NY-CO-58/KIF2C is overexpressed in a variety of solid tumors and induces frequent T cell responses in patients with colorectal cancer

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NY-CO-58/KIF2C has been identified as a tumor antigen by screening antibody responses in patients with colorectal cancer. However, expression had not consequently been examined, and nothing was known about its ability to induce spontaneous T cell responses, which have been suggested to play a role in the development of colorectal cancer. We analyzed 5 colorectal cancer cell lines, and tumor samples and adjacent healthy tissues from 176 patients with epithelial cancers for the expression of NY-CO-58/KIF2C by RT-PCR and Western Blot. T cell responses of 43 colorectal cancer patients and 35 healthy donors were evaluated by ELISpot following stimulation with 30mer peptides or full-length protein. All cell lines and tumor samples from colorectal cancer patients expressed NY-CO-58/KIF2C on the protein and RNA level, and expression levels correlated strongly with Ki-67 expression (r = 0.69; p = 0.0003). Investigating NY-CO-58/KIF2C-specific T cell responses, CD8⁺ T cells directed against 1 or more peptides were found in less than 10% of patients, whereas specific CD4⁺ T cells were detected in close to 50% of patients. These T cells were of high avidity, recognized the naturally processed antigen and secreted IFN-γ and TNF-α. Depletion of CD4⁺CD25⁺ T cells before stimulation significantly increased the intensity of the preexisting response. NY-CO-58/KIF2C is significantly overexpressed in colorectal and other epithelial cancers and expression levels correlate with the proliferative activity of the tumor. Importantly, NY-CO-58/KIF2C was able to induce spontaneous CD4⁺ T cell responses of the Th1-type, which were tightly controlled by peripheral T regulatory cells.

Key words: colorectal cancer, tumor antigen, tumor immunology, T cells, immunotherapy

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Colorectal cancer is the second leading cause of cancer-related death in Western countries with ~1 million new cases and over 500,000 deaths per year worldwide. In locally advanced colorectal cancer, surgery combined with the selective application of adjuvant chemotherapy represents the treatment of choice providing cure in a significant number of cases. However, about 40% of patients whose primary tumor has surgically been removed will eventually relapse and ultimately die from the disease. Therefore, alternative therapeutic strategies, especially in the adjuvant setting, are needed to improve the prognosis of patients with this malignancy. In the case of colorectal carcinoma, there is strong evidence for a role of the T cell-mediated immune system in controlling the development and progression of the disease. Thus, it has convincingly been demonstrated that infiltration
of malignant epithelia with CD8+ T cells represents a strong and independent prognostic factor in patients with colorectal carcinoma. Levels of circulating and local effector T cells in relation to regulatory T cells (Tregs) in the tumor microenvironment were shown to be important factors for survival and to be orchestrated by tumor-infiltrating antigen presenting cells. It has been suggested that T cell infiltration may contribute to improve the patients’ prognosis by eradicating micrometastases, and, accordingly, T cell responses within colorectal carcinoma are associated with the absence of signs of early metastatic invasion such as vascular emboli and lymphatic or perineural invasion. However, the molecular targets of these apparently clinically relevant tumor-infiltrating T cells have not yet been determined.

The development of antigen-specific cancer immunotherapy depends on the identification of immunogenic gene products expressed predominantly in tumor cells. Luckily, significant progress has been made in the past 15 years regarding the description of molecular targets for active immunotherapies of human cancer with cancer-testis (CT) antigens representing the most promising candidates. This group of close to 60 proteins have been considered attractive therapeutic targets based on their immunogenicity and restricted tissue expression. Unfortunately, colorectal cancer and other gastrointestinal malignancies are characterized by a very infrequent expression of CT antigens, calling for the identification of additional proteins with tumor-specific expression and sufficient immunogenicity.

Scanlan et al. first identified NY-CO-58 as a tumor antigen screening sera of colorectal cancer patients using SEREX (serological analysis of recombinant cDNA expression libraries). This initial analysis showed that the expression pattern of NY-CO-58 in 9 colorectal cancer samples closely resembled the typical expression of CT antigens—a strong overexpression in the malignant samples and healthy testis with only trace levels of expression in other normal tissues. Furthermore, applying a serum antibody detection array, IgG antibodies against NY-CO-58 were observed in 3 of 74 sera from colorectal cancer patients, suggesting that this antigen may also be capable of generating spontaneous immune responses in cancer patients.

On the basis of these previous observations, we hypothesized that NY-CO-58, which is identical to genes kinesin family member 2C (KIF2C), mitotic centromer-associated kinesin (MCAK) and kinesin-like 6 (KNL6), might represent an attractive target for antigen-specific immunotherapies in colorectal cancer and, possibly, other malignancies. However, NY-CO-58 expression had not been examined in detail in colorectal cancer, and nothing was known about the expression of NY-CO-58/KIF2C in other tumors. It was unclear whether this tumor antigen was able to induce spontaneous T cell responses in cancer patients. Therefore, we performed a comprehensive analysis of NY-CO-58/KIF2C expression in colorectal cancer and analyzed a variety of human solid tumors for its expression, and we examined cancer patients for CD4+ and CD8+ T cell responses against NY-CO-58/KIF2C.

