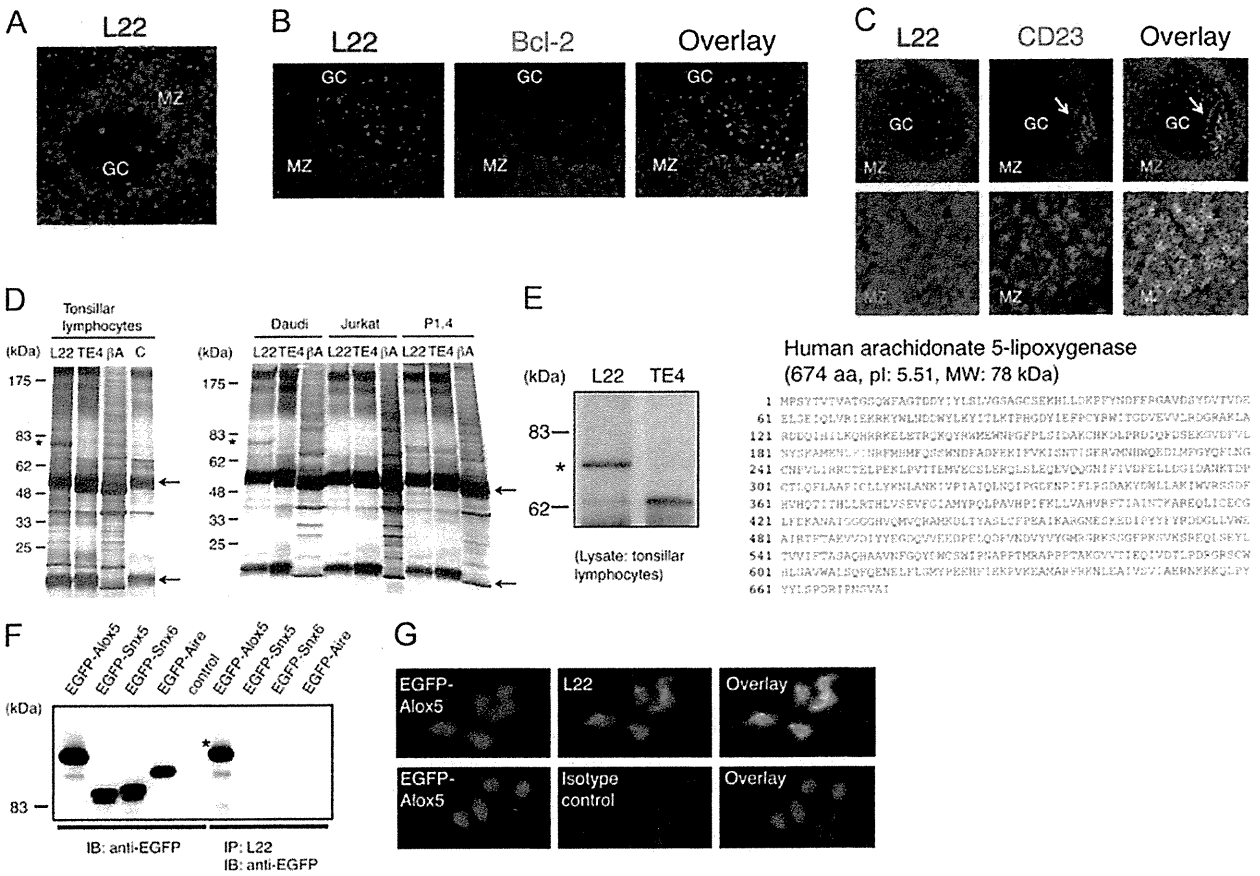

Figure 1. Mantle zone B cells of lymphoid tissues highly express Alox5. **A–C:** Immunohistochemical analysis of lymphoid follicles of tonsils with L22 mAbs. **A:** Mantle zone B cells around germinal centers express L22 Ag (green). Original magnification, ×200. **B:** Mantle zone B cells with L22 Ag (green) simultaneously express Bcl-2 (red). Original magnification, ×200. **C:** The mantle zone exhibits a mixed population of $L22^+CD23^+$ and $L22^+CD23^-$ B cells. **Upper panel** shows the lymphoid follicle containing follicular dendritic cells (**arrows**). **Lower panel** focuses on the mantle zone. Original magnification: ×200 (**upper panel**); ×400 (**lower panel**). In **A, B,** and **C,** the mantle zone and germinal center are represented as MZ and GC, respectively. The large L22-expressing cells within the GC are macrophages. **D:** Immunoprecipitation analysis of tonsillar lymphocytes and cell lines with L22 mAbs. After separation of immunoprecipitates, the proteins were visualized by silver staining. The **left** and **right panels** demonstrate bands that resulted from the lymphocytes of tonsils and cell lines, including Daudi B cells, Jurkat T cells, and P1.4 thymic epithelial cells. The band that specifically reacts to L22 mAbs is indicated by **asterisks** in each panel. L22, L22 mAbs; TE4, antithymic medullary epithelium mAbs; βA, anti–β-actin mAbs; C, isotype control. **Arrows** indicate light or heavy Ig chains bound to beads. **E:** Proteomics analysis of L22 Ags for identifying Alox5. Mass spectrometry of the band is indicated by an **asterisk** [**left panel**; same as (**D**)] revealed four different peptide sequences, including GVDFVLNYSK, AMENLFINR, YDWLLAK, and FTIAINTK. The protein sequence of Alox5 is shown in the **right panel**, where the four peptides are depicted in red, as directed by a Mascot search. **F:** Immunoprecipitation analysis of EGFP-tagged Alox5 and other human proteins with L22 mAb. HEK 293 cells were transiently transfected with a plasmid expressing EGFP-Alox5, EGFP–sorting