Unexpected Abundance of HLA Class II Presented Peptides in Primary Renal Cell Carcinomas

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Abstract

Purpose: To elicit a long-lasting antitumor immune response, $CD8^+$ and $CD4^+$ T cells should be activated. We attempted to isolate HLA-DR–presented peptides directly from dissected solid tumors, in particular from renal cell carcinoma, to identify MHC class II ligands from tumor-associated antigens (TAA) for their use in peptide-based immunotherapy.

Experimental Design: Tumor specimens were analyzed by immunohistochemical staining for their HLA class II expression. HLA class II peptides were subsequently isolated and identified by mass spectrometry. Gene expression analysis was done to detect genes overexpressed in tumor tissue. Peptides from identified TAAs were used to induce peptide-specific $CD4^+$ T-cell responses in healthy donors and in tumor patients.

Results: In the absence of inflammation, expression of MHC class II molecules is mainly restricted to cells of the immune system. To our surprise, we were able to isolate and characterize hundreds of class II peptides directly from primary dissected solid tumors, especially from renal cell carcinomas, and from colorectal carcinomas and transitional cell carcinomas. Infiltrating leukocytes expressed MHC class II molecules and tumor cells, very likely under the influence of IFNγ. Our list of identified peptides contains ligands from several TAAs, including insulin-like growth factor binding protein 3 and matrix metalloproteinase 7. The latter bound promiscuously to HLA-DR molecules and were able to elicit $CD4^+$ T-cell responses.

Conclusions: Thus, our direct approach will rapidly expand the limited number of T-helper epitopes from TAAs for their use in clinical vaccination protocols.

$CD4^+$ helper T cells play an important role in orchestrating the effector function of antitumor T-cell responses (1), and for this reason, the identification of $CD4^+$ T-cell epitopes derived from tumor-associated antigens (TAA) has recently been a major focus of attention (2, 3). Even in the absence of CTL effector cells, helper T cells in the mouse can inhibit tumor angiogenesis via IFNγ (4) and counteract tumor progression via the induction of an antibody response (5). In contrast to HLA class I ligands, only a small number of class II ligands of TAA has been described. Because HLA class II molecules are constitutively presented on cells of the immune system alone (6), the possibility of isolating class II peptides directly from primary tumors as opposed to class I ligands (7) has not been considered viable. Therefore, numerous strategies to target antigens into the class II processing pathway of antigen-presenting cells have been described (e.g., the incubation of antigen-presenting cells with the antigen of interest to enable it to be taken up, processed, and presented; ref. 8).

To identify HLA class II ligands from TAA for their use in peptide-based immunotherapy, we attempted to isolate HLA-DR–presented peptides directly from dissected solid tumors, in particular from renal cell carcinoma (RCC). Even if the majority of tumor cells were class II negative, with state-of-the-art mass spectrometers, it should be possible to identify class II peptides from minimal numbers of tumor cells, from infiltrating immune cells possibly cross-presenting TAA, and from stromal cells.

The reasons for concentrating on RCC are the following: Around 150,000 people worldwide are affected by RCC each year, resulting in ~78,000 deaths per annum (9). If metastasis is diagnosed, the 1-year survival rate decreases to ~60% (10), underlining the dissatisfactory therapeutic situation. Because RCC seems to be an immunogenic tumor, as indicated by the

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Received 11/11/05; revised 3/22/06; accepted 4/7/06.

Grant support: Deutsche Forschungsgemeinschaft grants SFB 510, SFB 685, and Graduiertenkolleg 794; NGFN2 grant BMBF 0313311; and Deutsche Krebshilfe grant 10-2189-St 2.

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-05-2470

existence of tumor-reacting and tumor-infiltrating CTL (11), clinical trials have been initiated to develop peptide-based antitumor vaccinations. However, due to the lack of helper T-cell epitopes from TAA, molecularly defined vaccines usually comprise class I ligands only.

We were able to isolate class II ligands from nine RCCs, three colorectal carcinomas, and two transitional cell carcinomas (urothelial carcinoma). Selected ligands of TAA promiscuously binding to HLA-DR molecules were found to be recognized by $CD4^+$ T cells.

Materials and Methods

Patient samples. The majority of patient samples were obtained from the Department of Urology, University of Tübingen. The local ethical committee approved the study, and informed consent was obtained from the patients. Patient information is provided in Supplementary Table S1.

MHC class II immunohistology. Tumors were fixed in 4% phosphate-buffered formaldehyde, embedded in paraffin, stained with H&E, and examined by light microscopy. Diagnosis of the RCC was carried out according to routine histopathologic and immunohistologic investigations (12).