Material and Methods

Patients and healthy controls
In total, 176 consecutive, consenting patients were included in this study. These consisted of patients with colorectal (N = 75), pancreatic (N = 17), gastric (N = 10), breast (N = 44) and head and neck cancer (N = 30). Patients had been admitted to the University Medical Center Hamburg-Eppendorf, and tumor samples and normal adjacent tissues were collected during routine surgery. Informed consent was obtained from each subject. One main inclusion criterion for a tissue sample was that the tumor made up at least 50% of the total sample volume as indicated by immunohistochemistry.

Tissues remaining from the initial immunohistochemical evaluation were stored in RNA later (Ambion, Austin, TX) at −80°C until further use. Peripheral blood mononuclear cells (PBMC) were obtained from colorectal cancer patients admitted for surgery or application of chemotherapy to the University Medical Center Hamburg-Eppendorf. PBMC from 35 healthy blood donors were obtained as controls. The study protocol had received approval by the local ethics committee (decision number OB-010/05).

RT-PCR analysis
Total RNA was extracted from cell lines applying the RNeasy Mini Kit (Qiagen, Hilden, Germany) and from tissue samples using guanidium isothiocyanate for denaturation followed by cesium chloride gradient ultracentrifugation over night (36,000 rpm, 4°C). Reverse transcription was performed using AMV reverse transcriptase (Promega, Madison, WI) and was run at 42°C for 45 min with heat inactivation of the enzyme at 95°C for 5 min. PCR primers for target genes were the same as for real-time PCR without the respective probe. Primers for housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were F(forward): 5'-TCTGGGACATCAAGAAGGTGCA-3' and R(reverse): 5'-TTTCTTA GTGACATCAAGAAGGTGG-3' and GAPDH (Ambion) as a positive control were integrated into all PCR reactions. To assess primer specificity, PCR products were run at 42°C for 5 min. Following 35 PCR cycles, products were separated on 1.5% agarose gels, stained with ethidium bromide, visualized with UV light, recorded using a CCD camera and assessed for expected size. Quality of cDNA was tested by RT-PCR using primers for housekeeping gene GAPDH. All RT-PCR experiments were performed at least twice. Negative controls without cDNA and cDNA derived from human testis RNA (Ambion) as a positive control were integrated into all PCR reactions. To assess primer specificity, PCR products were analyzed repeatedly by sequence analysis.
Real-time PCR
Patient samples and a panel of 20 healthy tissues pooled from 3 donors (Ambion) were analyzed using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) and appropriate primers and probes (Eurogentec, Seraing, Belgium) for NY-CO-58/KIF2C (F:5'-GTGTCCTGTGTTAGGAAACGCC-3', R:5'-CCTTGGAGAATGGAAATCAT-3', P(Probe): 5'-FAM-CTGAAATAAGCAGATTTGGGCAAAGAGAAAT TGAC-TAMRA-3'), Ki-67 (F:5'-GCCCAACCCAAAAGA AAGTCT-3', R:5'-AGCTTTGTGCTTCATTCACTTCA-3', P:5'-FAM-CATCAAACAGCCTCAAACTCAGGA-TAMRA-3'). Primers and probe for beta-glucuronidase were designed by Applied Biosystems (Assay-on-Demand Hs99999908). First, a master mix was prepared containing the following components: MgCl2 (5.0 mM), forward and reverse primers (1 μM), probe (500 nM), 200 nM dNTP (Invitrogen, Karlsruhe, Germany), DMP 1%, BSA at 250 μg/ml and 1 U FastStart taq polymerase (Roche Diagnostics, Branchburg, NJ) in a total volume of 20 μl. After an initial denaturation at 50°C for 2 min and at 95°C for 10 min, the PCR reactions were cycled 40 times as follows: 15 sec at 95°C and 60 sec at 60°C (annealing temperature). Fluorescence intensity was measured at the end of each annealing phase. A standard curve prepared of the specific PCR product cloned into a pcDNA2.1Vector (Invitrogen, Carlsbad, CA) was used to determine the concentration of target transcripts in cDNA samples. Relative quantification values were obtained from the threshold cycle number at which the increase in signal associated with exponential growth of PCR was first detected. Results are shown as expression of the target gene relative to housekeeping gene beta-glucuronidase. Corresponding clinical data for correlation with expression analyses were available for all patients with head and neck cancer, 39 patients with breast cancer, 12 colorectal cancer patients, 11 patients with pancreatic cancer and 10 patients with gastric cancer.

Western blot analysis
Protein lysates were prepared from cell lines and normal tissues using standard lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and were subsequently denatured for 10 min at 70°C. Lysates of malignant and corresponding healthy tissues from 4 patients with colorectal cancer were obtained from Protein Diagnostics, Ramona, CA. Samples of lysates containing 30 μg total protein were resolved on 4–12% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA) under reducing conditions. Proteins were blotted on Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), blocked overnight at 4°C with Top-Block (Fluka, Buchs, Switzerland) and incubated with 1 μg primary monoclonal antibody directed against NY-CO-58/KIF2C (clone 1G2; Abnova, Taipei City, Taiwan) or beta-actin ( clone SC-47778; Santa Cruz Biotechnology, Santa Cruz, CA) for 4 hr at room temperature. Next, secondary HRP-labeled anti-mouse monoclonal antibody (R&D Systems, Minneapolis, MN) was applied for 1 hr at room temperature. Specific binding was visualized by chemiluminescence (ECL Western Blotting Analysis System, Amersham Biosciences). In some experiments, blocking with recombinant NY-CO-58/KIF2C protein (Abnova) was performed to demonstrate specificity of staining with the NY-CO-58/KIF2C-specific antibody.

