Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma

Andreas Bonertz,[1] Jürgen Weitz,[2] Dong-Ho Kim Pietsch,[1] Nuh N. Rahbari,[2] Christoph Schlude,[1] Yingzi Ge,[1] Simone Juenger,[1] Israel Vlodavsky,[3] Khashayarsha Khazaie,[4] Dirk Jaeger,[5] Christoph Reissfelder,[2] Dalibor Antolovic,[2] Maximilian Aigner,[2] Moritz Koch,[2] and Philipp Beckhove[1]

[1]Translational Immunology Unit, The German Cancer Research Center, Heidelberg, Germany. [2]Department of Visceral Surgery, University Hospital of Heidelberg, Heidelberg, Germany. [3]Vascular and Tumor Biology Research Center, Technion, Israel Institute of Technology, Haifa, Israel. [4]Division of Gastroenterology, Northwestern University Feinberg School of Medicine, Robert Lurie Comprehensive Cancer Center, Chicago, Illinois, USA. [5]National Center of Tumour Diseases, Heidelberg, Germany.

Spontaneous antitumor T cell responses in cancer patients are strongly controlled by Tregs, and increased numbers of tumor-infiltrating Tregs correlate with reduced survival. However, the tumor antigens recognized by Tregs in cancer patients and the impact of these cells on tumor-specific T cell responses have not been systematically characterized. Here we used a broad panel of long synthetic peptides of defined tumor antigens and normal tissue antigens to exploit a newly developed method to identify and compare ex vivo the antigen specificities of Tregs with those of effector/memory T cells in peripheral blood of colorectal cancer patients and healthy subjects. Tregs in tumor patients were highly specific for a distinct set of only a few tumor antigens, suggesting that Tregs exert T cell suppression in an antigen-selective manner. Tumor-specific effector T cells were detectable in the majority of colorectal cancer patients but not in healthy individuals. We detected differences in the repertoires of antigens recognized by Tregs and effector/memory T cells in the majority of colorectal cancer patients. In addition, only effector/memory T cell responses against antigens recognized by Tregs strongly increased after Treg depletion. The selection of antigens according to preexisting T cell responses may improve the efficacy of future immunotherapies for cancer and autoimmune disease.

Introduction

Malignant transformation and cancer progression are immunologically relevant events in immunocompetent hosts (1). Molecular identification of human tumor-associated antigens (TAAs) in the last decade has led to the development of antigen-specific immunotherapy targeting these antigens. Despite promising results from animal studies, limited objective clinical responses were observed in cancer patients (2, 3). Recent advances in understanding the mechanisms underlying antigen presentation, notably the role of DCs, have led to the development of synthetic long peptides for vaccination purposes. Use of these peptides circumvents HLA restrictions (4), leads to more efficient peptide presentation (5), and minimizes induction of tolerance through antigen presentation on nonprofessional APCs, such as T and B cells (6, 7). Such peptides have been shown to elicit T cell responses against TAA in colorectal carcinoma (CRC) patients (8). However, several challenges remain to be overcome, including the insufficient antitumor responses due to immunosuppression driven by Tregs.

Tregs play a critical role in the maintenance of peripheral self tolerance. Naturally occurring $CD4^+CD25^{hi}$ Tregs are produced in the thymus (9) and express FoxP3, a transcriptional factor required for establishment and maintenance of Treg lineage identity and suppressor function (10, 11). Tregs accumulate at the tumor site, where they suppress the effector function of tumor antigen–specific T cells, resulting in tumor growth despite the presence of tumor antigen–specific T cells (12, 13). Increased densities of tumor-infiltrating $FoxP3^+$ Tregs have been associated with poor prognosis in various solid tumors, including pancreatic (14), ovarian (12, 15), and hepatocellular carcinoma (16, 17). Depletion of Tregs results in enhanced antitumor immunity and tumor rejection in murine models (18) but may also result in the development of autoimmune diseases (19, 20). Consistent with the enhanced antitumor immunity observed in mice, depletion of Tregs in the peripheral blood of patients with CRC was recently shown to boost $CD4^+$ T cell responses to TAAs (21).

Because of the superior suppressive capacities of antigen-specific Tregs over non-antigen-specific Tregs, as shown in mouse studies (22–24), it is critical to determine the TAA recognition patterns of Tregs in cancer patients (25). Previous studies demonstrated the presence of antigen-specific Tregs for restricted sets of antigens (26–28). However, thus far, there has been no comprehensive study comparing the actual distribution of Treg specificities for multiple tumor antigens in cancer patients with their impact on effector T cell (Teff) responses.

In this report, we demonstrate the presence of TAA-specific Tregs in CRC patients against multiple TAAs. Using a broad panel of long synthetic peptides of defined tumor antigens and normal tissue antigens, we here exploited a newly developed method to identify and compare ex vivo the antigen specificities of Tregs with those of memory/effector T cells (Tmems/Teffs). Depletion of Tregs led to increased recognition rates of TAAs as determined by IFN-γ ELISPOT analysis. The strongest increases in TAA recogni-

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Table 1
Tumor antigens and synthetic long polypeptides

TAA (ref.)	Peptide position in aa sequence	Peptide sequence (ref. for included HLA-A2 sequence)
EGFR (54)	479–528	KLFGTSGQKTKIISNRGENSCKATGQ VCHALCSPEGCWGPEPRDCVSCRN (53)
Her-2/neu (45)	351–384	REVRAVTSANIGEFAGCKKIFGSLAFL PESFDGD (49, 50)
MAGE-3 (45)	271–314	FLWGPRALVETSYVKVLHHMVKISG GPHISYPPLHEWVLREGEE (48)
MUC-1 signal sequence (45)	1–100	MTPGTQSPFFLLLLLTVLTVVTGSGHA SSTPGGEKETSATQRSSVPSSTEK NAVSMTSSVLSSHSPGSGSSTTQGQD VTLAPATEPASGSAATWGQDVTS (44)
MUC-1 tandem repeat (45)	137–157	(GVTSAPDTRPAPGSTAPPAH)x5 (44)
P53 (45)	118–167	TAKSVTCTYSPALNKMFCQLAKTCPV QLWVDSTPPPGTRVRAMAIYKQSQ (55)
Telomerase (45)	958–1007	LTFNRGFKAGRNMRRKLFGVLRLK CHSLFLDLQVNSLQTVCTNIYKILLL (52)
Survivin (45)	93–142	FEELTLGEFLKLDRERAKNKIAKETNN KKKEFEETAKKVRRAIEQLAAMD (51)
Heparanase 1(56)	1–50	MLLRSKPALPPPLMLLLLGPLGPLSPG ALPRPAQAQDVVDLDFFTQEPLH (50)
Heparanase 2 (56)	163–212	STYSRSSVDVLYTFANCSGLDLIFGLNA LLRTADLQWNSSNAQLLLDYCS (50)
CEA (45, 46)	569–618	YVCGIQNSVSANRSDPVTLDVLYGPD TPIISPPDSSYLSGANLNLSCHSA (47)

tion were seen for the same TAAs for which specific Tregs were frequently found in the patients. Strikingly, not all TAA-specific T cell responses were under the control of Tregs. The ability to determine Treg specificities in patients raises the possibility of choosing TAAs for vaccination purposes without further impairing tumor-specific immunity by inducing preexistent Tregs in patients.

Results

TAA-specific Teff/Tmem responses are found in CRC patients. Nine TAAs frequently overexpressed in CRCs were selected for the study (Table 1). Additionally, 3 normal self antigens that have not been described to be tumor specific (collagen IV, proinsulin, colon-specific intestinal membrane A4 protein [A4]) were chosen as normal self antigens for comparison (Table 2). From these antigens, long peptide sequences (30–50 amino acids in length) were chosen that contained at least one of the characterized HLA-A*0201–restricted nonapeptides. The remaining sequence was defined using the SYFPEITHI database (29) in such a way that the predicted sequence was optimal for presentation on a wide range of HLA types.

