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Comprehensive Analysis of HLA-DR- and HLA-DP4-Restricted CD4$^+$ T Cell Response Specific for the Tumor-Shared Antigen Survivin in Healthy Donors and Cancer Patients[1]

Xiao-Fei Wang,* Jerome Kerzerho,* Olivier Adotevi,† Hélène Nuyttens,* Cecile Badoual,† Gaetan Munier,* Stéphane Oudard,‡ Shuiping Tu,§ Eric Tartour,† and Bernard Maillère[2]*

Because of the wide distribution of the survivin Ag in a variety of tumors, we have investigated the survivin-specific CD4$^+$ T cell response in healthy donors and cancer patients. Screening of the entire sequence of survivin for HLA class II binding led to the identification of seven HLA-DR promiscuous peptides, including four HLA-DP4 peptides. All of the peptides were able to prime in vitro CD4$^+$ T cells of eight different healthy donors. The peptide-specific T cell lines were stimulated by dendritic cells loaded with the recombinant protein or with the lysates of tumor cells. The high frequency of responders (i.e., immunoprevalence) was provided by a wide reactivity of multiple peptides. Six peptides were T cell stimulating in at least half of the donors and were close to CD8$^+$ T cell epitopes. HLA-DR molecules were more frequently involved in T cell stimulation than were HLA-DP4 molecules, and hence immunoprevalence relies mainly on HLA-DR promiscuity in the survivin Ag. In two cancer patients a spontaneous CD4$^+$ T cell response specific for one of these peptides was also observed. Based on these observations, the tumor-shared survivin does not appear to be the target of immune tolerance in healthy donors and cancer patients and is a relevant candidate for cancer vaccine. ***The Journal of Immunology,*** **2008, 181: 431–439.**

Because of its widespread expression and its vital function in tumor cells, survivin appears as an ideal candidate for cancer immunotherapy. It is expressed during development and in proliferating cells during the G2/M phase, but it remains largely undetectable in differentiated tissue. In contrast, its expression is aberrantly elevated in most human solid malignancies, including lung, colon, breast, ovarian, renal, pancreatic, prostate, and gastric carcinomas and melanoma (1). It is also overexpressed in leukemia of various origins (1). Initially isolated as a member of the inhibitors of apoptosis protein family (2), survivin preferentially blocks mitochondrial-dependent apoptosis by interfering with the caspase pathway. It is also involved in the regulation of cell division and interacts with the spindle mitotic apparatus (3). It contributes to survival and proliferation of tumor cells. In agreement with its functions, survivin expression correlates with poor prognosis for the patients (4), while inhibition of its expression by antisense oligonucleotides induced tumor apoptosis and increased tumor sensitivity to chemotherapy (5).

Survivin-specific cytotoxic T cell response has been investigated by several groups. It was initially described for HLA-A2 donors (6, 7), and this type of study was extended to other haplotypes (8, 9). Cytotoxic response raised against survivin was demonstrated in breast cancer, leukemia, and melanoma patients (6) and was induced in vitro from PBMC derived from colorectal carcinoma (10) and leukemia patients (11). Based on these data, vaccination trials have been engaged (12–15). However, despite these immunological data and the requirement of CD4$^+$ T lymphocytes to sustain cytotoxic responses (16), the CD4$^+$ T cell response specific for survivin remains largely unknown (17). This response is expected to exist because spontaneous humoral response has been detected in tumor-bearing patients (18, 19) and because it can be induced in mice by using appropriate epitope vectors (20). However, because survivin is an overexpressed Ag as telomerase and P53, a status of tolerance may limit the CD4$^+$ T cell response and give rise to low-affinity CD4$^+$ T cells. Moreover, because it is relatively small, the CD4$^+$ T cell response may be limited to particular haplotypes and hence may not be effective in all individuals. To account for the frequency of responders of an epitope or an Ag, we use the term immunoprevalence, while immunodominance is used to qualify peptides recognized in the context of the presentation of the native Ag. Clearly, a high immunoprevalence is needed for a candidate vaccine and is thought to be achieved by the presence of promiscuous HLA-DR-restricted peptides or peptides that are presented by preponderant HLA class II (HLA II)[3] molecules as HLA-DP4. However, the respective roles of HLA-DR and HLA-DP4 molecules in CD4$^+$ T cell responses in humans remain poorly documented. We have therefore addressed the question of whether Survivin can sustain an efficient CD4$^+$ T cell