Immunohistochemical analysis
Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections, which had been obtained for routine diagnostics. Briefly, slides were deparaffinized and pretreated with 10 mmol/l citrate, pH 6.0 (Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking Chamber; BioCare Medical, Walnut Creek, CA) followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pretreated with peroxidase block (Dako) followed by blocking with goat serum diluted 1:1 in 20 mmol/l Tris-HCl, pH 7.4, for 20 min. Primary murine anti-human NY-CO-58/KIF2C monoclonal antibody (Abnova) was applied at a 1:10 dilution in 50 mmol/l Tris-HCl, pH 7.4, with 3% goat serum for 1 hr. Slides were washed in 50 mmol/l Tris-HCl and goat antimouse horse-radish peroxidase-conjugated antibody (Envision detection kit; Dako) was applied for 30 min. After further washing, immunoperoxidase staining was developed using a diaminobenzidine chromogen kit (Dako), as per the manufacturer's instructions.

Immunofluorescence
To examine the subcellular localization of NY-CO-58/KIF2C protein in colorectal cancer cell lines COLO320 and DLD1, cells were seeded at 2 × 10⁴/chamber in an 8-chamber slide. After 48 hr, cells were fixed for 12 min with PBS containing 4% paraformaldehyde and were permeabilized with PBS + 0.1% Triton X for 2.5 min at room temperature. Blocking was performed using PBS + 3% BSA for 12 hr at 4°C, and cells were subsequently stained applying monoclonal mouse anti-NY-CO-58/KIF2C antibody ( Abnova) at 5 μg/ml. After washing with PBS, a secondary FITC-conjugated goat antimouse monoclonal antibody (Dako, Hamburg, Germany), which was diluted 1:100 in PBS, was applied for 45 min at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Boehringer Ingelheim, Ingelheim, Germany) dissolved in Vectashield medium (Vector Laboratories, Burlingame, CA), and fluorescence was analyzed using an Axioskop 2 microscope (Zeiss, Jena, Germany).

Target cells and tumor cell lines
For the generation of T-APC, 16 CD4⁺ T cells were cultured in RPMI medium with 10% human AB serum, glutamine, antibiotics, nonessential amino acids, IL-2 (10 U/ml; Roche, Basel, Switzerland), IL-7 (20 ng/ml, R&D Systems, Minneapolis, MN) and 10 μg/ml phytohemagglutinin (PHA HA15,
Murex, UK). On Day 3, half of the medium was replaced with complete medium containing IL-2 (20 U/ml) and IL-7 (40 ng/ml), and this was repeated every 3 to 4 days. T-APCs were usually harvested and used as target cells around Day 20 of the culture. Epstein-Barr virus (EBV)-transformed B lymphocytes (EBV-B cells) and tumor cell lines were cultured in RPMI medium supplemented with 10% FCS (Summit Biotechnology), glutamine, penicillin, streptomycin and nonessential amino acids.

**Analysis of CD4⁺ and CD8⁺ T cell responses**

Analyses of T cell responses against NY-CO-58/KIF2C were conducted in parallel at the New York branch of the Ludwig Institute for Cancer Research and the Department of Oncology/Hematology at the University Medical Center Hamburg-Eppendorf. Based on publicly available prediction software and algorithms for CD4⁺ and CD8⁺ T cell epitope binding to HLA molecules (http://www-bimas.cit.nih.gov/molbio/hla_bind/; http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm; http://www.imtech.res.in/raghava/propred/) and proteasome processing (http://www.cbs.dtu.dk/services/NetChop/; http://www.paproc.de), we designed 9 different 30 mer peptides predicted to be rich in epitopes, covering more than 40% of the complete amino acid sequence of NY-CO-58/KIF2C (see Supporting Information Table S1, peptides from Bio-Synthesis, Lewisville, TX). We applied a brief stimulation with pools of NY-CO-58. Read-out-assays were performed following a single in vitro presensitization, as previously described.¹⁶,¹⁷ Briefly, CD4⁺ and CD8⁺ T cells were sequentially purified from PBMC applying antibody-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway). In some experiments, CD4⁺ T cells were further depleted of CD25⁺ cells using appropriate magnetic beads (Miltenyi Biotec, Auburn, CA). T cells were stimulated once with irradiated CD8⁺ CD4⁻ cells either pulsed with individual or with pools of 9 overlapping 30 mer peptides (Bio-Synthesis, Lewisville, TX) covering 40% of the entire NY-CO-58/KIF2C sequence. After 10–20 days of culture in RPMI containing 10% SAB supplemented with glutamine, antibiotics, nonessential amino acids, IL-2 (10 U/ml) and IL-7 (20 ng/ml), CD8⁺ and CD4⁺ T cells were harvested and tested by ELISPOT on autologous or histocompatible EBV lymphoblastoid cells or T-APC pulsed over night with cognate or control peptides or full-length protein (made by Ivan Gout and Valeriy Filonenko, Kiev, Ukraine). As controls, influenza nucleoprotein peptide 206–229, irrelevant SSX-2 peptide 1–20 and irrelevant NY-ESO-1 protein were used. In some experiments, target cells were infected with recombinant adenovirus (1000 IU/cell) or vaccinia virus (5 IU/cell; both viruses made by Vincenzo Cerundolo and Khoon-Lin Ling, Oxford, UK) encoding NY-CO-58/KIF2C or control protein NY-ESO-1. Numbers of IFN-γ producing spots were revealed on plates (Millipore, Bedford, MA) using a specific antibody kit (Mabtech, Stockholm, Sweden) and counted using AID EliSpot reader and EliSpot software version 3.2.3 (Autoimmun Diagnostika, Strassberg, Germany). Average from duplicates was calculated and a positive response was defined as >20 spots and >2 × number of spots for irrelevant control target. Results were representative of at least 2 repeat experiments.