For immunohistologic detection of MHC class II molecules or CD68 molecules, respectively, 5-μm paraffin-embedded tissue sections were pretreated with 10 mmol/L citrate buffer (pH 6) followed by incubation either with a mouse anti-HLA-DR α-chain monoclonal antibody (clone TAL.1B5, 1:50) or CD68 antibody (Clone PGM1, 1:50; DAKO, Hamburg, Germany) or mouse IgG1 (2 μg/mL, BD Biosciences PharMingen, San Diego, CA) and visualized using the Ventana iView 3,3′-diaminobenzidine detection kit (Nexes System, Ventana Medical Systems, Illkirch, France). For the detection of $CD4^+$ T lymphocytes, the tissue sections were pretreated with 1 mmol/L EDTA (pH 8) for 5 minutes in a pressure cooker before incubation with a monoclonal mouse anti-CD4 antibody (Ventana, clone 1F4) and further processing with the Nexes System as described above. Tissue sections were counterstained with hematoxylin and finally embedded in Entellan.

Elution and molecular analysis of HLA-DR–bound peptides. Frozen tumor samples were processed as previously described (7), and peptides were isolated according to standard protocols (13) using the HLA-DR–specific monoclonal antibody L243 (14). Natural peptide mixtures were analyzed by a reversed-phase Ultimate HPLC system (Dionex, Amsterdam, the Netherlands) coupled to a Q-TOF I mass spectrometer (Waters, Eschborn, Germany), or by a reversed-phase CapLC HPLC system coupled to a Q-TOF Ultima API (Waters) as previously described (15). Fragment spectra were analyzed manually and automatically.

Gene expression analysis by high-density oligonucleotide microarrays. RNA isolation from tumor and autologous normal kidney specimens as well as gene expression analysis by Affymetrix Human

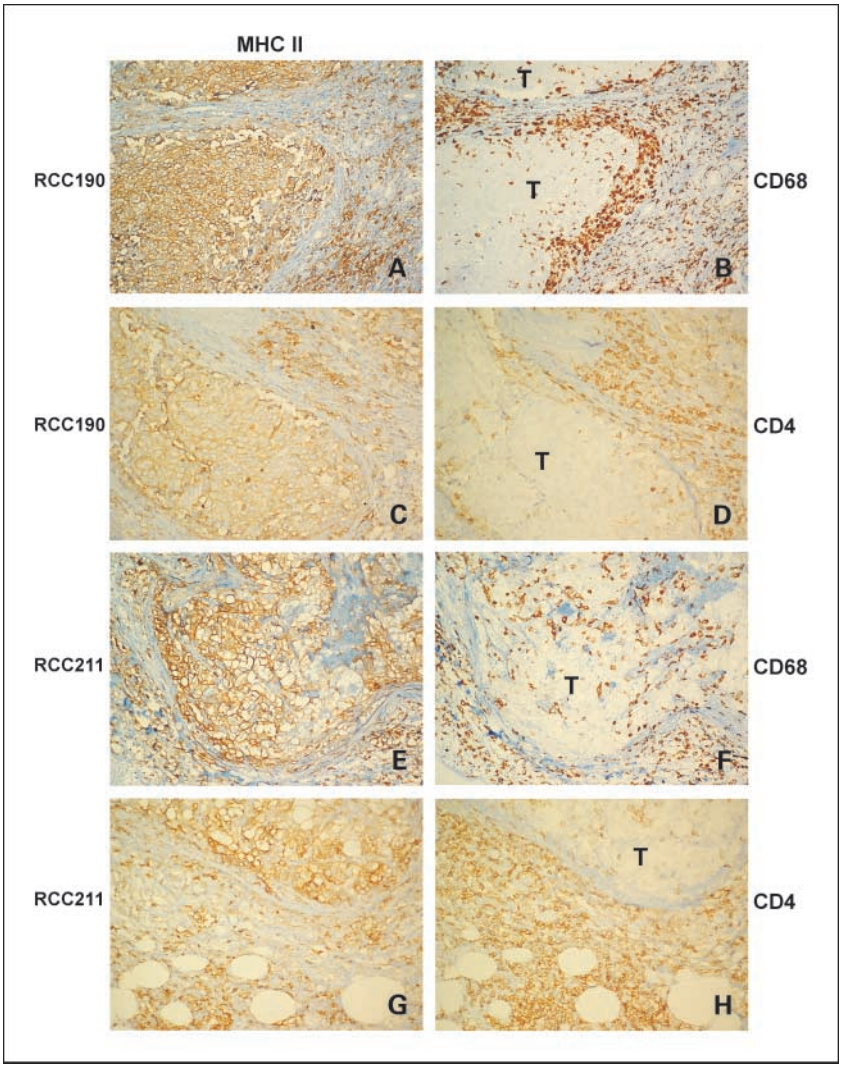

Fig. 1. Expression of HLA class II molecules in RCC of two patients. The HLA class II expression patterns of the tumors from patient RCC190 and RCC211, revealing a papillary structure, were evenly spread (*A*, *C*, *E*, and *G*). The visualization of $CD68^+$ macrophages (*B* and *F*) and $CD4^+$ Tcells (*D* and *H*) in serial tissue sections illustrate a close spatial relationship of tumor-infiltrating immune cells and HLA II – expressing tumor cells. Incubation with mouse IgG instead of specific antibodies consistently revealed negative staining results (Supplementary Fig. S1*E* and *F*). T, tumor.