Peripheral blood was taken from 170 patients before tumor resection. Median age of the tumor patients was 66 years (mean, 63.7 ± 12.0 years), ranging from 22 to 88 years. International Union Against Cancer (UICC) staging was available for 142 patients. Of these patients, 21 (15%) were classified as UICC stage I, 33 (23%) UICC stage II, 29 (20%) UICC stage III, and 59 (42%) UICC stage IV. The lymph node status was 0 in 68 patients (49%), 1 in 35 patients (25%), and 2 in 35 patients (25%). Hepatic metastases were detectable in 50% of the patients at the time of analysis.

Autologous DCs loaded with respective peptides were used for stimulation of ex vivo isolated purified peripheral blood T cells and tested in 40-hour IFN-γ ELISPOT assays. T cells stimulated similarly with irrelevant human IgG served for determination of the unspecific background. Primary data from one representative patient are shown in Figure 1A. Antigen-specific responses, defined by significantly increased spot numbers in triplicate wells of test antigen relative to negative control antigen, are indicated by asterisks. To verify that our findings on TAA-reactive T cell responses were not due to a decrease in IFN-γ spots in control wells, we compared total IFN-γ spots in responding and nonresponding patients. Low background spot numbers were not correlated with a higher probability of achieving positive results. Instead, positive results were characterized by significantly increased reactivity against TAAs (Figure 1B). These TAA-specific T cell responses were confined to the T cell compartment of $CD45RO^+$ Tmems/Teffs since we did not detect any TAA-specific reactivity in the population of $CD45RO^-$ naive T cells (Figure 1C). Frequencies of TAA-reactive T cells were calculated by subtracting mean background values from mean test values in case of significant antigen-specific responses (Figure 1D). The highest frequencies of tumor-specific T cells in responding patients were seen against autologous tumor lysate and the polypeptides derived from mucin-1 and heparanase. Figure 1E shows the percentage of CRC patients and healthy individuals in whom T cell responses against the respective TAAs or normal self antigens were measured. Interestingly, the recognition rates of the individual TAAs varied strongly. Whereas we found frequent responses against p53 (21.3%), carcinoembryonic antigen (CEA; 17.2%), HER2/neu (17.6%), and heparanase pp1

Table 2
Normal self antigens and synthetic long polypeptides

Antigen	Peptide position in antigen sequence	Peptide sequence (ref. for included HLA-A2 sequence)
IgG1	40–89	SWNSGALTSGVHTFPAVLQSSGLYS LSSVVTVPSSSLGTQTYICNVNHKP
Proinsulin	1–50	MALWMRLLPLLALLALWGPDPAAA FVNQHLCGSHLVEALYLVCGERGFFY (57)
Collagen IV	1–50	MKLRGVSLAAGLFLLALSLWGQPAE AAACYGCSPGSKCDCSGIKGEKGER (58)
Intestinal membrane A4 protein	101–150	VVLVERGNHSKIVAGVLGLIATCLFG YDAYVTFPVRQPRHTAAPTDPADG (59)

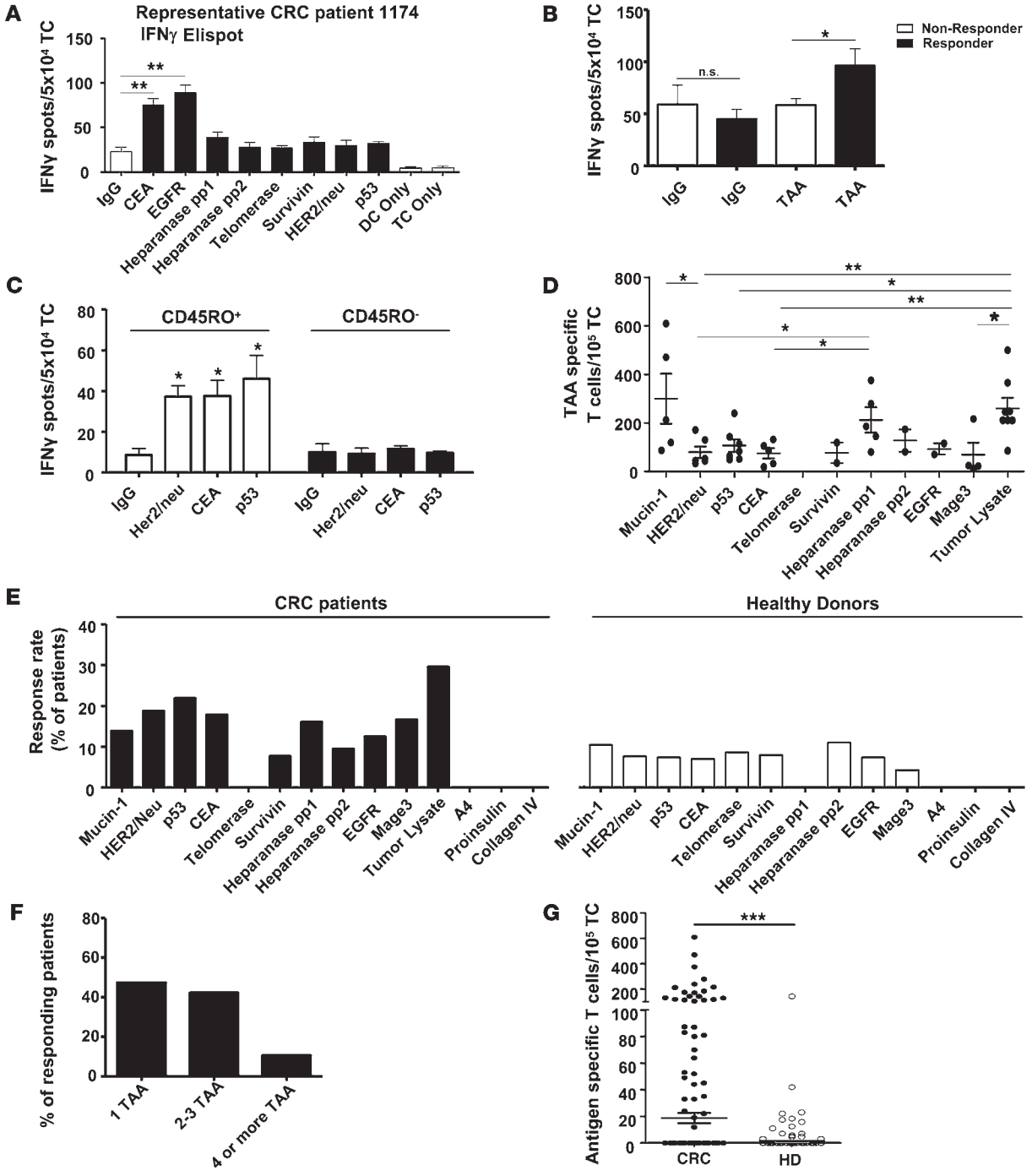

Figure 1

T cell responses against TAAs in CRC patients and healthy donors. (**A**) Primary data from an exemplary IFN-γ ELISPOT assay. Peripheral blood T cells of 120 patients and 32 healthy donors were stimulated with polypeptides derived from negative control antigen (human IgG), normal self antigens, or the respective test tumor antigens. Data represent mean spot number of 3 wells per antigen ± SEM. $^{**}P < 0.05$, spot numbers in test wells compared with pooled spot numbers of negative control antigen (2-tailed Student's *t* test). TC, T cells. (**B**) Cumulative total IFN-γ spots from Treg-undepleted T cells for control antigen (IgG) in responding and nonresponding patients and responding and nonresponding TAAs. Mean ± SEM; $^{*}P < 0.05$ (2-tailed Student's *t* test); IgG, $n = 21–28$; TAAs, $n = 29–127$. (**C**) Primary data of an IFN-γ ELISPOT assay with CD45RO^{+} Tmems or CD45RO^{-} naive T cells. Mean ± SEM; asterisks indicate significant differences between those groups that are connected by horizontal lines. $^{*}P < 0.05$. (**D**) Frequency of TAA-specific T cells from responding CRC patients for single TAAs. Mean ± SEM; asterisks indicate significant differences between those groups that are connected by horizontal lines; $^{*}P < 0.05$; $^{**}P < 0.01$; $n = 2–8$. (**E**) Proportions of CRC patients and healthy donors among all individuals tested that exerted significant T cell reactivity against TAAs from autologous tumor or against polypeptides derived from the respective tumor antigens. Data represent the percentage of patients or healthy donors with TAA-specific T cells; $n = 10–36$. (**F**) Number of different TAAs recognized in individual responding patients; $n = 25$. (**G**) Frequency of TAA-specific T cells from individual CRC patients and healthy donors (HD). Mean ± SEM. $^{***}P < 0.0001$; $n = 238–269$.