For the measurement of intracellular cytokines, pulsed targets were stained with 0.2 μM 5-(and-6) -carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) for 10 min at 37°C. Target cells were then washed and were incubated with presensitized effector T cells at a 1:2 ratio in 200 μl X-VIVO-15 at 37°C for 7 hr. Brefeldin-A (Sigma-Aldrich, St. Louis, MO) at 10 μg/ml was added after the first 2 hr of culture. Cells were then fixed using FACS Lysing Solution (Becton Dickinson) diluted 1:10, permeabilized using permeabilizing solution 2 (BD Biosciences, San Jose, CA) and stained with appropriate antibodies against CD4, IFN-γ, TNF-α, IL-5 and IL-10 (BD Biosciences). Cells were analyzed by flow cytometry with gating on morphologically defined lymphocytes, CD4-positive and CFSE-negative cells.

For a preliminary determination of the HLA restriction of the investigated NY-CO-58 epitopes, we used EBV blasts from partially HLA-matched blood donors pulsed with the respective peptides as previously described.¹⁶

**Statistical analysis**

The Wilcoxon test was applied to results from tumor samples versus autologous healthy tissues. Spearman’s rank correlation test was used to analyze correlations between gene expression and clinicopathological characteristics of the patients. Statistical analysis was performed using SPSS software (SPSS, Chicago, IL). Results were considered significant if p < 0.05.

**Results**

**Tumor antigen NY-CO-58/KIF2C is overexpressed in colorectal carcinoma**

NY-CO-58/KIF2C has previously been described as a tumor antigen in colorectal cancer; however, only few patient samples were analyzed and expression was not described in detail.¹⁴ Analyzing NY-CO-58/KIF2C expression in 5 colorectal cancer cell lines by RT-PCR and Western blot, we found that all cell lines strongly expressed the tumor antigen on the RNA and protein level (Fig. 1a). Analyzing 2 of the latter colon cancer cell lines for the subcellular expression of NY-CO-58/KIF2C applying immunofluorescence, we observed strong staining of the nuclear membrane, significant nuclear expression and a comparably weaker cytoplasmic staining with the NY-CO-58/KIF2C-specific monoclonal antibody (Fig. 1b). When we compared expression levels in 20 different normal human tissues applying real-time PCR, we found that healthy human testis expressed the highest levels of NY-CO-58/KIF2C mRNA, whereas thymic tissue showed an intermediate level of expression. In contrast, the remaining healthy tissues evidenced only trace levels of NY-CO-58/KIF2C mRNA (Fig. 1c). Accordingly, examining malignant...
Figure 1. NY-CO-58/KIF2C is overexpressed in colorectal cancer on the RNA and protein levels. (a) Five colon cancer cell lines and human testis were analyzed for expression of NY-CO-58/KIF2C using conventional RT-PCR and Western blot (WB). GAPDH and beta-actin (ACTB) served as internal controls. Sizes of the PCR products and the target proteins are indicated. (b) Immunofluorescence was analyzed in colon cancer cell lines COLO320 and DLD1 applying a FITC-conjugated monoclonal antibody against NY-CO-58/KIF2C and counterstaining with DAPI. An appropriate isotype was used to control for unspecific staining. (c) Twenty different normal human tissues were analyzed for mRNA expression of NY-CO-58/KIF2C using real-time PCR, and results were normalized to expression levels of housekeeping gene beta-glucuronidase. (d) Expression of NY-CO-58/KIF2C protein was analyzed by Western blot in lysates derived from malignant (P1-4) and healthy (N1-4) colon tissue from 4 patients with colorectal cancer. Blocking experiments with recombinant NY-CO-58/KIF2C protein were performed to demonstrate specificity of staining with the NY-CO-58/KIF2C-specific antibody. (e) Tumors and nonmalignant colon tissues of 22 patients with colorectal cancer were analyzed regarding expression levels of NY-CO-58/KIF2C applying real-time PCR, and results were normalized to expression levels of housekeeping gene beta-glucuronidase. Black dots represent relative copy numbers of the target gene for each benign or malignant sample, respectively, and bars represent medians calculated. Benign and malignant samples were compared using Wilcoxon's test. Asterisks indicate significant differences (***p < 0.001). (f) Immunohistochemistry was applied to tumor tissues of 10 patients with colorectal cancer and to tissue derived from healthy human testis as a control. Staining was performed using a NY-CO-58/KIF2C-specific monoclonal antibody, and results are shown for 2 representative tumor samples and normal testis.
and autologous healthy colonic tissues from 4 patients with colorectal cancer, Western blot analysis revealed NY-CO-58/KIF2C protein expression in all malignant samples, whereas normal colorectal tissues showed no or very little NY-CO-58/KIF2C expression (Fig. 1d). To verify this finding in a larger number of clinical samples, we next analyzed NY-CO-58/KIF2C expression levels in malignant and normal colorectal tissues from 22 patients with colorectal cancer using real-time PCR. We found that NY-CO-58/KIF2C was indeed strongly and significantly overexpressed in malignant compared with autologous healthy colorectal tissues (Fig. 1e). Immunohistochemistry revealed that, as in the case of classical CT antigens, NY- CO-58/KIF2C expression within tissue derived from healthy testis was restricted to spermatogonia (Fig. 1f). However, within colorectal cancer samples, NY- CO-58/KIF2C was strongly and homogeneously expressed in the malignant epithelial tissue. These collected findings led us to the conclusion that NY- CO-58/KIF2C is highly and specifically overexpressed in human colorectal cancer at the protein and RNA levels.