Genome U133 Plus 2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA) were done as described previously (16). Data were analyzed with the GCOS software (Affymetrix). Pairwise comparisons between tumor and autologous normal kidney were calculated using the respective normal array as baseline. For RCC149 and RCC211, no autologous normal kidney array data were available. Therefore, pooled healthy human kidney RNA was obtained commercially (Clontech, Heidelberg, Germany) and used as the baseline for these tumors.

Maturation of dendritic cells. Dendritic cells were prepared using blood from healthy donors. Briefly, peripheral blood mononuclear cells (PBMC) were isolated using standard gradient centrifugation (Lymphocyte Separation Medium, PAA Laboratories GmbH, Pasching, Austria) and plated at a density of 7×10^6/mL in X-Vivo 15 medium. After 2 hours at 37°C, nonadherent cells were removed, and adherent monocytes were cultured for 6 days in X-Vivo medium with 100 ng/mL granulocyte macrophage colony-stimulating factor and 40 ng/mL interleukin-4 (AL-ImmunoTools, Friesoythe, Germany). On day 7, immature dendritic cells were activated with 10 ng/mL tumor necrosis factor-α (R&D Systems, Wiesbaden, Germany) and 20 μg/mL poly(IC) (Sigma-Aldrich, Steinheim, Germany) for 3 days.

Generation of antigen-specific CD4$^+$ T cells. PBMCs (10^6 per well) were stimulated with 2×10^5 peptide-pulsed (5 μg/mL) autologous dendritic cells. Cells were incubated in 96-well plates (seven wells per donor and per peptide) with T-cell medium: supplemented RPMI 1640 in the presence of 10 ng/mL interleukin-12 (Promocell, Heidelberg, Germany). After 3 to 4 days of coincubation at 37°C, fresh medium with 80 units/mL interleukin 2 (Proleukin, Chiron Corp., Emeryville, CA) and 5 ng/mL interleukin-7 (Promocell) was added. Restimulations were done with autologous PBMCs plus peptide every 6 to 8 days.

Intracellular IFNγ staining. After three and four rounds of stimulation, PBMCs were thawed, washed twice in X-Vivo 15 medium, resuspended at 10^7 cells/mL in T-cell medium, and cultured overnight. On the next day, PBMCs pulsed with 5 μg/mL peptide were incubated with effector cells in a ratio of 1:1 for 6 hours. Golgi-Stop (Becton Dickinson, Heidelberg, Germany) was added for the final 4 hours of incubation.

Cells were analyzed using a Cytofix/Cytoperm Plus kit (Becton Dickinson) and CD4-FITC (Immunotools), IFNγ-PE, and CD8-PerCP clone SK1 antibodies (Becton Dickinson). For negative controls, cells of seven wells were pooled and incubated either with irrelevant peptide or without peptide, respectively. Stimulation with phorbol 12-myristate 13-acetate/Ionomycin was used for positive control. Cells were analyzed on a three-color FACSCalibur (Becton Dickinson).

Results

HLA class II expression by RCC. Under normal, noninflammatory conditions, class II molecules should only be expressed by cells of the hematopoietic system and by the thymic epithelium (6). The situation changes during inflammation. MHC II expression can be induced in most cell types and tissues by IFNγ (17). As RCC incidence is often accompanied by inflammatory events (18, 19), it has been reported that class II molecules might be expressed in the vicinity of or by tumors (20).

We analyzed HLA class II expression of nine RCC specimens comprising histologic clear cell and papillary renal carcinoma (Supplementary Table S1) by immunohistochemical staining and found that all investigated samples revealed class II–positive tumor cells. In RCC revealing a papillary architecture, the expression of HLA class II molecules was evenly distributed throughout the tumor (Fig. 1A, C, E, and G). At the margin of the tumor, we observed a close spatial correlation of HLA-positive tumor cells with tumor-infiltrating immune cells as illustrated by the visualization of CD68-positive macrophages and CD4-positive T cells in serial tissue sections (Fig. 1B, D, F, and H). The comparison of the HLA class II, CD68, and CD4 immunohistochemical staining patterns in serial tissue sections clearly shows that in addition to macrophages and T cells,