nexin 5 (Snx5), EGFP–sorting nexin 6 (Snx6), or EGFP–autoimmune regulator (Aire), with expected molecular weights of 118, 86, 88, and 98 kDa, respectively. L22 mAbs bind to EGFP-Alox5 (**asterisk**) but not to other EGFP-tagged proteins. **G:** Immunohistochemical analysis of HEK 293 cells expressing EGFP-Alox5 with L22 mAb. L22 mAb (red) reacts to cells transiently expressing EGFP-Alox5 (green). Original magnification, ×400.

Alox5 Is Required for Specific Humoral Immune Responses

Next, we immunized *Alox5*$^{-/-}$ mice with various antigens and investigated their sera and immune cells. When SRBCs were administered as T-cell–dependent foreign antigens, these mice showed impaired production of SRBC-specific antibodies at the initial and recall phases (Figure 3, A and B). To further examine the role of Alox5 in B cells, we established bone marrow chimeras of *Rag1*$^{-/-}$ mice lacking B and T cells. Lin^-Scal^+ cells from the bone marrow of *Alox5*$^{+/+}$or *Alox5*$^{-/-}$ mice were transplanted into *Rag1*$^{-/-}$ mice (termed *Rag1*^{WT} or *Rag1*^{Alox5} mice, respectively). When immunized with SRBC, *Rag1*^{Alox5} could not fully produce SRBC-specific antibodies, whereas *Rag1*^{WT} mice could (Figure 3D). These results strongly suggested the requirement of Alox5 to produce specific antibodies from B cells.

Most Ig subclasses examined were at normal serum levels in unimmunized *Alox5*$^{-/-}$ mice, suggesting that Alox5 was not necessary to produce naturally occurring Igs (Figure 3C). To our surprise, however, IgG2a levels dependent on type 1 helper T cells were significantly reduced in *Alox5*$^{-/-}$ mice even after SRBC immunization (Figure 3, A and C). Similarly, *Rag1*^{Alox5} mice demonstrated low levels of IgG2a compared with *Rag1*^{WT} mice (data not shown). The inability of IgG2a production by *Alox5*$^{-/-}$ and *Rag1*^{Alox5} mice suggests the possible involvement of Alox5 in an unknown mechanism of class switching of the IgG2a heavy chain within B cells or of insufficient effects from neighboring cells such as helper T cells.[32,33]