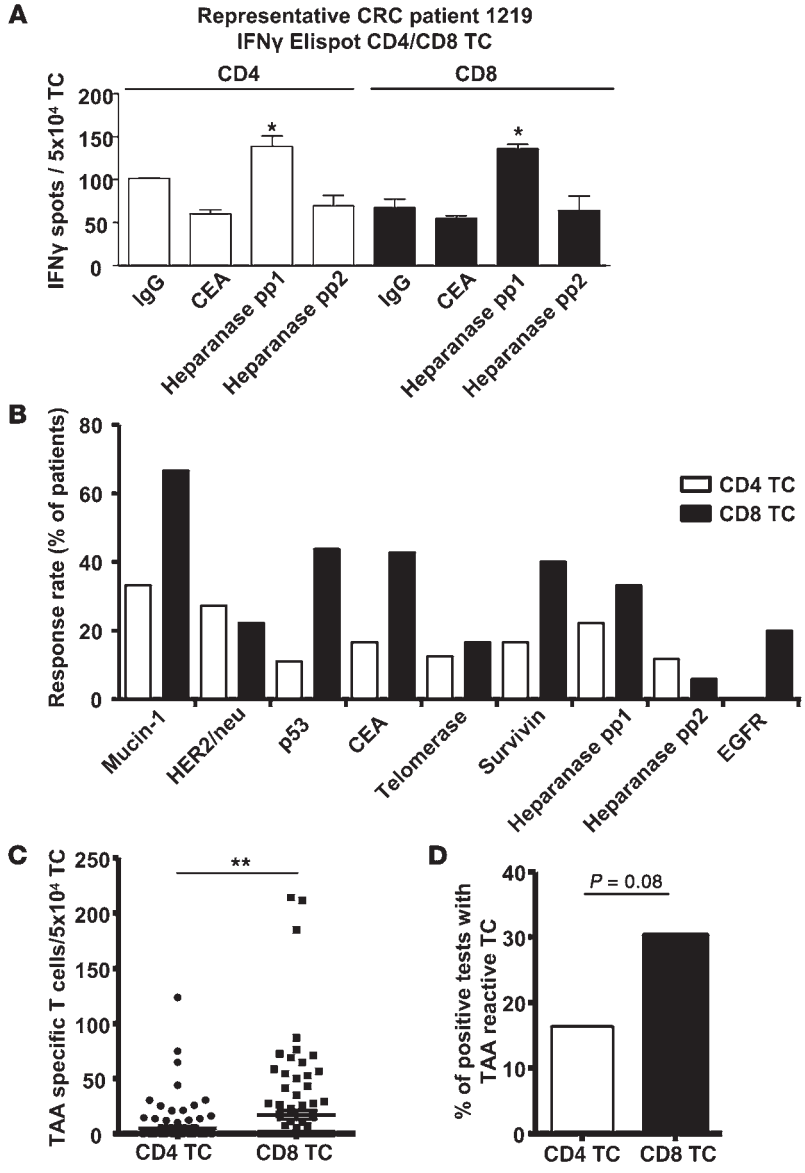

Figure 2

CD4+ and CD8+ T cell responses against TAAs in CRC patients. (**A**) Primary data of an exemplary IFN-γ ELISPOT assay with separated CD4+ and CD8+ T cell populations. Peripheral blood T cells of CRC patients were separated into a CD4+ and CD8+ T cell fraction and subsequently stimulated with TAA-derived polypeptides. Data represent mean spot number of 3 wells per antigen ± SEM. *$P < 0.05$, spot numbers in test wells compared with pooled spot numbers of negative control antigen (2-tailed Student's t test). (**B**) Proportions of CRC patients among all individuals tested that exerted significant CD4+ or CD8+ T cell reactivity against polypeptides derived from the respective tumor antigens. Data represent the percentage of patients with TAA-specific T cells; $n = 5–18$. (**C**) Frequency of TAA-specific CD4+ and CD8+ T cells from individual CRC patients. Mean ± SEM. **$P < 0.01$; $n = 99–110$. (**D**) Comparison of TAA-specific CD4+ and CD8+ T cell reactivity in CRC patients. Data represent the percentage of tests where significant T cell reactivity against a TAA was measured (Fisher's exact test); $n = 99–110$.

(16.1%), we did not find any telomerase-specific T cells in any of the tested patients. Most frequently, we detected T cell responses against autologous tumor lysate (29.6%). The response rates in healthy donors were significantly lower than in the CRC patients, and we did not find self antigen–specific T cell responses in CRC patients or healthy donors. Recognition of TAAs was very individual and mostly oligovalent, with responding patients on average recognizing 1.7 TAAs (Figure 1F). Only rarely did the patients recognize more than 3 TAAs. To assess the strength of spontaneous antitumor T cell responses, we compared the frequencies of TAA-reactive T cells in CRC patients and healthy donors (Figure 1G). TAA-specific T cells were observed at significantly higher frequencies in CRC patients than in healthy donors, showing the selective presence of TAA-specific Tmem/Teff responses in tumor patients.

TAA-derived synthetic long peptides are recognized by CD4+ and CD8+ T cells. The polypeptide sequences used in our analysis contained sequences that have been shown to be presented on HLA-A*0201 MHC class I alleles. To test whether these polypeptides can also be presented on MHC class II complexes, we assessed the ability of CD4+ T cells to recognize the same tumor antigens. For this, T cells were separated into a CD4+ T cell fraction and a CD8+ T cell fraction using magnetic beads. Both fractions were then tested separately for their ability to secrete IFN-γ upon incubation with TAA-pulsed DCs in an ELISPOT assay. Primary data from 1 representative experiment are shown in Figure 2A. Response rates against TAAs and the frequencies of TAA-specific T cells were higher in the CD8+ cytotoxic T cell compartment as compared with the CD4+ T helper cell compartment for most antigens tested (Figure 2, B–D). However, CD4+ T cell responses were found for all antigens, except for EGFR, demonstrating that the length of the polypeptides is sufficient for presentation on MHC class II alleles and subsequent recognition by CD4+ T cells.