Table 1. mRNA expression of IFN-inducible genes

Gene symbol	Entrez gene ID	Gene title	Fold overexpression: tumor vs normal			
			RCC149	RCC180	RCC190	RCC211
HLA-DPA1	3113	MHC, class II, DP α1	3.5	3.7	4.9	13.9
HLA-DPB1	3115	MHC, class II, DP β1	2.6	2.5	2.8	14.9
HLA-DQB1	3119	MHC, class II, DQ β1	4.3	4.0	6.5	5.3
HLA-DRB1	3123	MHC, class II, DR β1	1.2	1.9	2.8	4.3
CXCL10	3627	Chemokine (C-X-C motif) ligand 10	1.1	3.2	10.6	24.3
FCGR1A	2209	Fc fragment of IgG, high-affinity Ia, receptor for (CD64)	6.5	2.6	12.1	29.9
IFI16	3428	IFNγ-inducible protein 16	8.6	3.0	4.3	11.3
IFI44	10561	IFN-induced protein 44	2.8	1.4	2.5	2.8
OAS1	4938	2′,5′-Oligoadenylate synthetase 1, 40/46 kDa	3.5	2.3	2.6	5.3
PSMB8	5696	Proteasome subunit, β type, 8 (LMP7)	2.6	4.3	6.1	6.5
PSMB9	5698	Proteasome subunit, β type, 9 (LMP2)	4.3	7.5	6.5	16.0
PSMB10	5699	Proteasome subunit, β type, 10 (MECL1)	3.2	2.5	5.3	13.0
SP100	6672	Nuclear antigen Sp100	4.0	1.1	1.5	2.8
TAP1	6890	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	2.5	2.8	6.5	8.0
VCAM1	7412	Vascular cell adhesion molecule 1	5.7	5.3	3.2	12.1

NOTE: Expression in tumor samples was compared with autologous normal kidney (RCC180 and RCC190) or pooled healthy kidney (RCC149 and RCC211). All genes showed an "increase" in the change-call algorithm of the GCOS software for all four tumors and have been described as IFN inducible.

tumor cells also express HLA class II. The same could be observed for transitional cell carcinomas (Supplementary Fig. S1) and colorectal carcinomas (Supplementary Fig. S2).

To further elucidate the mechanism of class II expression on RCC, we did comparative gene expression analysis using oligonucleotide microarrays. With this technique, we were able to asses the overall class II expression in the dissected tumors regardless of the expressing cell types. We analyzed differential expression in four tumors (RCC149, RCC180, RCC190, and RCC211) compared with normal reference kidney. In all four tumors, HLA class II genes were overexpressed (Table 1). One possible reason for this might be an induced expression by IFNγ, and for this reason, we looked for other genes known to be up-regulated by IFNs (21). Interestingly, a considerable number of such genes were found to be overexpressed in one or more tumor samples. Table 1 shows IFN-inducible genes that were up-regulated reproducibly in all four samples, in accordance with our earlier findings (7). Among them are *LMP2*, *LMP7*, and *MECL1*: proteins that are exchanged against constitutive proteasomal subunits to form the immunoproteasome, a hallmark process in an IFN-rich environment. Additionally, IFNγ was directly assessed by quantitative real-time reverse transcription-PCR (Taqman). The tumors displayed in Table 1 showed at least a 5-fold IFNγ mRNA overexpression compared with their autologous normal RNA samples (data not shown). Thus, our results show that IFNγ might play an important role in RCC and is most likely the reason for abundant class II expression.

HLA-DR ligands isolated from dissected carcinomas. Class II peptides from primary solid tumors have thus far not been

Table 2.