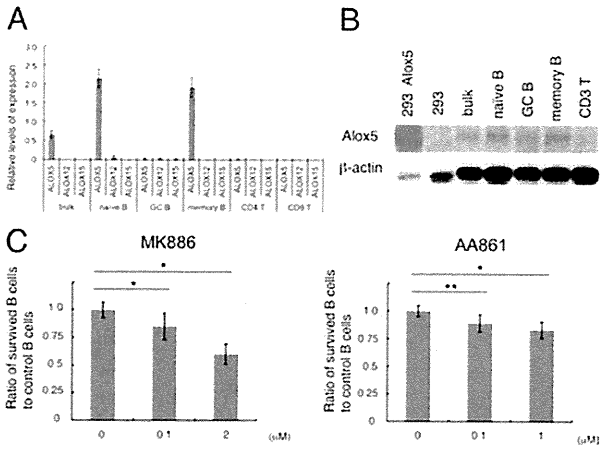

Figure 2. Expression and function of Alox5 in primary resting B cells. **A:** Quantitative PCR analysis of Alox5 in tonsillar lymphocytes. On the basis of the oxidation site of arachidonic acid, lipid oxidation enzymes are categorized into three types: Alox5, Alox12, and Alox15. Naive and memory B cells preferentially express transcripts of Alox5 but not Alox12 and Alox15. **B:** Immunoblotting analysis of Alox5 in tonsillar lymphocytes. Alox5 is abundantly expressed in naive and memory B cells. HEK 293 cells and their transient transformants expressing Alox5 were used as controls. **C:** B cells require Alox5 for their survival. 2×10^6 $CD19^+$ B cells from tonsils were cultured in serum-free AIM V medium (Invitrogen) in each well of a 24-well plate with or without MK886 or AA861 for 96 hours, and then flow cytometric analysis of B cells was performed. The data are demonstrated as the ratios of the $CD19^+$ population in the control media to these cells in the inhibitor-containing media, where the cells were counted as $CD19^+$ cells. Results are representative of three to four independent experiments. $^*P < 0.1$, $^{**}P < 0.05$.

Furthermore, we investigated the role of Alox5 in the regulation of memory-specific humoral responses. When the mice were administered NP36-CGG, although memory B cells with the $B220^+IgG1^+NP^+$ phenotype were indeed found in *Alox5*$^{-/-}$ mice, the number of memory B cells in *Alox5*$^{-/-}$ mice was almost half the number in *Alox5*$^{+/+}$ mice (Figure 3E). The serum titer of NP25-BSA or NP3-BSA specific IgG1 increased in *Alox5*$^{+/+}$ mice in response to the second challenge of NP36-CGG; however, this titer did not increase to the same extent in *Alox5*$^{-/-}$ mice, as observed in the SRBC immunization experiment (Figure 3F). Meanwhile, there were no significant differences in a ratio of the titer of NP25-BSA to that of NP3-BSA in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice at each date of investigation (Figure 3G). Therefore, Alox5 might regulate the number of memory B cells, whereas Alox5 would not be required for the processes of affinity maturation.[23]

Alox5 Regulates Survival and Function of Follicular B Cells

As previously reported, the spleen is relatively smaller in *Alox5*$^{-/-}$ mice than in wild-type mice (Figure 4A).[19] When immunized with SRBCs, the spleen of *Alox5*$^{+/+}$ mice gradually increased in weight up to approximately double the original weight. Immunization also increased the spleen weight in *Alox5*$^{+/-}$ and *Alox5*$^{-/-}$ mice; however, the spleens were smaller and their weights did not reach the weight attained by *Alox5*$^{+/+}$ mice. Inverted microscopy revealed that lymphoid nodules of the spleen did not develop well in *Alox5*$^{+/-}$ and *Alox5*$^{-/-}$ mice even after administration of SRBCs (Figure 4B). Indeed, immunohistochemical studies revealed fewer mature germinal centers in the lymphoid follicles in the spleen of SRBC-immunized *Alox5*$^{-/-}$ mice (Figure 4C). Furthermore, flow cytometry analysis indicated reduced numbers of germinal center cells ($B220^+PNA^+IgD^-$) in SRBC-immunized *Alox5*$^{-/-}$ mice (Figure 4D). These results suggest that the weak humoral responses to foreign antigens in *Alox5*$^{-/-}$ mice may be caused by inability to generate sufficient germinal centers.

Freshly isolated spleens of *Alox5*$^{-/-}$ mice had slightly fewer B cells ($B220^+$) than did those of *Alox5*$^{+/+}$ mice, even after SRBC immunization. Primary resting B cells are generally classified into 2 major subpopulations—follicular B cells ($B220^+CD21^{int}CD23^+$) and marginal zone B cells ($B220^+CD21^{hi}CD23^-$), each of which plays a unique role in the humoral immune response.[34,35] Follicular B cells play an important role in antibody production in a T-cell–dependent manner, and marginal zone B cells are considered to be innate-like cells. Interestingly, *Alox5*$^{-/-}$ mice demonstrated a significantly decreased number of follicular B cells, ie, approximately 60% of the number of cells in *Alox5*$^{+/+}$ mice (Figure 4E). We also observed the same tendency of follicular B cells in the spleen of *Rag1*^{Alox5} mice compared with those of *Rag1*^{WT} mice (Figure 4F). Note that Alox5 deficiency could lead to apoptosis of follicular B cells, as assessed by the cell surface expression of annexin V (Figure 4G).[36] These evidences imply that follicular B cells depend on Alox5 for maintaining the B-cell population; ie, the B-cell repertoires might be preserved by Alox5 to effectively produce specific antibodies.