Treg depletion results in stronger TAA-specific Tmem/Teff function. To examine the impact of Tregs on the TAA-specific Tmem/Teff responses, we first looked at the presence of Tregs in the peripheral blood of CRC patients. CD4+CD25+Foxp3+ Tregs accounted for approximately 5% of total CD3+CD4+ cells (Figure 3, A and B). We then developed a protocol for the depletion of CD4+CD25+ Tregs from the blood. For this, purified CD3+ cells were subjected to CD4 followed by CD25 magnetic bead isolation. Depletion of Tregs resulted in the loss of Foxp3+ cells as determined by flow cytometry (Figure 3, C and D). Next, we compared TAA-specific T cell responses in Treg-undepleted T cell fractions with T cell responses in Treg-depleted T cell fractions (Figure 4A). As seen for the Treg-undepleted fraction, there was no correlation between low background spot numbers and a higher probability of positive test results in Treg-depleted fractions (Figure 4B). We found a significant increase in IFN-γ spots only for TAAs for which reactive T cells were found in comparison with nonresponding TAAs. For most tested TAAs, we saw an increase in the response rates of CRC patients after deple-

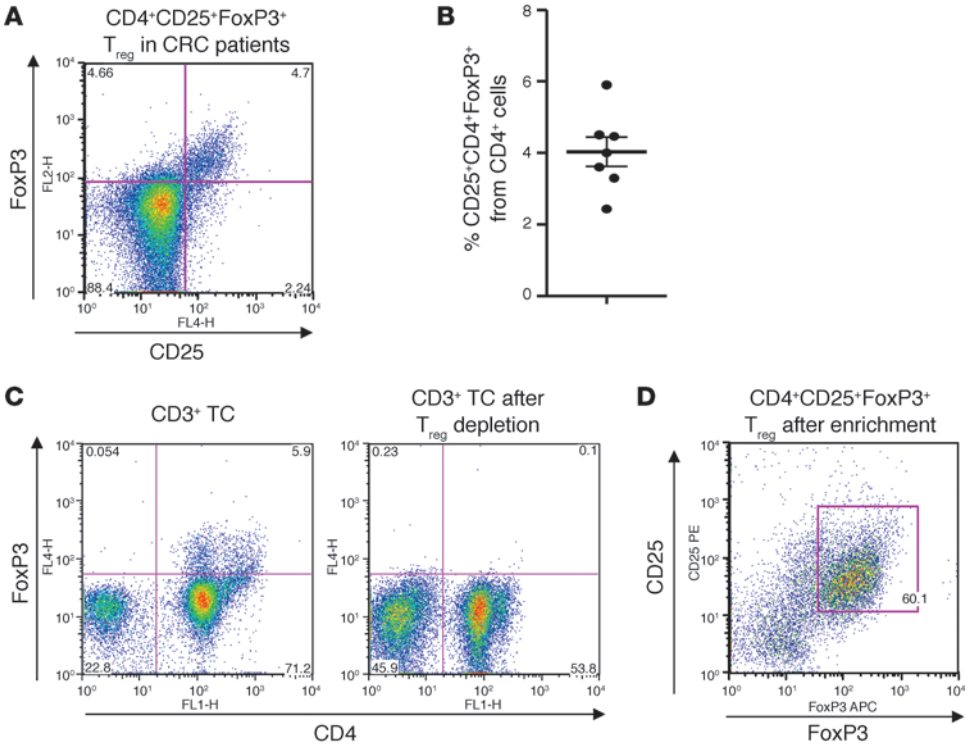

Figure 3

Presence of Tregs in CRC patients. (**A**) Exemplary flow cytometry of CD25+FoxP3+ Tregs gated on CD3+CD4+ T cells from peripheral blood of 1 CRC patient. (**B**) Percentage of CD4+CD25+FoxP3+ Tregs of CD4+ T cells in the peripheral blood of individual CRC patients. Mean ± SEM. $n = 7$. (**C**) Foxp3 expression in CD3+ cells before and after depletion of CD4+CD25+ Tregs. (**D**) Enrichment of CD4+CD25+FoxP3+ Tregs after isolation of CD4+CD25+ cells.

tion of Tregs (Figure 4, C and D). Such an increase was not seen in healthy donors. However, some TAAs in CRC patients were not influenced by a depletion of Tregs and only showed minor increases in response rates (p53, survivin, MAGE-3) or no increase (heparanase). As with the Treg-undepleted T cells, no T cell responses against the tested normal self antigens in CRC patients were measured in the absence of Tregs. Only in healthy donors, we rarely found T cell responses against proinsulin after Treg depletion. In addition to increased response rates in the patients, we measured stronger TAA-specific responses after depletion of Tregs as determined by an increase in the frequencies of TAA-reactive T cells (Figure 4E). Furthermore, depletion of Tregs led to an increased fraction of responding patients that displayed reactivity against more than 3 TAAs, as compared with the Treg-undepleted T cells (data not shown). Responding patients on average recognized 2.1 TAAs after Treg depletion. Our analyses suggest that not all TAAs are under the control of Tregs. As the depletion of Tregs did not result in any increases in TAA recognition of more than 10% in healthy donors, we considered any change below this threshold as background. We therefore classified the TAAs into a group that showed in CRC patients a more than 10% increase in T cell response rate after Treg depletion (Treg-dependent TAAs) and a group with Treg-independent Tmem/Teff responses (less than 10% increase in response rate after Treg depletion). According to this classification, the increase in IFN-γ ELISPOT response rate was primarily found in the Treg-dependent Tmem/Teff response group (Figure 4F).

Recognition of long synthetic TAA-derived polypeptides is independent of HLA type. Since long synthetic polypeptides contain a large number of sequences that could potentially be presented on MHC complexes, we investigated the dependency of polypeptide recognition in patients on the HLA type of these patients. All polypeptides used contained at least 1 sequence that has been described to be efficiently presented by APCs on HLA-A*0201–positive cells. We therefore performed HLA typing for all patients and classified them accordingly in an HLA-A*0201–positive and an HLA-A*0201–negative cohort. Comparison of these groups revealed no difference in the ability to recognize TAAs, suggesting that the length of the peptides used is sufficient to allow antigen presentation independent of the patients' HLA type (Figure 4G). In line with this, we did not see differences in antigen recognition between HLA-A*0201–positive and –negative cohorts for any of the respective antigens (data not shown).

TAA-specific Tregs are found in CRC patients but not in healthy donors. To investigate whether the differential influence of Tregs on TAA-specific T cell responses is due to an unequal distribution of Treg specificities, we developed an assay to investigate the TAA recognition capabilities of Tregs. As displayed in Figure 5A, we pulsed DCs with polypeptides derived from the TAAs or with irrelevant human IgG as control and added Tregs to these cells in order to selectively activate Tregs specifically recognizing a TAA sequence presented by the DCs. The proliferation of polyclonally activated Teffs was then suppressed in comparison to test wells with irrelevant antigens. As an additional control, we also used unpulsed DCs since all antigens tested are self antigens, including the control antigen IgG, and might therefore lead to an activation of Tregs and subsequent suppression of T cell proliferation. Primary data of 2 representative patients are shown in Figure 5, B and C. Antigen-specific Treg suppression, defined by significantly decreased proliferation in triplicate wells of test antigen compared with negative control antigen, is indicated by asterisks. TAA-specific Tregs were most prevalently observed in CRC patients for CEA, telomerase, HER2/neu, and MUC-1, whereas Tregs specific for survivin were only rarely detected (Figure 5D). Furthermore, we did not detect any Tregs specific for p53 or either one of the 2 heparanase-derived polypeptides. No TAA-specific Tregs were detected in healthy donors. Interestingly, we frequently detected Tregs specific for the normal self antigen proinsulin in both CRC patients and healthy donors.