*Commissariat à l'Energie Atomique, Institut de Biologie et Technologies de Saclay, Service d'Ingénierie Moléculaire des Protéine, Gif Sur Yvette; †EA 4054 Université Paris-Descartes-Ecole Nationale Vétérinaire d'Alfort, Hôpital Européen Georges Pompidou, Unité d'Immunologie Biologique et Unité d'Anatomie Pathologique Assistance Publique-Hôpitaux de Paris, Paris; ‡Service d'Oncologie Médicale, Hôpital Européen Georges Pompidou, EA 4054 Université Paris-Descartes, Paris, France; and §Department of Gastroenterology, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China

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[2] Address correspondence and reprint requests to Dr. Bernard Maillere, Commissariat à l'Energie Atomique, Institut de Biologie et Technologies de Saclay, Service d'Ingénierie Moléculaire des Protéines, Gif Sur Yvette, F-91191, France. E-mail address: bernard.maillere@cea.fr

[3] Abbreviations used in this paper: HLA II, HLA class II; DC, dendritic cell; NSLC, non-small cell lung carcinoma; RCC, renal cell carcinoma; rh, recombinant human.

Table I. *Binding capacities to HLA-DR and HLA-DP4 molecules of overlapping peptides from survivin*[a]

Peptides	Relative Activity												Bound HLA II
	DR1	DR3	DR4	DR7	DR11	DR13	DR15	DRB3	DRB4	DRB5	DP401	DP402	
1–15	8,308	>289	>3,074	>6,140	**7**	>164	1,429	>6,262	>4,110	1,046	1,022	302	1
4–18	34,615	>289	>3,074	>6,140	**3**	**62**	>2,420	>6,262	>4,110	716	530	157	2
8–22	5,056	**40**	1,314	404	**4**	**4**	**93**	546	725	286	2,333	456	4
11–25	4,615	163	1,406	267	**35**	**4**	**0.4**	230	1,875	295	3,667	1,667	3
17–31	**8**	>289	288	**1**	**24**	**1**	**0.1**	>6,262	188	**30**	**4**	**2**	8
19–33	**8**	>289	**18**	**32**	**15**	**15**	**2**	>6,262	110	494	**2**	**1**	8
20–34	**4**	>289	163	667	**21**	**4**	**26**	>6,262	138	500	**23**	**5**	6
23–37	**29**	>289	163	2000	3,700	>164	>2,420	>6,262	>4,110	447	>6,773	247	1
36–50	1,581	>289	531	567	589	>164	>2,420	>6,262	1,225	496	282	126	0
41–55	842	>289	156	161	115	>164	125	667	**17**	442	>6,773	>3,989	1
52–66	3,154	**34**	688	287	**10**	**35**	**54**	792	>4,110	699	>6,773	200	4
56–70	495	>289	>3,074	433	**35**	>164	**54**	800	>4,110	1,538	>6,773	>3,989	2
59–73	1,800	>289	1657	300	194	>164	143	>6,262	>4,110	3,462	>6,773	>3,989	0
62–76	>22,567	>289	>3,074	>6,140	>10,034	>164	1,161	1,933	>4,110	>12,934	>6,773	>3,989	0
65–79	>22,567	>289	>3,074	>6,140	>10,034	>164	>2,420	>6,262	>4,110	>12,934	>6,773	>3,989	0
72–86	73,077	>289	>3,074	>6,140	>10,034	**17**	>2,420	>6,262	>4,110	477	>6,773	>3,989	1
84–98	1,028	**7**	543	**12**	**1**	**1**	**15**	741	**49**	231	**10**	**5**	8
87–101	115	>289	193	**43**	217	>164	121	426	346	784	216	279	1
90–104	**30**	>289	**28**	**6**	248	>164	134	3,200	>4,110	**37**	**3**	**1**	6
91–105	**9**	>289	**41**	**2**	248	>164	**80**	2,000	>4,110	**19**	**1**	**1**	7
93–107	**99**	>289	172	120	**55**	**13**	**12**	3,000	>4,110	**69**	**1**	**3**	7
96–110	**14**	**16**	**44**	243	**1**	**1**	**12**	>6,262	180	**13**	172	**27**	8
99–113	**37**	**13**	**56**	278	**4**	**15**	**43**	>6,262	550	**3**	4,444	>3,989	7
102–116	4,804	>289	>3,074	1,667	943	>164	>2,420	>6,262	>4,110	**2**	>6,773	>3,989	1
111–125	2,500	>289	>3,074	>6,140	3,000	108	1,607	>6,262	1,750	**2**	>6,773	>3,989	1
122–136	3,508	**17**	>3,074	667	**33**	**2**	**43**	>6,262	>4,110	**58**	>6,773	>3,989	5
128–142	**5**	**30**	**11**	**58**	**3**	>164	**7**	>6,262	**64**	141	1,444	1,333	7