We further investigated the features of follicular B cells of *Alox5*$^{-/-}$ mice. After examination of various molecules related to antibody production, the expression levels of the interleukin-21 (IL-21) receptor were profoundly reduced in follicular B cells of *Alox5*$^{-/-}$ mice (Figure 4H).[37,38] However, levels of IL-6, interferon γ receptors, and TLRs were not significantly altered in these cells. Similarly, there were no differences in the major histocompatibility complex class II expression of follicular B cells in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice, suggesting that Alox5 of follicular B cells might not affect the cellular presentation of foreign antigens to helper T cells (Figure 4I). Because Alox5 regulates the cell fate and IL-21–mediated responses of follicular B cells, Alox5-deficient mice might become incapable of producing antibodies specific to foreign antigens.

Alox5 Is Involved in the Generation of Tfh Cells

As noted, IgG2a is preferentially regulated by type 1 helper T cells; therefore, we considered the possible functional effect of Alox5 in the development of effector helper T cells. This might also be suggested by evidence that professional APCs other than primary B cells, such as macrophages or dendritic cells, possess Alox5.[11,39] Before this investigation, we analyzed the status of thymic selection in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice. Results revealed no significant differences between the populations of developing thymocytes in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$

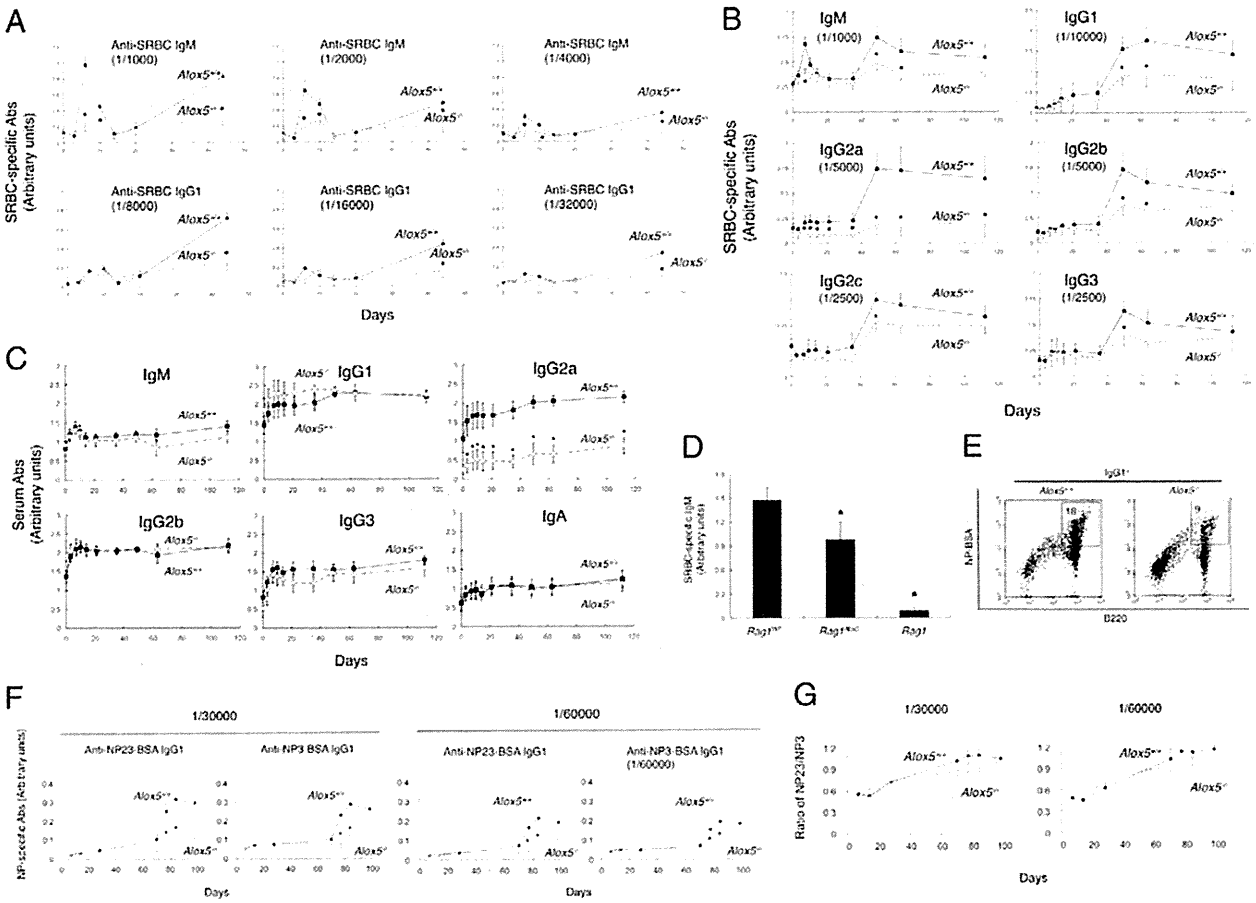

Figure 3. Functional defects of antibody responses specific to foreign antigens of Alox5-deficient mice. **A:** SRBC-specific serum titers of IgM and IgG1 of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice. Mice were immunized with SRBCs on days 0 and 28. SRBC specific IgM and IgG1 titers on consecutive days are shown in different dilutions. $n = 6$ to 8 mice per group. **B:** SRBC specific serum Ig titers of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice. The mice were immunized with SRBCs on days 0 and 42. Titers of SRBC specific IgM, IgG1, IgG2a, IgG2b, IgG2c, and IgG3 on consecutive days are shown in different dilutions. $n = 7$ to 10 mice per group. **C:** Impairment of IgG2a production in *Alox5*$^{-/-}$ mice. Serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA of the specimens studied in **B** are demonstrated. **D:** SRBC specific serum titers of IgM of *Rag1*^{WT} and *Rag1*^{Alox5} mice. Before immunization, *Rag1* chimera mice were examined for whether they produced Igs. Then mice were immunized with SRBCs on day 0, and the titers of SRBC