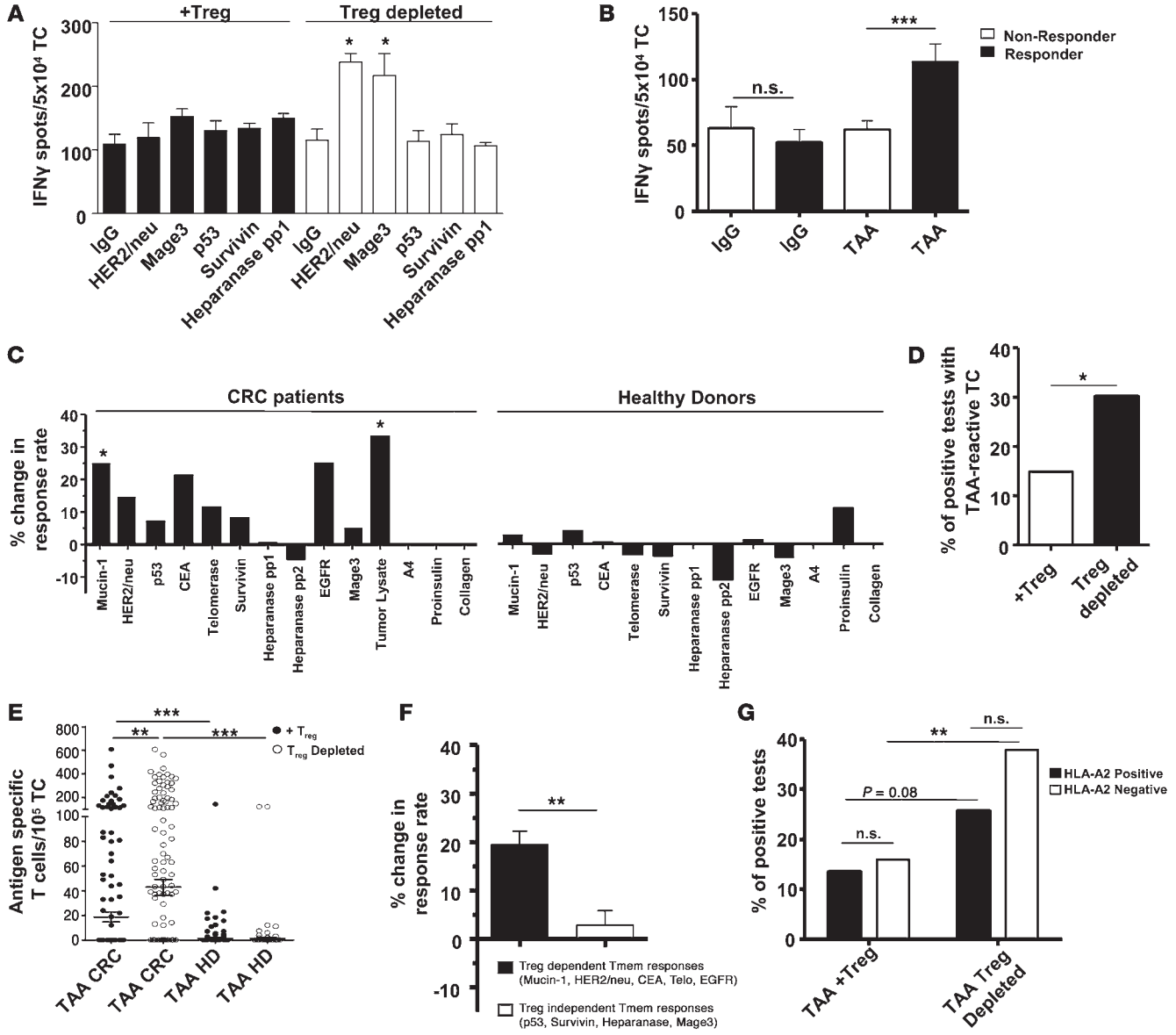

Figure 4

Influence of Tregs on TAA-specific T cell responses in CRC patients and healthy donors. (**A**) Primary data of an exemplary IFN-γ ELISPOT assay (CRC patient 1056) with T cell fractions before and after depletion of $CD4^+CD25^+$ Tregs. Mean ± SEM; horizontal lines indicate samples containing (black bars) or lacking (white bars) Tregs; $^*P < 0.05$ (2-tailed Student's *t* test). (**B**) Cumulative total IFN-γ spots from Treg-depleted T cells for control antigen (IgG) in responding and nonresponding patients and responding and nonresponding TAAs. Mean ± SEM; $^{***}P < 0.0001$ (2-tailed Student's *t* test); IgG, $n = 21–28$; TAAs, $n = 50–106$. (**C**) Data represent the percentage change in tested individuals that exerted significant T cell reactivity against the tested TAAs after depletion of Tregs relative to the response rates before Treg depletion. $^*P < 0.05$ (Fisher's exact test); $n = 10–34$. (**D**) Comparison of TAA reactivity in CRC patients before and after depletion of Tregs. Data represent the percentage of tests where significant T cell reactivity against a TAA was measured. $^*P < 0.05$ (Fisher's exact test); $n = 246–254$. (**E**) Frequencies of TAA-specific Tmems before and after Treg depletion in individual CRC patients and healthy donors. Mean ± SEM; $^{**}P < 0.01$; $^{***}P < 0.001$ (2-tailed Student's *t* test); $n = 209–269$. (**F**) TAAs were classified into Treg-dependent Tmem responses (>10% change in response rate) or Treg-independent Tmem responses (<10% change in response rate). Mean ± SEM; asterisks indicate significant difference between the groups indicated by horizontal line; $^{**}P < 0.01$. Telo, telomerase. (**G**) Patients were tested for the presence of HLA-A*0201 and grouped into an HLA-A*0201–positive and an HLA-A*0201–negative cohort. Depicted are the proportions of CRC patients for both groups among all individuals of the respective group tested that exerted significant T cell reactivity against tested polypeptides. $^{**}P < 0.01$; NS, $P > 0.05$ (Fisher's exact test); $n = 93–189$.

High individuality of Treg specificities and TAA-specific Tmem/Teff responses. To examine whether the recognition of TAAs by Tregs directly correlates with TAA-specific spontaneous Tmem/Teff responses, we compared Treg specificity analyses with IFN-γ ELISPOT analyses in 12 patients (Figure 6). A proportion of patients displayed TAA-specific Tregs but no Tmem/Teff responses against any of the tested antigens (patients 1296, 1317, 1333). Specific Treg and Teff responses after depletion of