[a] Peptides encompassing the whole sequence of the survivin Ag were submitted to binding assays specific for HLA-DR and HLA-DP4 molecules. Reference peptides were used to validate each assay. These peptides are the nonbiotinylated forms of the biotinylated peptides used in the assay and correspond to very good binders. Data are expressed as relative activity (ratio of the IC_{50} of the peptides to the IC_{50} of the reference peptide) and are the means of three experiments. Good binders have a relative activity <100 and are in boldface type.

response in multiple donors and how this response is supported by the HLA II molecules.

Materials and Methods

Peptides and proteins

Overlapping peptides and biotinylated peptides were synthesized using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Advanced ChemTech Apex synthesizer (Advanced ChemTech Europe) and cleaved from the resin by 95% trifluoroacetic acid. If necessary, peptides were purified by reversed phase-HPLC on a C_{18} Vydac column (Interchim). Their purity was ~90% as indicated by analytical HPLC. Peptides HA255–270 (RGYFKMRTGKSSIMRS) and HA306–318 (PKYVKQNTLKLAT) were from NeoMPS. Protein survivin (Bir5) and HIV Nef protein were produced in *Escherichia coli* as fusion proteins with the GST protein, purified on glutathione column, and separated from GST by proteolytic cleavage.

Cell transfection

The pcDNA3-survivin (pBir5+) (21) (20 μg) was introduced into HeLa cells (3×10^6 cells) by electroporation (0.9 kV, 250 μF, 0.4-cm gap) with a Gene Pulser II (Bio-Rad). The treated cells were suspended in 40 ml of prewarmed DMEM medium with 10% fetal serum and incubated under 5% CO_2/air at 37°C. Forty-eight hours later, transfection efficacy was assessed by flow cytometry. Briefly, cells were permeabilized with 75 μl of fixation/permeabilization solution (BD Biosciences) for 20 min at 4°C and washed twice with BD Perm/Wash buffer plus 2% FCS (BD Biosciences). They were stained for 20 min on ice with anti-survivin-PE (Cell Signaling Technology) or anti-isotype control. Cell staining was assessed on a FACSCalibur flow cytometer (BD Biosciences).

HLA II peptide binding assays

HLA-DR and HLA-DP4 molecules were immunopurified from homologous EBV cell lines by affinity chromatography using the monomorphic mAbs L243 and B7/21, respectively (22–24). The binding to HLA-DR and HLA-DP4 molecules was assessed by competitive ELISA as previously reported (22–24). Binding specificity for each HLA II was ensured by the choice of the biotinylated peptides as described previously (23, 24). Unlabeled forms of the biotinylated peptides were used as reference peptides to assess the validity of each experiment. Their sequences and IC_{50} values were as follows: HA 306–318 (PKYVKQNTLKLAT) for DRB1*0101 (4 nM), DRB1*0401 (33 nM), DRB1*1101 (10 nM), and DRB5*0101 (8 nM); YKL (AAYAAAKAAALAA) for DRB1*0701 (16 nM); A3 152–166 (EAEQLRAYLDGTGVE) for DRB1*1501 (40 nM); MT 2–16 (AK TIAYDEEARRGLE) for DRB1*0301 (340 nM); B1 21–36 (TERVR LVTRHIYNREE) for DRB1*1301 (600 nM); LOL 191–210 (ESWGAVWRIDTPDKLTGPFT) for DRB3*0101 (16 nM); E2/E168 (AGDLLAIETDKATI) for DRB4*0101 (24 nM); and Oxy 271–287 (EKKYFAATQFEPLAARL) for DPB1*0401 (15 nM) and HLA-DPB1*0402 (25 nM).