Gene symbol	Entrez gene ID	Peptide sequence	Gene title
A. HLA-DR ligands isolated from RCC190			
ACTG1	71	WISKQEYDESGPSIVHRKCF	Actin, γ 1 propeptide
ALB	213	LKKYLYEIARRHP	Albumin precursor
ALB	213	TLVEVSRNLGKVG	Albumin precursor
ALB	213	TPTLVEVSRNLGKVGS	Albumin precursor
APOA2	336	EKSKEQLTPLIKKAGTELVNF	Apolipoprotein A-II precursor
APOB	338	YPKSLHMYANRLLDHR	Apolipoprotein B precursor
C1R	715	EPYYKMQTRAGSRE	Complement component 1, r subcomponent
C4B	721	APPSGGPGFLSIERPDSRPP	Complement component 4B proprotein
C4BPA	722	FGPIYNYKDTIVFK	Complement component 4 binding protein, α
CALR	811	SPDPSIYAYDNF	Calreticulin precursor
CALR	811	EPPVIQNPEYKGEWKPRQIDNPD	Calreticulin precursor
CFL1	1072	GVIKVFNDMKVRK	Cofilin 1 (non-muscle)
CPE	1363	APGYLAITKKVAVPY	Carboxypeptidase E precursor
FCGBP	8857	ASVDLKNTGREEFLTA	Fc fragment of IgG binding protein
FCN1	2219	GNHQFAKYKSFKVADE	Ficolin 1 precursor
FTL	2512	VSHFFRELAEEKREG	Ferritin, light polypeptide
FTL	2512	TPDAMKAAMALEKK	Ferritin, light polypeptide
GAPD	2597	FVMGVNHEKYDN	Glyceraldehyde-3-phosphate dehydrogenase
GAPD	2597	TGVFTTMEKAGAH	Glyceraldehyde-3-phosphate dehydrogenase
GAPD	2597	ISWYDNEFGYSNRVVDLMAHMASKE	Glyceraldehyde-3-phosphate dehydrogenase
HIST1H1C	3006	GTGASGSFKLNKKAASGEAKPK	H1 histone family, member 2
HLA-DQB1	3119	DVGVYRAVTPQGRPD	MHC, class II, DQ β1 precursor
HLA-DRB1	3123	DVGEFRAVTELGRPD	MHC, class II, DR β1 precursor
IGFBP3	3486	HPLHSKIIIIKKGHAK	Insulin-like growth factor binding protein 3
KNG1	3827	DKDLFKAVDAALKK	Kininogen 1
NPC2	10577	KDKTYSYLNKLPVK	Niemann-Pick disease, type C2 precursor
S100A8	6279	VIKMGVAAHKKSHEESHKE	S100 calcium-binding protein A8
SERPINA1	5265	MIEQNTKSPLFMGKVVNPTQK	Serine (or cysteine) proteinase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1
SOD1	6647	GPHFNPLSRKHGGPK	Superoxide dismutase 1, soluble
TF	7018	DPQTFYYAVAVVKKDS	Transferrin
B. Natural presented HLA-DR ligands used for generating antigen-specific CD4$^+$ T cells			
IGFBP3	3486	HSKIIIIKKGHAK	Insulin-like growth factor binding protein 3
MMP7	4316	SQDDIKGIQKLYGKRS	Matrix metallo proteinase 7
CCND1	595	NPPSMVAAGSVVAAV	Cyclin D1

NOTE: The core sequences of HLA-DR ligands isolated from RCC190 (HLA-DRB1*11, DRB1*15, and DRB3, DRB5) are shown. The complete list of identified ligands is displayed in Supplementary Table S2.

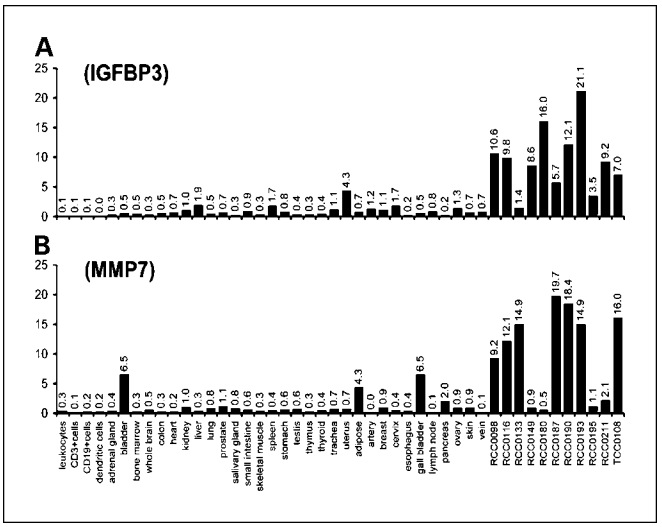

Fig. 2. mRNA expression profiles of IGFBP3 (*A*) and MMP7 (*B*). Ten RCCs, one transitional cell carcinoma, and various human tissues were analyzed. Relative expression values are normalized to kidney (expression = 1). IGFBP3 seemed overexpressed in eight RCCs and MMP7 in six RCCs compared with normal tissues.

isolated and identified. This has only been achieved for cultured cell lines (22–24). We analyzed nine different RCC, three colorectal carcinoma, and two transitional cell carcinoma specimens and were able to isolate HLA-DR ligands from all samples: 452 peptides in total (Supplementary Table S2). Table 2A shows a representative list of peptides and corresponding source proteins identified from RCC190. The specimens differed in their HLA genotypes, in weight, and in the number of identified ligands. There was no correlation between tumor weight and number of identified ligands. On one hand, ligands that should be presented by leukocytes were found, such as peptides from complement components C3, C4A, C4 binding protein α, CD14, and Fc fragment of IgG binding protein. On the other hand, we found peptides probably presented by tumor cells from overexpressed TAA [e.g., from vimentin, matrix metalloproteinase 7 (MMP7), eukaryotic translation elongation factor 1 α 1, and nicotinamide *N*-methyltransferase]. This observation is in accordance with immunohistochemistry data (Fig. 1) and shows that class II–positive tumor cells and infiltrating leukocytes were present in analyzed specimens. A clear distinction between different peptide pools is not possible. However, our data suggest that the eluted peptides derive from these distinct cell types.