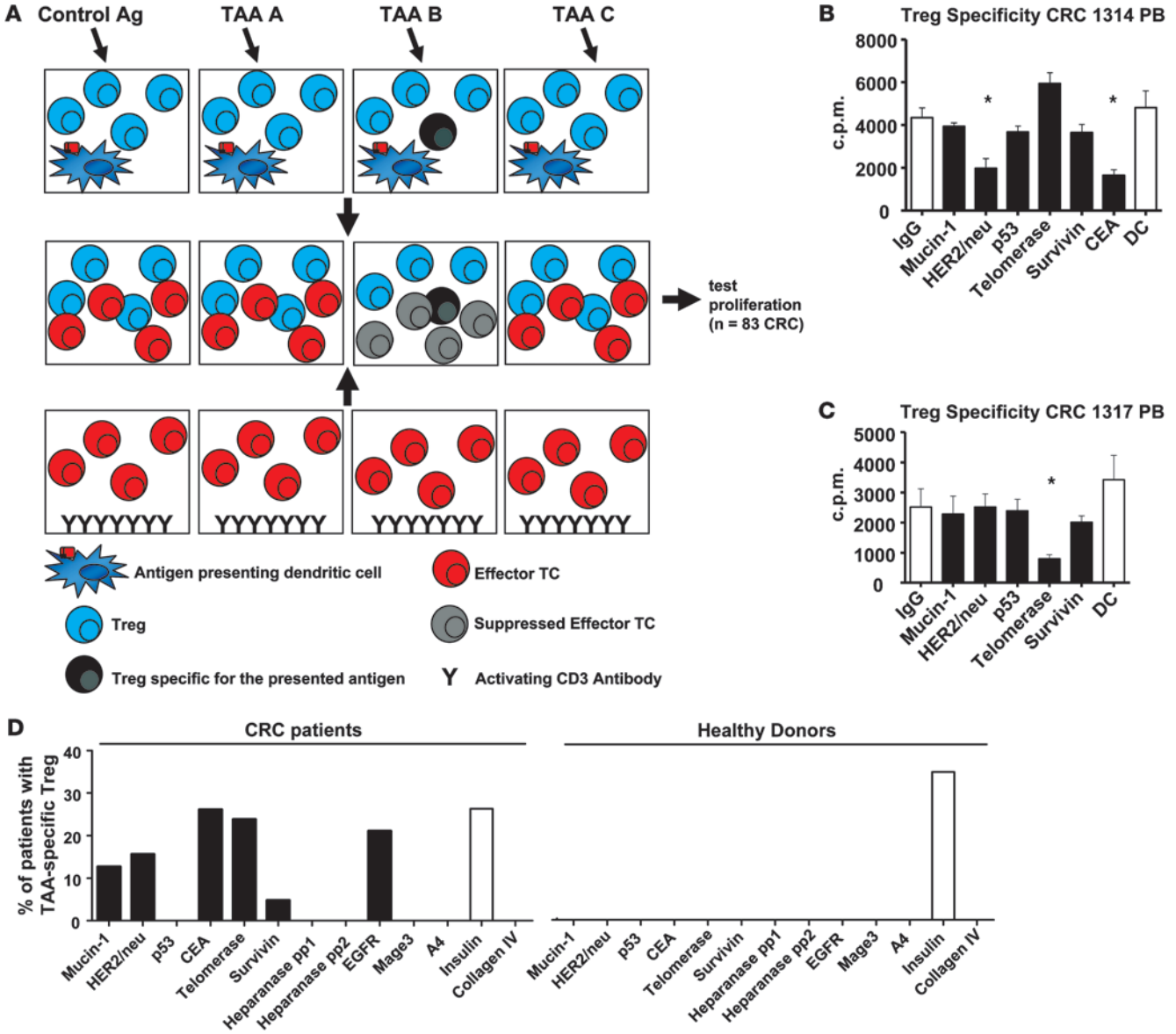

Figure 5

Specificity of Tregs for TAAs in CRC patients and healthy donors. (**A**) Scheme for determination of Treg specificity. DCs were pulsed with TAA polypeptide or control antigen. Autologous Tregs were added. Tregs specific for an antigen presented by the DCs were selectively activated. Polyclonally activated Teffs were added to the DCs and Tregs. Tregs activated by their respective antigen then suppressed the proliferation of the Teffs, which was measured by [^{3}H]thymidine incorporation. (**B** and **C**) Primary data of 2 representative Treg specificity experiments displaying selective suppression of proliferation in wells where DCs were pulsed with HER2/neu and CEA (**B**) or telomerase (**C**). DC, no antigen added. Data represent mean counts per minute of 3 wells per antigen ± SEM; *$P < 0.05$, counts in test wells compared with pooled counts of negative control antigen (2-tailed Student's *t* test). (**D**) CRC patients and healthy donors were tested for the presence of TAA-specific Tregs. Data represent the percentage of individuals with Tregs specific for the respective antigen; CRC patients, $n = 14–56$; HD, $n = 10–14$.

Tregs against the same antigens were detected in patients 1314, 1341, 1299, and 1295. Furthermore, some patients displayed Treg specificities or TAA-reactive Teffs without noticeable correlation. Thus, Treg specificities and TAA-specific spontaneous Tmem/Teff responses were highly individual in CRC patients.

Treg specificities correlate with Treg influence in IFN-γ ELISPOT. For TAA-reactive T cell responses, we classified the tested TAAs based on the differential influence of Tregs (Figure 4F). We then applied the same classification on the Treg specificities that we measured in the peripheral blood of CRC patients. As shown in Figure 7A, 96% of the Treg specificities measured were against TAAs in which a strong influence of Tregs was also seen in the IFN-γ ELISPOT experiments, but only 4% of the specificities were in the TAA group in which no or only minor Treg influence was present in these experiments. The unequal recognition of TAAs by Tregs was not due to a general bias of T cells to recognize only

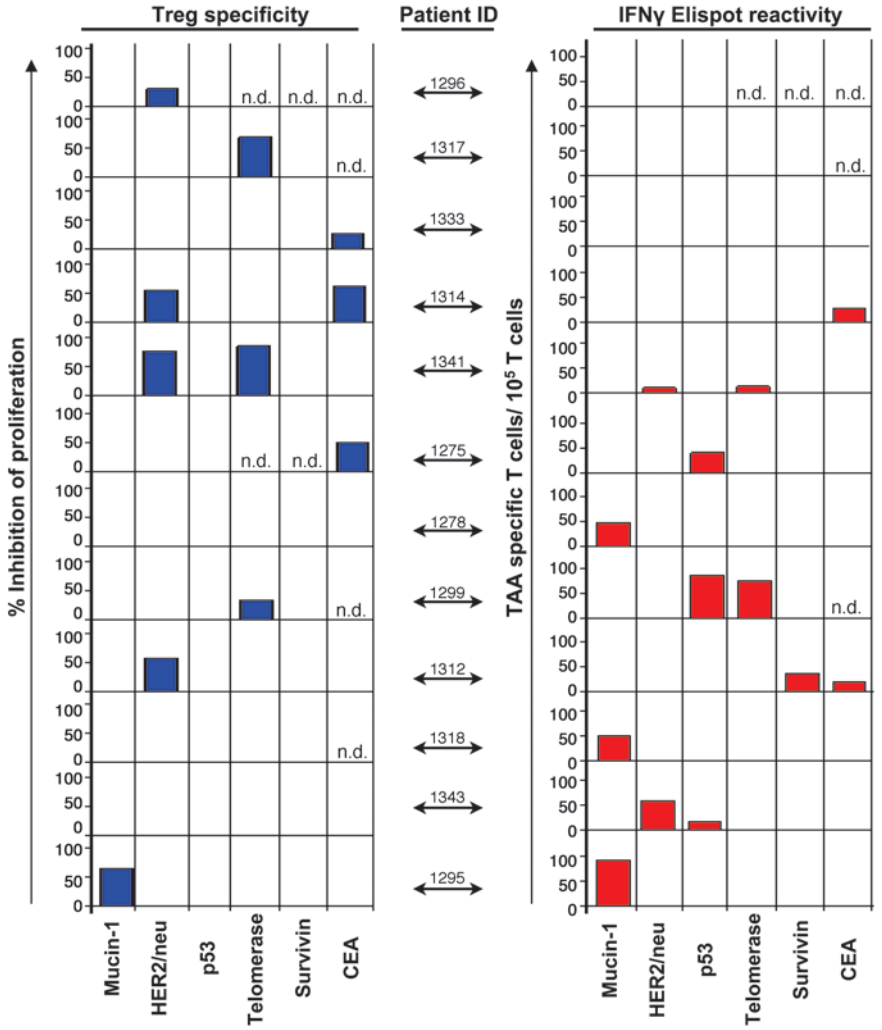

Figure 6

Individuality of Treg specificities and T cell responses against TAA-derived polypeptides. Displayed is the pattern of reactivity in Treg specificity analysis and IFN-γ ELISPOT analysis in 12 patients. For Treg specificity assays, data represent mean inhibition of proliferation of 3 wells per antigen compared with control antigen for antigens that exerted significant inhibition of proliferation. For IFN-γ ELISPOT analysis, data show the mean number of TAA-specific T cells of 3 wells per antigen for those antigens that showed significant difference in spot numbers compared with control antigen. n.d., not determined.

Discussion

This study provides what we believe to be the first detailed examination of the influence of Tregs on TAA-specific immune responses in a large cohort of cancer patients. Using a protocol developed by our group, we found TAA-specific Tregs for a broad range of antigens that have been previously described as relevant TAAs in CRC. The identification of tumor-specific Tregs did not require cloning of Tregs and was readily done from peripheral blood samples. Interestingly, we frequently found Tregs specific for the same antigens for which we saw strong increases in Teff responses after depletion of Tregs.