specific IgM on day 7 are presented. Results from *Rag1* are also shown as a control. $n = 5$ to 6 mice per group. **E:** Low numbers of memory B cells (B220$^+$IgG1$^+$NP$^+$) in *Alox5*$^{-/-}$ mice. Mice were immunized with NP36-CGG on days 0 and 56. FACS analysis was used to assess B cells in the spleen on day 64. After gating IgG1$^+$ cells, B220$^+$NP$^+$ cells were found to compose 18% and 9% of IgG1$^+$ cells in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice, respectively. $n = 4$ to 6 mice in each group. **F:** Recall antibody responses of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice. The mice were immunized with NP36-CGG on days 0 and 63. Titers of NP23-BSA or NP3-BSA specific IgG1 at consecutive days are shown at different dilutions. $n = 6$ mice per group. **G:** Ratio of the titer of NP23 specific IgG1 to NP3 specific IgG1 obtained in **F**. No significant differences were observed between the ratios in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice. Results of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice are depicted as solid and dashed lines, respectively. $n = 6$ to 10 mice per group. $^*P < .05$ compared with the wild-type control.

mice (Figure 5A). Previous studies have indicated the diversification of peripheral naive helper T cells into different types of helper T cells, among which Tfh cells (CD3$^+$CD4$^+$CD25$^-$CXCR5$^+$ICOS$^+$) have the distinguished property of driving specific antibody responses.[14,15] When a population of Tfh cells was studied, *Alox5*$^{-/-}$ mice unexpectedly lost their capacity to develop Tfh cells after SRBC immunization (Figure 5B). We next investigated the expression of receptors for Alox5-related lipid mediators in Tfh cells derived from human tonsils in two cases (Figure 5C). In these cases, Tfh cells rather than control helper T cells (CD3$^+$CD4$^+$) up-regulated the expression of v-maf musculoaponeurotic fibrosarcoma oncogene (MAF), Bcl-6, and IL-21, as reported in previous studies.[40–42] The control helper T cells expressed leukotriene receptors of BLT1 and BLT2 for leukotriene B4 and CysLTR1 or CysLTR2 for cysteinyl leukotrienes. In contrast, the levels of these receptors in Tfh cells seemed to be down-regulated or similar to those in control helper T cells. In fact, when human tonsillar T cells were examined by means of flow cytometry, CD4$^+$ T cells frequently presented BLT1 on the cell surface, but BLT1 was lost in Tfh cells (data not shown). These results imply that the signaling of Alox5-related lipid mediators would be required during the initiation of differentiation of naive helper T cells to Tfh cells, probably while in contact with professional APCs.