Tumor antigen NY-CO-58/KIF2C is overexpressed in a variety of epithelial cancers

On the basis of our finding of a strong overexpression of NY- CO-58/KIF2C in colorectal cancer, we next analyzed the expression of this tumor antigen in other epithelial cancers. Applying real-time PCR to malignant and autologous healthy tissues from a total of 101 patients with pancreatic, gastric, breast and head and neck cancer, we found that NY- CO-58/KIF2C was significantly overexpressed in all these tumor types (Fig. 2a). Comparing expression levels between tumor types, we observed that NY- CO-58/KIF2C was most strongly overexpressed in gastric, breast and colorectal cancer, while overexpression was less pronounced in pancreatic and head and neck cancer (Fig. 2b).

When we next analyzed whether NY- CO-58 expression was related to common clinicopathological characteristics of the tumor samples, we did not find an association of TNM status, stage and grading (and hormone receptor status breast cancer cases) with the expression of the tumor antigen in any of the 5 patient groups (data not shown). NY- CO-58/KIF2C, however, plays an important role in the mitosis of normal human cells,18 and, therefore, we hypothesized that the expression level of this gene might still be related to the proliferative activity of human cancers despite the lacking association with classical clinicopathological characteristics of tumor progression. On the basis of this idea, we next determined the proliferation rate of the tumors of our 30 patients with head and neck cancer applying a Ki-67-specific real-time PCR. We chose patients with this diagnosis because they represented the largest group of patients undergoing analysis of NY- CO-58/KIF2C tumor expression and the only group of patients with sufficient volumes of tumor tissue allowing for additional studies. We found that expression levels of Ki-67, which is a well-established proliferation marker, and those of NY- CO-58/KIF2C correlated strongly and significantly (Fig. 2c), suggesting that expression of NY- CO-58/KIF2C is indeed specifically and strongly related to the proliferative activity of human cancers.

Tumor antigen NY-CO-58/KIF2C evokes spontaneous and frequent T cell responses in colorectal cancer patients and healthy subjects

Because our study had shown that NY- CO-58/KIF2C is overexpressed in human cancers and based on the fact that this tumor antigen was first identified by analyzing tumor-specific antibody responses, we reasoned that NY- CO-58/KIF2C might also be able to induce spontaneous T cell responses in vivo. On the basis of publicly available prediction software and algorithms for CD4+ and CD8+ T cell epitope binding to HLA molecules and proteasome processing, we designed 9 different 30 mer peptides predicted to be rich in epitopes, covering more than 40% of the complete amino acid sequence of NY- CO-58/KIF2C to perform a comprehensive analysis of T cell responses against this tumor antigen. We applied a brief stimulation with pools of NY- CO-58/KIF2C peptides to peripheral CD4+ and CD8+ T cells separated from PBMC of 43 patients with colorectal cancer and 35 healthy controls. Following 1 cycle of antigen-specific stimulation, autologous PHA blasts (T-APC) pulsed with peptide pools or individual peptides were used as targets in an IFN-γ-ELISPOT read-out assay to quantify the number of NY- CO-58/KIF2C-specific T cells.

CD8+ T cell-mediated responses against the 9 NY- CO-58/KIF2C peptides were only detected in less than 10% of patients or healthy controls (Fig. 3a) and were generally weak (mean spot number per NY- CO-58/KIF2C-specific response: 39/25,000 CD8+; mean background: 13/25,000 CD8+). In contrast, we found CD4+ T cell responses against 1 or more NY- CO-58/KIF2C peptides in close to 50% (20/43) of patients with colorectal cancer (Fig. 3a). Surprisingly, we observed equally frequent NY- CO-58/KIF2C-specific CD4+ T cell responses in healthy blood donors with the majority (21/35) of subjects evidencing a response against at least 1 NY- CO-58/KIF2C peptide. CD4+ responses in patients and healthy subjects were comparably strong with a mean spot number of 147 for the NY- CO-58/KIF2C-specific responses over a mean background of only 7 spots per 25,000 CD4+ T cells.