Preexisting T cell responses in the peripheral blood of cancer patients were found against all tested TAAs, except for telomerase, whereas no T cell responses against the normal self antigens were measured (Figure 1E). T cell reactivity was confined to the CD45RO$^+$ population of Tmems and Teffs (Figure 1C). Therefore, it is possible that our approach of using total T cell populations in the tests underestimated to some degree the presence of tumor-reactive T cells, which might have consequences for our estimation of the impact of Tregs on these responses. However, the procedure of separating Tmems/Teffs from naive T cells is associated with a major loss of approximately 50% of cells. The limited amount of cells that could be obtained from a patient's blood sample, the further cell loss caused by the procedure of Treg depletion, and the need to test in parallel T cell responses against various TAAs forced us to use total T cell populations instead of Tmem purification. In healthy donors, TAA-specific T cell responses were only rarely observed. Furthermore, the frequency of TAA-specific T cells as determined by IFN-γ ELISPOT analysis was massively increased in cancer patients as compared with healthy individuals (Figure 1G), demonstrating that the observed TAA responses are limited to cancer patients. T cell responses against TAAs were very individual and oligovalent.

the TAAs in the Treg-dependent TAA group. The TAA-reactive T cell responses were similar in the Treg-dependent TAA group and the Treg-independent TAA group (53% and 47%, respectively) (Figure 7B). From some patients, we isolated sufficient numbers of T cells and Tregs to evaluate simultaneously TAA-specific Tmem/Teff responses and Treg responses. While this group was too small for generalized conclusions, the data obtained confirm on an individual level our observation that the presence of TAA-specific Tregs controls the reactivity of T cells against the same antigens and that such Treg-mediated T cell inhibition is predominantly directed against the group of "Treg-dependent" antigens (MUC-1, Her-2, telomerase, CEA, and EGFR) but not against the group of "Treg-independent antigens" (heparanase, p53, survivin, or MAGE) (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI39608DS1).

The use of long synthetic polypeptides has been previously described to be advantageous over short peptides with exact MHC binding properties (4). In line with this, TAA-derived polypeptides were recognized by both CD4$^+$ and CD8$^+$ T cells (Figure 2). Interestingly, most TAA were recognized more frequently by CD8$^+$ than CD4$^+$ T cells. This is consistent with previous studies demonstrating that an increase in the length of the peptide used for vaccination resulted in more robust and effective CD8$^+$ T cell responses than vaccination with a minimal T cell epitope (2, 3). In these studies, an increase in CD8$^+$ T cell responses was also observed in the absence of CD4$^+$ T cell help, as shown by significantly higher CD8$^+$ T cell responses in MHC class II–knockout

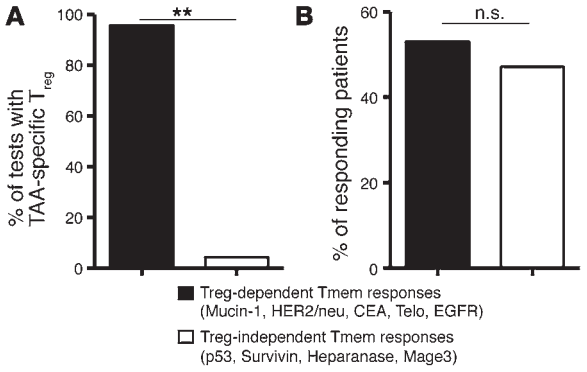

Figure 7

Correlation of Treg specificities with Tregs' influence on TAA-specific Tmem responses. (**A**) Distribution of Treg specificities to TAAs in Treg-dependent Tmem responses group and Treg-independent Tmem responses group based on IFN-γ ELISPOT analysis. Data represent the percentage of tests with TAA-specific Tregs. $^{**}P < 0.01$ (Fisher's exact test); $n = 80–152$. (**B**) Distribution of TAA-specific Tmem responses before depletion of Tregs in Treg-dependent Tmem responses group and Treg-independent Tmem responses group. Data represent the percentage of responding patients; $n = 23$.

mice and in CD40-knockout mice when vaccinated with a long-peptide vaccine in contrast to a minimal MHC class I epitope.

Furthermore, the recognition of TAA-derived polypeptides was independent of the patients' HLA type, demonstrating that the length of the polypeptides was sufficient to allow for efficient presentation on various HLA alleles (Figure 4G). Testing of TAA-specific T cell reactivities is therefore not confined to certain HLA alleles present only in a subset of patients. Furthermore, this lack of dependence on restricted HLA types might circumvent the potential immunogenic escape of cancer cells in a vaccination setting through downregulation of individual HLA alleles or the mutation of T cell epitopes.

On average, 5% of $CD4^+$ T cells were $Foxp3^+$ Tregs in CRC patients (Figure 3, A and B). Depletion of Tregs led to increased recognition rates of TAA by TAA-specific Tmems (Figure 2, C and D). Interestingly, Treg depletion did not lead to increased T cell recognition rates for some TAAs, such as p53 and heparanase, suggesting that Treg suppression is not equal for all tested antigens. Our data revealed that Tregs suppress preexisting TAA-specific immune responses in some patients. In line with this, Treg depletion did not result in increased response rates in healthy donors. Of note, no T cell responses were observed against normal self antigens in the absence of Tregs in CRC patients. Only rarely did we detect T cell responses against proinsulin in healthy individuals in the absence of Tregs. Classification of TAAs, depending on the influence of Treg depletion on T cell responses against these antigens, revealed that the increase in patient response rates were due to a specific set of TAAs, including MUC-1, EGFR, CEA, and HER2/neu.

Examination of Treg specificities in CRC patients demonstrated that TAA-specific Tregs are frequently present in these patients (Figure 5D). This is in line with the observation that in the presence of Tregs, Teff responses were dampened in some patients. Preexisting Tregs with specificities for CEA, telomerase, HER2/neu, MUC-1, and EGFR were prevalent. However, we did not find specific Tregs for p53 or heparanase in any of the tested patients. The discriminative recognition of TAAs by Tregs was not due to the fact that the polypeptides that were not or were only poorly recognized did not contain any sequences that can be presented on MHC class II complexes (Figure 2) or to an overall increased recognition of the TAAs for which specific Tregs are found (Figure 7, A and B). The recognition rate of TAAs by memory T cells was similar for both the TAAs that were recognized by Tregs and for those that were not. One explanation for this finding may be that the different environments in which the proteins are expressed may account for an unequal capacity to induce Tregs specific for a particular antigen. Heparanase is a proteolytic enzyme that degrades polymeric heparin sulfate molecules in the extracellular matrix and is primarily abundant at inflammatory sites (30). The microenvironment of these sites may inhibit the induction of Tregs against this protein. The tumor suppressor p53 is involved in the regulation of the cell cycle. p53 is inactive in normal cells and is bound to the protein MDM2, which prevents its action and promotes its degradation by acting as a ubiquitin ligase. The low abundance of this protein in normal cells may also lead to a weakened induction of Tregs against p53. The impact of the antigen-specific Treg-derived suppressive function on antitumor immunity is further emphasized by reports from mouse studies that $CD4^+CD25^+$ Tregs inhibited $CD4^+CD25^-$ conventional T cells with the same antigen specificity when cotransferred at similar numbers (31). The presence of preexisting Tregs in patients is of eminent importance when TAA-derived peptides are used in a vaccination setting that may induce or reactivate preexisting Tregs and might therefore further hamper an effective immune response, as was observed in a study by van der Burgh and colleagues (32).

Strikingly, we commonly found Tregs specific for proinsulin, a precursor of insulin produced by β-islet cells in the pancreas. These Tregs were present in CRC patients and in healthy individuals alike. Several reports have shown the presence of insulin-specific Teffs (33, 34) and that depletion of Tregs can lead to destruction of β-islet cells and development of a diabetes phenotype in mice (35). Our data indicate that Tregs with specificity for β-islet–specific proteins, such as proinsulin, are commonly present in the peripheral blood and may be involved in the prevention of autoimmune-mediated destruction of β-islet cells and sequential development of type 1 diabetes.

Although we found Tregs specific for the same antigens for which we measured increased memory T cell response rates after Treg depletion, the individual TAA-specific T cell responses and Treg specificities did not always correlate within single patients (Figure 5). Several factors may be responsible for this observed high individuality, including the possibility that the time frame of in vitro culture in the absence of Tregs may not be long enough to unmask all T cell responses that have been suppressed by Tregs. In addition, since the TAA-derived polypeptides used in this study represent only part of the total protein sequence, it is possible that we detected Treg responses against some epitopes within these sequences, while preexisting memory T cell responses against epitopes that are not present in the sequence may have remained undetected, and vice versa. TAA-specific memory T cell response rates and TAA recognition by Tregs against a chosen antigen may therefore increase when overlapping polypeptides are used that cover the full protein sequence. Therefore, the grouping of the TAAs into Treg-dependent Tmem/Teff and Treg-independent Tmem/Teff responses is not inalterable and may differ depending on the exact antigen sequence tested.