To identify peptides from TAA, we compared ligand source proteins with overexpressed genes detected by microarray analysis of tumors (7, 16). We identified a peptide from insulin-like growth factor binding protein 3 ($IGFBP3_{166-181}$), on RCC190. In addition, two variants of this peptide ($IGFBP3_{169-181}$ and $IGFBP3_{169-184}$) were found on TCC108. From the same tumor, a peptide from MMP7 ($MMP7_{247-262}$) could be isolated (Supplementary Table S2). At the mRNA level, IGFBP3 was overexpressed in 8 and MMP7 in 6 of 10 analyzed RCCs (Fig. 2), and both have been described to be tumor associated (25–27). To test these peptides for their capacity to stimulate specific T-cell reactivities, the shortest variant of the IGFBP3 peptides ($IGFBP3_{169-181}$) and the MMP7 peptide were used (Table 2B).

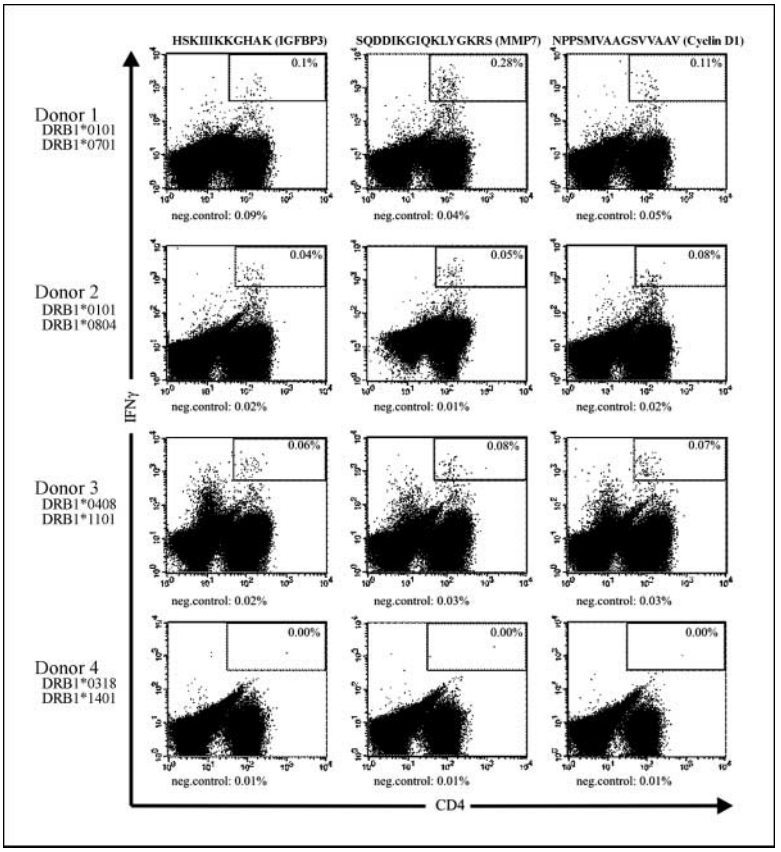

Fig. 3. $CD4^+$ T cells specific for $IGFBP3_{169-181}$, $MMP7_{247-262}$, and $CCND1_{198-212}$. Representative dot plots of intracellular IFNγ staining against CD4-FITC. Percentages of negative control responses (irrelevant peptide) are given below respective plot. For negative controls, cells of seven wells were pooled and incubated with irrelevant peptide or without peptide, respectively (Supplementary Fig. S3).

Antigen-specific CD4$^+$ T cells against IGFBP3$_{169-181}$ and MMP7$_{247-262}$. The peptides IGFBP3$_{169-181}$ and MMP7$_{247-262}$ were analyzed with respect to their binding characteristics by the SYFPEITHI class II epitope prediction algorithm (http://www.syfpeithi.de). The HLA-DR genotypes of the source tumors were HLA-DRB1*11 and DRB1*15 (Supplementary Table S2) in both cases. According to the epitope predictions for DRB1*1101 and DRB1*1501, both peptides received a high binding score for HLA-DRB1*11 (data not shown). To generate antigen-specific CD4$^+$ T cells and to test the peptides for promiscuous binding, PBMCs of four healthy donors with different HLA-DR alleles (Fig. 3), one of them carrying *DRB1*1101*, were stimulated using peptide-pulsed autologous dendritic cells. In addition, the peptide CCND1$_{198-212}$, a known T-cell epitope (28), was used as positive control (Table 2B). As a read-out system for the generation of antigen-specific CD4$^+$ T cells, IFNγ levels were assessed by flow cytometry. T cells were analyzed after the third and fourth weekly stimulation by intracellular IFNγ staining plus CD4-FITC and CD8-PerCP staining to determine the percentage of IFNγ-producing cells in specific T-cell subpopulations. In all experiments, stimulations with irrelevant peptide and without peptide were done as negative controls. IFNγ response was considered as positive if the percentage of IFNγ producing CD4$^+$ T cells was >2-fold higher compared with negative controls (29).