We were able to identify responses against individual NY- CO-58/KIF2C peptides in all healthy responders and 14 of the 20 patient responders. Patients with colorectal cancer evidenced simultaneous responses against a mean number of 1.6 epitopes (range 1–3) and healthy subjects against 2.0 epitopes (range 1–3). Looking at the distribution of these NY- CO-58/KIF2C epitopes among patients and healthy subjects (Fig. 3b), we found that most subjects in both groups reacted against peptide 1–32. However, although ~50% of healthy responder also evidenced CD4+ T cells directed against NY- CO-58/KIF2C peptide 692–721, only 1 responder with
colorectal cancer reacted against this epitope (Fig. 3b). On the other hand, patient responders more frequently evidenced CD4+ T cells specific for 3 neighboring epitopes within the NY-CO-58/KIF2C region 375–539. Although different HLA expression patterns might bias the result of this particular analysis, we believe that this still suggests a certain difference regarding NY-CO-58/KIF2C-specific T cell epitopes present in patients versus healthy donors. A preliminary analysis of the HLA restriction of the investigated epitopes in our patients using partially matched EBV blasts from healthy donors.
donors suggests that peptides 1–32 and 403–432 were HLA-DP*03 restricted, whereas peptide 510–539 was most likely HLA-DR*11 restricted.

NY-CO-58/KIF2C-specific CD4+ T cells are of high avidity and recognize the naturally processed antigen

Peptide titration experiments showed that although NY-CO-58/KIF2C-specific CD4+ T cells of patients seemed to be of slightly higher avidity, CD4+ T cells in both groups of subjects recognized NY-CO-58/KIF2C peptides at the nanomolar level (Fig. 4a). In some cases, we were able to define minimal epitopes using shorter 12 mer peptides of NY-CO-58/KIF2C, which were also efficiently recognized by the NY-CO-58/KIF2C-specific CD4+ T cells stimulated with the longer 30 mer peptides (Fig. 4b). Importantly, the same NY-CO-58/KIF2C-specific CD4+ T cells did not only react toward synthetic peptides of NY-CO-58/KIF2C but also recognized the naturally processed antigen. Thus, healthy donor- (Fig. 4c) and patient-derived (Fig. 4d) CD4+ T cells efficiently recognized autologous targets infected with vaccinia or adenovirus recombinant for full-length NY-CO-58/KIF2C or target cells pulsed with recombinant NY-CO-58/KIF2C protein (Fig. 4c).

NY-CO-58/KIF2C-specific CD4+ T cell responses are of Th1-type and are controlled by peripheral T regulatory cells

It has become clear that peripheral tolerance is largely maintained by immunosuppressive Tregs, such as CD4+CD25+ T cells. Unfortunately, in addition to their role in suppressing autoimmune responses, Tregs also represent a main obstacle of an effective antitumor T cell response. Accordingly, previous studies have indicated that T cell responses against CT antigens such as NY-ESO-119,20 or tumor antigen ST421 are tightly controlled by Tregs in most cancer patients and healthy donors. In the case of CD4+ T cell responses against NY-CO-58/KIF2C, depletion of CD4+CD25+ Tregs before stimulation with NY-CO-58/KIF2C led to a dramatic increase in the intensity of pre-existing responses and to an unmasking of previously undetectable responses against this tumor antigen (Fig. 5a).

Further investigating the quality of the CD4+ T cell responses against NY-CO-58/KIF2C in patients with colorectal cancer, we performed cytoplasmatic staining of various cytokines upon reexposure to the respective target peptide. Traditionally, T cells producing T-helper-1 (Th1)-type cytokines have been suggested to play a role in supporting an antibody-mediated immune response while preventing an effective cellular antitumor immune response. In contrast, Th1 cytokines have been considered critically important for the induction of cellular immunity and the generation of a relevant antitumor response in vivo. We observed that the NY-CO-58/KIF2C-specific CD4+ T cells specifically produced Th1-type (IFN-γ, TNF-α) but not Th2-type (IL-5, IL-10) cytokines (Fig. 5b), clearly indicating that the NY-CO-58/KIF2C-specific T cell responses in patients with colorectal cancer were of the Th1-type.

Discussion

Expression of NY-CO-58/KIF2C has previously only been analyzed in a small number of colorectal cancer patients and has not been compared to the expression level observed in autologous nonmalignant colon tissue.14 When we analyzed NY-CO-58/KIF2C expression in a large variety of healthy tissues, we detected only trace levels of this protein in organs other than thymus and testis. Our study was not designed to address the question whether nonmalignant tissues

Figure 3. NY-CO-58/KIF2C frequently induces CD4+ T cell responses in patients with colorectal cancer. (a) Patients with colorectal cancer and healthy blood donors were analyzed in an ELISPOT assay for T cell responses against 9 different 30 mer peptides of NY-CO-58/KIF2C following a single cycle of antigen-specific stimulation. Percentages of subjects in both groups evidencing a CD4+ and/or CD8+ T cell response against at least 1 NY-CO-58/KIF2C epitope are shown. (b) CD4+ responses against different NY-CO-58/KIF2C epitopes are shown as percentage of responders reacting against the respective epitope (gray bars). Black dots indicate the individual intensity of the responses.
Figure 4. NY-CO-58/KIF2C-specific CD4+ T cells are of high avidity and recognize the naturally processed antigen. (a) Peptide titration experiments were performed with CD4+ T cells of patients and donors following a single cycle of antigen-specific stimulation with NY-CO-58/KIF2C peptides 1–32, 151–180 and 692–721. Black dots indicate spot numbers in an IFN-γ ELISPOT at different peptide concentrations. The gray area indicates the mean background level at a peptide concentration of 10 μmol/l. (b) We were able to define minimal epitopes for some NY-CO-58/KIF2C-specific CD4+ responses. Thus, T cells specific for NY-CO-58/KIF2C peptide 1–32 also recognized NY-CO-58/KIF2C 12 mer 15–26. For some responses, such as those against NY-CO-58/KIF2C peptide 692–721, minimal epitopes differed between responding subjects, probably depending on the given HLA context. Thus, CD4+ T cells specific for NY-CO-58/KIF2C peptide 692–721 also recognized NY-CO-58/KIF2C peptide 699–710 in some responders, whereas the same specificity contained the smaller epitope NY-CO-58/KIF2C 703–714 in others. Bars indicate the mean spot number of duplicate ELISPOT experiments.