Alox5 Ensures Survival of Mice from Chronic Enterocolitis under Conventional Conditions

Our observations suggest the novel role of Alox5 in the regulation of adaptive humoral responses. To investigate

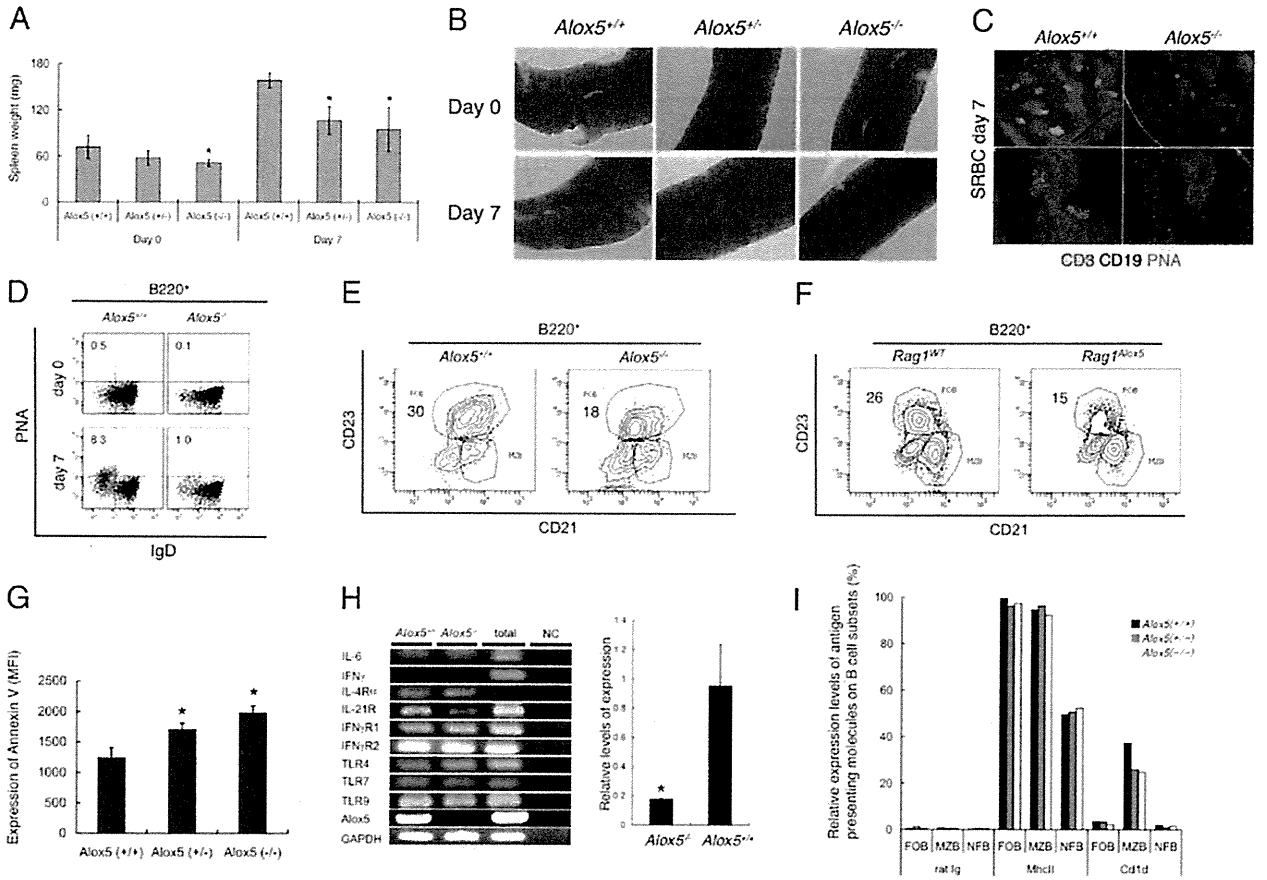

Figure 4. Analysis of antibody-producing cells in Alox5-deficient mice. **A:** Spleen weights of *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice before (day 0) and after immunization with SRBCs (day 7). **B:** Features of the spleens of *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice before (day 0) and after (day 7) immunization with SRBCs as examined using a stereomicroscope (SZX7, Olympus). Original magnification, ×15. **C:** Immunohistochemical analysis of the spleen of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice after immunization with SRBCs (day 7). CD3$^+$ T cells (green), CD19$^+$ B cells (blue), and PNA$^+$ germinal center cells (red) are visualized. Original magnification: ×200 (upper panel) and ×400 (lower panel). **D:** FACS analysis of spleen cells of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice before (day 0) and after (day 7) immunization with SRBCs. After immunization, the population of germinal center cells (B220$^+$IgD$^-$PNA$^+$) of *Alox5*$^{+/+}$ mice increased from 0.5% to 8.3% of the total B220$^+$ cells, whereas that of *Alox5*$^{-/-}$ mice increased from 0.1% to only 1.0%. **E, F:** A FACS analysis of follicular B cells (B220$^+$CD21^{int}CD23$^+$) of the spleen. **E:** Follicular B cells compose 30% and only 18% of the total B220$^+$ cell population in *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice, respectively. **F:** Follicular B cells compose 26% and 15% of the total B220$^+$ cell population in *Rag1*^{WT} and *Rag1*^{Alox5} mice, respectively. **G:** The expression levels of annexin V on follicular B cells of the spleen in *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice after administration of SRBCs (day 7) as assessed by means of FACS analyzer. The mean fluorescence intensity (MFI) of annexin V on follicular B cells is shown. **H:** Expression levels of the transcripts of cytokines and receptors of follicular B cells sorted from the spleen of *Alox5*$^{+/+}$ and *Alox5*$^{-/-}$ mice as assessed by means of PCR. The **left panel** shows the RT-PCR results (25 cycles) investigating various molecules, including Alox5, and determines the down-regulation of the IL-21 receptor transcripts