In three of four donors, we were able to generate specific CD4$^+$ T cells for both peptides (Figs. 3 and 4). T-cell responses could not be observed in donor 4 after any stimulation. In donor 1, 0.06% to 0.13% IFNγ-producing CD4$^+$ T cells (Fig. 4) were detected in six of seven stimulation attempts after the fourth stimulation with peptide IGFBP3$_{169-181}$. IFNγ-producing CD4$^+$ T cells specific for the peptide IGFBP3$_{169-181}$ were also observed in donors 2 and 3, with maximal frequencies of 0.11% and 0.07%.

Donors 1, 2, and 3 also showed CD4$^+$ T cells reactive to peptide MMP7$_{247-262}$. The highest frequencies of IFNγ-producing CD4$^+$ T cells specific for the MMP7 peptide were found in donors 1 and 2, respectively. Donors 1, 2, and 3 showed IFNγ responses to peptide CCND1$_{198-212}$, which has already been described as an MHC class II–restricted T-cell epitope (28).

Thus, peptides from IGFBP3, MMP7, and CCND1 are promiscuous HLA class II binders that are able to elicit CD4$^+$ T-cell responses in three of four healthy donors carrying different HLA alleles. Comparing the HLA alleles of the two tumor patients from which the IGFBP3 and MMP7 peptides were derived with those of the four healthy donors, it seems very likely that the peptides are presented by HLA-DRB1*01, HLA-DRB1*04, and HLA-DRB1*11. All three allotypes have a glycine residue at position 86 and an aspartic acid residue at position 57 of their β chains (see http://www.anthony-nolan.com/HIG). Therefore, they have very similar binding characteristics for their binding pockets P1 and P9 (30). For peptide CCND1$_{198-212}$, a T-cell epitope known to be presented by HLA-DRB1*0401 and HLA-DRB1*0408 (28), the same holds true. Donor 4 carries *HLA-DRB1*0318* and *HLA-DRB1*1401*, alleles with peptide motifs that probably differ from those described above. This could explain why it was not possible to elicit T-cell responses against the three peptides using cells from this donor.

Interestingly, IFNγ-producing CD8$^+$ T cells were detected in two donors after stimulations with the three peptides, in particular in donor 3, but also to a lesser extent in donor 1 (data not shown). These observations suggest the presence of CD8 T-cell responses directed against class I epitopes included in the long class II peptides (31).

We also analyzed patient tumor-infiltrating T cells and PBMCs *ex vivo* or after one *in vitro* presensitization in the presence of the relevant peptides for reactivity against peptides IGFBP3$_{169-181}$ and MMP7$_{247-262}$ by intracellular IFNγ staining. Only in one of 12 cases (RCC149) we were clearly able to detect