It is likely that other Treg-mediated effector functions, besides the suppression of Teff proliferation, add to a confinement of antitumor immune responses. Several suppression modalities have been recently shown to be involved in Treg function (36–42).

Investigation of these mechanisms using the protocol described here might bear valuable information on Treg activity in cancer patients. However, the strong correlation between the effect of Treg depletion on Teff function (antigen-specific IFN-γ production) and the specificities found for Tregs suggests that the measured suppression of Teff proliferation by Tregs, as determined in our analysis, is a reliable readout for Treg involvement.

In conclusion, CRC patients develop multivalent and individual T cell responses against a broad variety of different CRC-associated TAAs. Some, but not all of these T cell responses are kept under the control of TAA-specific Tregs. TAA-specific Tregs are predominantly present in the blood of patients but are not detectable in healthy individuals. Differences between Tregs and Teffs in the repertoires of recognized antigens can be detected in a majority of patients. Using selected sets of TAAs for tumor vaccinations that induce optimal Teff responses but minimal Treg activity may improve the efficacy of vaccination protocols.

Methods

Patient details. Peripheral blood samples were obtained from 170 patients with CRC and from 32 healthy donors. Informed consent was obtained from all participants. The protocol was approved by the Ethical Committee of the University of Heidelberg. Heparanized peripheral blood was subjected to Ficoll gradient centrifugation (Biochrom), and cells at interphase were collected.

Cell purification and culture. T cells were cultured for 7 days in RPMI medium containing 10% AB serum, 100 U/ml IL-2, and 60 U/ml IL-4. Afterward, T cells were transferred into cytokine-free medium for 12 hours and subsequently separated from contaminating cells by T cell negative isolation (Invitrogen). Some T cells were removed as Treg-undepleted T cell fraction. The remaining T cells were isolated for CD4 and CD25 expression by magnetic bead isolation ($CD4^+CD25^+$ Regulatory T Cell Isolation Kit; Miltenyi Biotec). $CD4^+CD25^+$ cells were collected separately. $CD4^+CD25^-$ and $CD8^+$ T cells depleted of $CD4^+CD25^+$ cells were mixed and used as the Treg-depleted T cell fraction. For CD45RO purification, T cells were stained with CD45RO-PE monoclonal antibody (BD), followed by magnetic bead isolation using anti-PE microbeads (Miltenyi Biotec).

DCs were generated as described previously (43). In brief, adherent cells from peripheral blood samples were cultured for 7 days in serum-free X-VIVO 20 (Cambrex) containing 50 ng/ml recombinant human GM-CSF, and 1,000 U/ml IL-4. DCs were enriched using anti-CD3–, anti-CD56–, and anti-CD19–coupled magnetic beads and pulsed for 14 hours with test or control peptides.

Antigens. As test tumor antigens, we used 11 different polypeptides derived from 9 different tumor antigens — MUC-1 (44, 45), CEA (45–47), MAGE-3 (45, 48), Her-2/neu (45, 49, 50), Survivin (45, 51), telomerase (45, 52), EGFR (53, 54), p53 (45, 55), and heparanase (50, 56) — expressed in CRC cells. All peptides were designed in such a way that they contain a previously described immunogenic HLA-A*0201 T cell epitope.

The tumor antigens and amino acid sequences of respective peptides are summarized in Table 1. In addition to TAAs, we used polypeptides derived from 3 non-malignancy-associated antigens: proinsulin (57), collagen IV (58), and the colon-specific intestinal membrane A4 protein (59) expressed by epithelial cells and stromal cells in normal colon mucosal tissue. As negative control antigens, we used human IgG. In Treg specificity analyses, we also used synthetic polypeptides derived from human IgG1. In some cases, DCs were pulsed with lysates of autologous colorectal tumor cells as a source of TAAs or with lysates of autologous PBMCs as a source of respective control antigen. Cell lysates were generated from freshly excised and mechanically dissected colorectal tumor biopsies or from PBMCs by 5 cycles of freezing and thawing, followed by filtration through an 0.3-μm filter as previously described (43).

IFN-γ ELISPOT assay. ELISPOT assays were done as described previously (43), with modifications. Briefly, peptide-pulsed DCs were incubated with autologous T cells at a 1:5 ratio for 40 hours in ELISPOT plates. IFN-γ spots were measured using KS ELISPOT software (Zeiss). Spots induced by control peptides were considered as background. Individuals were designated as responders if spot numbers in the presence of test peptides were significantly greater ($P < 0.05$) than in pooled wells of the negative control human IgG. In some cases, DCs were pulsed overnight with lysates from autologous colorectal tumor cells (test antigen) or autologous PBMCs (negative control antigen) at a concentration of 200 μg/ml and used for T cell stimulation during IFN-γ ELISPOT assay as described above. The frequency of tumor-reactive T lymphocytes was calculated as follows: (spots in test wells – spots in control wells)/T cell numbers per well. Each test group consisted of 3 wells. Low background spot numbers were not correlated with a higher probability of achieving positive results.

Flow cytometry. PBMCs, enriched $CD3^+$ T cells, enriched $CD3^+$ T cells depleted of $CD4^+CD25^+$ Tregs, or enriched $CD4^+CD25^+$ Tregs (1.0×10^5 to 1.0×10^7 cells per well) were blocked with polyclonal human immunoglobulins (Endobulin, 2.5 mg/ml; Baxter Oncology) and incubated with the following mouse anti-human monoclonal antibodies: anti-CD3–PECy5, anti-CD4–FITC, anti-CD25–PE (all 1:20; BD Biosciences —Pharmingen) for 30 minutes on ice. For intracellular FoxP3 staining, the manufacturer's protocol was followed (anti-FoxP3–APC, clone PCH101; eBioscience). Recordings were made on a FACSCalibur flow cytometer (BD) using FlowJo software (TreeStar).

HLA typing was done using mouse anti-human monoclonal HLA-A2–specific antibody (clone BB7.2; produced in-house) and FITC-labeled goat anti-mouse IgG secondary antibody (eBioscience).

Treg specificity assay. DCs (5×10^4) were incubated in RPMI medium containing 10% AB serum in round-bottom 96-well plates. Polypeptides or human IgG were added to the wells at a concentration of 200 μg/ml. Next, 2.5×10^4 $CD4^+CD25^+$ Tregs were added to the pulsed DCs. Treg-depleted Teffs were polyclonally stimulated with 0.5 μg/ml plate-bound anti-CD3 for 14 hours. Then, 2.5×10^4 activated Teffs were added to the Tregs and DCs. After 72 hours at 37°C, [^{3}H]thymidine at 1 μCi per well was added for an additional 16 hours of culture, and proliferation of T cells was measured by determining the amount of incorporated [^{3}H] using a scintillation counter (Liquid Scintillation Counter [1450 MicroBeta]; Perkin-Elmer) in triplicate wells. Tregs were designated as TAA-specific if count numbers in the presence of test peptides were significantly lower ($P > 0.05$) than in pooled wells of the negative control human IgG.

Statistics. ELISPOT results and differences between test groups were analyzed using 2-tailed Student *t* test. Differences in proportions of responders between test groups were analyzed with 2-sided Fisher's exact test. Treg specificity results and differences between test groups were analyzed using 2-tailed Student's *t* test. Differences in [^{3}H]thymidine total counts between test groups and control groups were analyzed using a paired 2-tailed Student's *t* test. Differences were considered significant when *P* was less than 0.05.

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Address correspondence to: Philipp Beckhove, Translational Tumour Immunology Unit, The German Cancer Research Center, Heidelberg, INF 280, 69120 Heidelberg, Germany. Phone: 49-6221-423745; Fax: 49-6221-423702; E-mail: p.beckhove@dkfz.de.

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