In healthy donors (c) and colorectal cancer patients (d), CD4+ T cells specific for different epitopes of NY-CO-58/KIF2C not only recognized the respective peptide but also produced IFN-γ in response to the naturally processed antigen in the form of autologous EBV-B cells or T-APC infected with vaccinia virus (VV) or adenovirus (Ad) recombinant for full-length NY-CO-58/KIF2C or target cells pulsed with recombinant NY-CO-58/KIF2C protein. Irrelevant virus recombinant for CT antigen NY-ESO-1 and recombinant NY-ESO-1 protein were used as controls.
characterized by higher rates of cell proliferation would show an increased expression of NY-CO-58/KIF2C, and future studies will analyze this in detail. However, we show here that NY-CO-58/KIF2C mRNA and protein were strongly overexpressed in colon cancer cell lines and in the malignant tissue of patients with colorectal cancer. Performing a comprehensive expression analysis in other human cancers, we observed overexpression of NY-CO-58/KIF2C in gastric, pancreatic, head and neck and breast cancer. Interestingly, the latter finding is in agreement with a recent study detecting upregulation of KIF2C expression in a genome-wide microarray analysis of breast cancer tissues.22

Our collected expression analyses suggest that NY-CO-58/KIF2C might represent a target for the active immunotherapy of a variety of human cancers. The usefulness of NY-CO-58/KIF2C as a tumor target would further be underscored, if it played a central role in tumor survival and/or progression. Antigen-specific eradication of malignant cells expressing such proteins would potentially hit an “Achilles’ heel” of the malignancy and, therefore, has the potential to result in long-lasting remission or even cure. NY-CO-58/KIF2C is a member of the kinesin family of motor proteins and plays a central role in chromosome segregation during mitosis. During cell division, assembly of the mitotic spindle and accurate

Figure 5. NY-CO-58/KIF2C-specific CD4⁺ effector T cells display a Th1 phenotype and are tightly controlled by peripheral CD4⁺ Tregs. (a) Depletion of CD25⁺ Tregs before antigen-specific stimulation resulted in dramatically amplified CD4⁺ T cell responses or even in an unmasking of previously undetectable responses against NY-CO-58/KIF2C in healthy subjects. (b) Representative CD4⁺ T cell response against NY-CO-58/KIF2C in a colorectal cancer patient as indicated by FACS analysis of intracellular cytokine expression. NY-CO-58/KIF2C-specific CD4⁺ T cells were expanded in a single cycle of peptide-driven stimulation. FACS analysis of intracellular concentration of Th1-type (IFN-γ and TNF-α) and Th2-type (IL-5 and IL-10) cytokines was performed on Day 20 postculture initiation. To identify effector cells, CD4⁺ T-APC were stained using the intracellular dye CFSE. Targets were later excluded from the analysis using double gating on morphologically defined lymphocytes and gating on CFSE-negative and CD4⁺ cells. Percentages of cytokine-expressing CD4⁺ T cells are indicated. Background levels were determined using T-APC pulsed with the irrelevant NY-ESO-1 peptide 80–109 and are shown in brackets.
chromosome segregation require the proper regulation of microtubule dynamics. The length of microtubules is controlled by the activity of microtubule polymerases and depletases such as NY-CO-58/KIF2C,18 indicating a major role of this protein in cell proliferation and, possibly, tumor growth. A central role of NY-CO-58/KIF2C in promoting the proliferation of malignancies is also supported by our observation of a strong correlation between NY-CO-58/KIF2C expression and the expression of Ki-67, which is an excellent marker to determine the growth fraction of the malignancy and represents a significant prognostic factor in a variety of human malignancies.23 Adding further support to a central role of NY-CO-58/KIF2C in tumor proliferation, Shimo et al. recently reported the treatment of breast cancer lines with small interfering RNA against KIF2C to growth inhibition in vitro.22 Finally, the activity of NY-CO-58/KIF2C is regulated by Aurora-B kinase,24 and levels of Aurora-B are increased in tumors such as colorectal cancer. Aurora kinase inhibitors are currently being evaluated as promising therapeutic agents in clinical trials including patients with a variety of malignancies such as breast and colorectal cancer.25 Therefore, our results are not only emphasizing the value of NY-CO-58/KIF2C as a tumor antigen but are also supporting the investigation of agents directly targeting the function of this gene in human cancer.