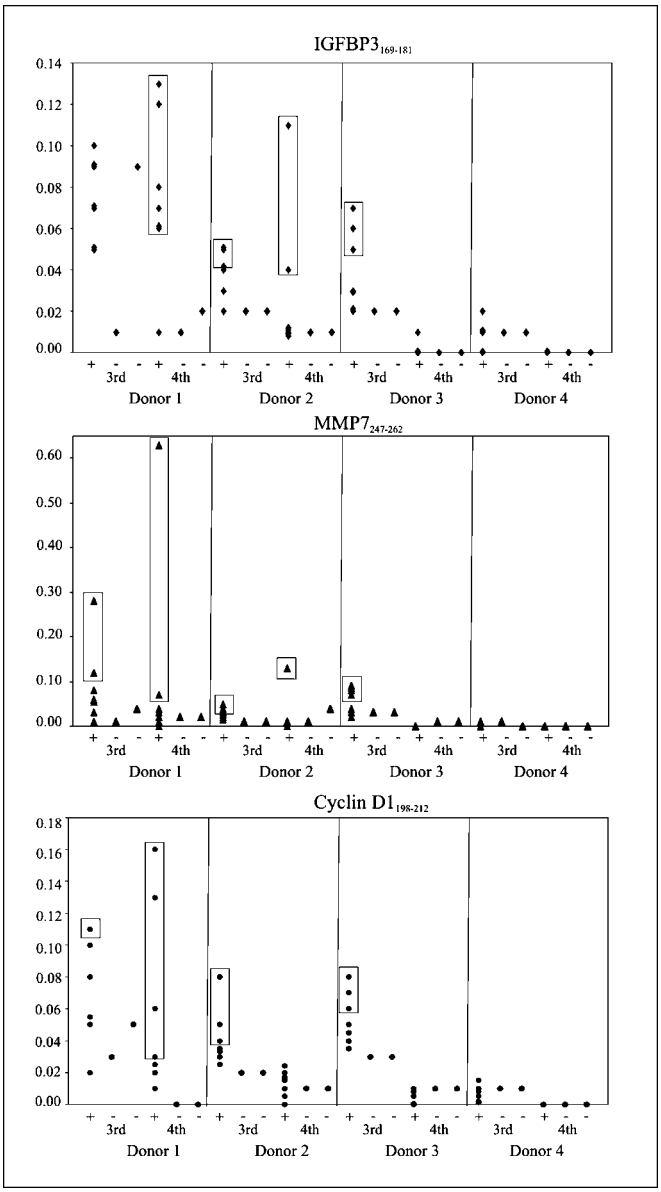

Fig. 4. Schematic illustration of antigen-specific IFNγ producing CD4$^+$ T cells detected in each donor and for each peptide. Percentage of IFNγ-producing CD4$^+$ T cells for each donor and peptide used for stimulation. Cells were incubated in 96-well plates: seven wells per donor and per peptide. Each symbol represents IFNγ response of CD4$^+$ T cells generated in one well. Boxed are values considered as positive: percentage of IFNγ-producing CD4$^+$ T cells was >2-fold higher compared with negative control with irrelevant peptide (*third column*). Percentages of IFNγ-producing CD4$^+$ T cells detected after stimulation with irrelevant peptide (*third column*) correlated with values after stimulation without peptide (*second column*), with the exception of donor 1 after the 3rd stimulation with IGFBP3$_{169-181}$ (see Supplementary Fig. S3). However, this effect was not seen anymore after the 4th stimulation.

MMP7-specific $CD4^+$ T cells after 8 days of culture (Supplementary Fig. S4; Supplementary Table S3). In this donor, the restriction element was most probably HLA-DRB1*01. We did not detect IGFBP3-specific T cells. These results suggest that spontaneous $CD4^+$ T-cell responses against the two MMP7 and IGFBP3 peptides are rare in RCC patients and/or happen at very low frequencies, which were not detected after one or two *in vitro* stimulations with peptides.

Discussion

The identification of helper T-cell epitopes of TAA remains an important task in antitumor immunotherapy. To elicit a long-lasting antitumor immune response, $CD8^+$ and $CD4^+$ T cells should be activated (32). The isolation and identification of tumor-associated class I peptides from solid carcinomas has been successfully employed (7). However, as class II molecules should be constitutively presented exclusively on cells of the immune system, this approach has not been used for the identification of class II peptides. Laborious strategies for the characterization of class II peptides from TAA have been carried out until now, ranging from the incubation of antigen-presenting cells with the antigen of interest to be taken up and processed (8) to various transfection strategies with fusion proteins (28). All these methods are very time consuming, and it often remains unclear if the identified ligands are presented *in vivo*. We could show for the first time that it is possible to isolate class II ligands directly from dissected solid tumors, thus identifying the peptides that are presented by tumors and surrounding tissue *in vivo*. Among the source proteins, several housekeeping and immunologic relevant proteins were present. However, peptides from TAA could also be detected, proving our method to be a straightforward approach for the identification of *in vivo* relevant class II ligands of TAA.

Under inflammatory conditions, MHC II expression can be induced in most cell types and tissues by IFNγ (17). We analyzed nine different RCCs by immunohistology and found abundant class II expression on all tumor samples. As class II–positive tumor cells were found predominantly in outer parts of dissected tumors, one could speculate that leukocytes attracted by the tumor produce IFNγ, which acts on neighboring malignant cells. It has been shown that IFNγ-producing $CD4^+$ Th1 cells and natural killer cells infiltrate RCC (33). IFNγ may also activate tumor associated macrophages, which in turn may produce proinflammatory cytokines, such as tumor necrosis factor-α and interleukin-1β, supporting tumor angiogenesis (34). Indeed, we could show that CD68-positive macrophages and CD4-positive T cells were also present in the analyzed sections, and that IFNγ mRNA expression was profoundly up-regulated in tumor compared with normal samples. This observation was further supported by a general up-regulation of IFN-inducible genes (21) in tumor samples. Thus, our results indicate that IFNγ might play an important role in RCC and be the reason for abundant class II expression. It also contradicts the widely held assumption of a prevailing MHC down-regulation in tumors.

In the search for peptides from TAA, we identified three ligands accounting for one core sequence from IGFBP3 and one ligand from MMP7. We found these proteins overexpressed in RCC; in addition, they have been described as tumor associated (25–27). These peptides bound promiscuously to HLA class II molecules and were able to activate $CD4^+$ T cells from different healthy donors and more rarely from tumor patients. Consequently, we consider our approach a breakthrough in the identification of new class II peptide candidates from TAA for use in clinical vaccination protocols.

Acknowledgments

We thank P. Hrstić for expert technical assistance and L. Yakes for carefully reading the article.

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Clin Cancer Res 2006;12:4163-4170.