in the *Alox5*$^{-/-}$ cells. The **right panel** presents results from quantitative PCR of the IL-21 receptor of follicular B cells, indicating that the IL-21 receptor levels in these cells of *Alox5*$^{-/-}$ cells are approximately 20% of those in *Alox5*$^{+/+}$ cells. Total, total spleen cells; NC, no template control. **I:** The expression levels of major histocompatibility complex class II on spleen cells of *Alox5*$^{+/+}$, *Alox5*$^{+/-}$, and *Alox5*$^{-/-}$ mice. FOB, follicular B cells; MZB, marginal zone B cells (B220$^+$CD21^{hi}CD23$^-$); NFB, nonfollicular B cells (B220$^+$CD21^{lo}CD23$^-$). The results presented are representative of three to four independent experiments. In each experiment, 3 to 10 mice per group were used. *$P < 0.05$ compared with the wild-type control.

whether humoral immunity regulated by Alox5 would work as a defense mechanism against microorganisms, an experimental colitis model was used in which DSS was orally administered to the mice in a conventional facility.[24] The results indicated that loss of Alox5 led to exaggerated enterocolitis (Figure 6A). Histologic examinations revealed severe erosion and an inflammatory reaction of the mucosa in *Alox5*$^{-/-}$ mice (Figure 6, B and C). Similar results were observed in *Rag1*^{Alox5} mice, implying that the Alox5 of B cells plays a pivotal role in establishing humoral immunity against pathogens under conventional conditions.

Alox5 Enhances the Growth of MCLs

Finally, we investigated the expression profiles of Alox5 in B-cell lymphomas composed of small lymphoid cells, including MCL, small lymphocytic lymphoma (SLL), and marginal zone lymphoma. Currently, naive B cells of the mantle zone, antigen-experienced B cells, and postgerminal center B cells are considered to be the postulated cell origins of MCL, SLL, and marginal zone lymphoma, respectively.[43] Immunohistochemical studies using L22 mAbs on frozen sections revealed the high expression of Alox5 in MCL and SLL but not in marginal zone lymphoma (Figure 7, A–I). These results seem to be in agreement with our observations that Alox5 was preferentially presented in naive and memory B cells. When the MCL cell lines were examined, three cell lines, including G519, MINO, and REC1, possessed Alox5 (Figure 7J). Interestingly, AA861, an Alox5 inhibitor, possessed the capacity to reduce the growth of MINO cells (Figure 7K). Moreover, AA861 combined with vincristine, an antitumor re-