Although studies have demonstrated that antibody responses against CT antigens and related proteins such as NY-CO-58/KIF2C are a comparably rare event in colorectal cancer patients,11,14,26 very little is known about the occurrence of T cell responses against such antigens in the same patient group.13 We performed a comprehensive analysis of T cell responses against NY-CO-58/KIF2C including 78 cancer patients and healthy controls analyzing them for CD4+ and CD8+ responses against 9 different NY-CO-58 peptides. We found that NY-CO-58/KIF2C was indeed capable of inducing spontaneous T cell responses in vivo resulting in frequently occurring and highly functional NY-CO-58/KIF2C-specific T cells in both patients with colorectal cancer and healthy donors. In contrast to other tumor antigens such as NY-ESO-1 that elicits integrated T cell and antibody responses, we observed T cell responses to NY-CO-58 in a much higher frequency of individuals than would be expected based on antibody responses alone.14 The occurrence of T cell responses against tumor antigens in healthy donors has been documented27 and appears to vary depending on the antigen. In the case of NY-ESO-1, healthy donors have specific T cells derived that are suppressed by Tregs,20 whereas in the case of MAGE-A3, specific T cells can sometimes be readily detected at low frequency in healthy donors.28 The CD4 T cell repertoire against NY-CO-58 therefore appears less stringently controlled in healthy donors compared with other tumor antigens. Although these results support the immunogenicity of NY-CO-58/KIF2C, the question remains why the cellular immune response was mainly mediated by CD4+ rather than CD8+ T cells, which have traditionally been regarded as the main effectors against tumor cells. One possible explanation for this finding could be related to the method we used to detect NY-CO-58/KIF2C-specific T cell responses. We applied pools of 30 mer peptides to stimulate T cells of colorectal cancer patients, and previous findings suggest that the application of peptides with a minimum length of 20 amino acids might bias T cell responses toward CD4+ rather than CD8+ responses.29 However, we and others have used a similar monitoring approach in previous studies resulting in the detection of strong CD4+ and CD8+ responses.30,31 Therefore, we believe that our methodological approach alone cannot be responsible for the distinct immunological findings described in this study.

NY-CO-58/KIF2C has been identified by SEREX, and SEREX-defined as well as a variety of other tumor antigens have been indicated to stimulate responses of immunosuppressive CD4+ FOXP3+ Tregs in cancer patients.32–34 On the basis of very limited material per patient, we were not able to perform assays testing the inhibitory potential of the NY-CO-58/KIF2C-specific CD4+ T cells; however, we believe for several reasons that these cells rather represent effector type than regulatory cells. NY-CO-58/KIF2C-specific CD4+ T cells in our study expressed Th1-type cytokines such as IFN-γ and TNF-α, which are usually not secreted by Tregs.32–35 In addition, Tregs in general and particularly Tregs specific for tumor antigens typically express immunoregulatory cytokines such as IL-10,33,36,37 a cytokine that was not expressed in the NY-CO-58-specific CD4+ T cells in colorectal cancer patients. Most importantly, however, our study shows that NY-CO-58-specific T cells are themselves strongly regulated by peripheral CD4+CD25+ Tregs. This finding also suggests that it might be possible to amplify the number and effectiveness of T cells directed against NY-CO-58/KIF2C by eliminating the immunosuppressive effect of Tregs in vivo.

Recent results suggest that the role of CD4+ effector T cells in antitumor immunity has long been underestimated.38 Although a main function of CD4+ T cells in this setting is indeed to provide help for the initiation, the amplification and the maintenance of CD8+ T cell responses,39 CD4+ T cells are also capable of activating effector cells other than CD8+ cells. Thus, tumor-infiltrating cells, such as NK cells, eosinophils and macrophages, mediate an effective antitumor response following activation by neighboring tumor-specific CD4+ T cells.38,40–46 Furthermore, CD4+ T cells also have the potential for an immediate effector function against tumors in vitro47 and in vivo.48 Finally, IFN-γ secreted by tumor-infiltrating CD4+ T cells or activated bystander cells promotes tumor recognition and elimination by upregulating expression of MHC molecules and inhibition of tumor angiogenesis.49,50

Although the effect of tumor-infiltrating CD4+ T cells on the course of colorectal carcinoma has not been examined in detail, one study demonstrated a positive effect of tumor-infiltrating CD4+ T cells, particularly CD45RO+ memory-type cells, on the clinical course of colorectal cancer.8 This finding is in line with other studies indicating an improved
survival in patients with colorectal cancer who show an increased intratumoral expression of the costimulatory molecule OX-40, which is found on activated CD4\(^+\) T cells. Furthermore, Galon et al. found the combined expression of Th1-type genes to be associated with reduced recurrence rates in patients with colorectal carcinoma.\(^4\) Finally, expression of HLA class II molecules on colorectal cancer has repeatedly been shown to represent a favorable prognostic marker,\(^51–53\) pointing again to a significant influence of CD8\(^+\) T cells on the progression of this malignancy.

In conclusion, it seems at least conceivable that the NY-CO-58/KIF2C-specific CD4\(^+\) T cells, which secreted Th1-type cytokines, properly primed and activated in vivo might have the potential to induce a meaningful antitumor immune response without the contribution of CD8\(^+\) T cells. Therefore, a potential hypothesis regarding the high frequency of NY-CO-58/KIF2C-specific CD4\(^+\) T cells in patients with colorectal cancer and in healthy subjects is that these responses detected in our study simply represent remnants of immunosurveillance “at work.” We suggest that this broad repertoire of preexisting T cell responses could further be strengthened and shaped by an antigen-specific immunotherapy using NY-CO-58/KIF2C as a tumor target and modifying the immunosuppressive environment in colorectal cancer which is, at least in part, mediated by Tregs.

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References


49. Qin Z, Blankenstein T. CD4+ T cell-mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity 2000;12:677–86.