Blood samples and HLA-DR genotyping

Blood cells were collected from anonymous healthy donors at the Etablissement Français du Sang (EFS, Rungis, France) as buffy-coat preparations after informed consent and following EFS guidelines. Eight cancer patients (four renal cell carcinomas (RCC), two melanomas, two non-small cell lung carcinomas (NSLC)) were recruited at the Hôpital Européen Georges Pompidou (Paris, France). With respect to cancer patient characteristics: the four RCC patients were staged as pT2N1M0 ($n = 2$) and pT1N2M0 ($n = 2$), the NSLC patients were stage IIIB ($n = 1$) and stage IV ($n = 1$), and the two melanoma patients were classified as having stage IV metastatic melanomas. All patients were men with a mean age of 58 years. These cancer patients were not treated by any immunomodulator before blood collection. This study was conducted in accordance with French laws and after approval by the local ethics committee. PBMC were isolated by density centrifugation on Ficoll-Hyperpaque gradients (Sigma-Aldrich). HLA-DR genotyping was performed by using the Olerup SSP DRB1 typing kit.

Generation of Ag-specific T cell lines from healthy donors

Monocyte-derived dendritic cells (DC) were generated from plastic-adherent cells of PBMC after 5–7 days of culture in AIM V medium (Invitrogen) supplemented with 1000 U/ml of recombinant human (rh)IL-4 and of

rhGM-CSF (Tebu). Immature DC were collected at day 5 or 6. Mature DC were generated after addition of 1 μg/ml of LPS (Sigma-Aldrich). $CD4^+$ T lymphocytes were isolated from nonadherent phase by positive selection using an anti-CD4 mAb coupled to magnetic microbeads (Miltenyi Biotec) and by magnetic cell sorting, as recommended by the manufacturer. Mature DC (5×10^5) were incubated at 37°C, 5% CO_2, for 4 h in 1 ml complete IMDM medium containing a mixture of survivin peptides, with each peptide being at a concentration of 10 μg/ml. Pulsed cells were washed and added at 10^4 per round-bottom microwell to 10^5 autologous $CD4^+$ lymphocytes in 200 μl IMDM supplemented by 1000 U/ml of IL-6 (R&D Systems) and 10 ng/ml IL-12 (R&D Systems). The $CD4^+$ T lymphocytes were restimulated on days 7, 14, and 21 with autologous DC freshly loaded with the survivin peptides and were grown in complete IMDM medium supplemented with 10 U/ml IL-2 (R&D Systems) and 5 ng/ml IL-7 (R&D Systems). Flow cytometry analysis confirmed that growing cells were $CD4^+$ T lymphocytes. Specificity of the T cell lines was investigated by IFN-γ ELISPOT assays at days 28 and 29 as described previously (25). APCs were autologous PBMC, or autologous immature DC or L cells transfected with HLA II molecules (26). For statistical evaluation, a *t* test was used. Values of $p < 0.05$ were considered significant.

Assessment of survivin-specific T cell response in cancer patients

PBMC were cultured for 6 days at 2×10^6 cells/ml in 2 ml per well with complete RPMI medium supplemented with 10% FCS. In each well, a pool of viral hemagglutinin or survivin peptides was added at a concentration of 20 μg/ml. On day 2 after the beginning of the culture, IL-2 (Chiron) was added at 20 IU/ml in standard conditions. After 6 days of culture, the ELISPOT assay was performed using peptide-pulsed PHA-activated cells as APCs as described previously (27). Briefly, PHA-activated cells were obtained by a culture of autologous PBMC in RPMI 1640 medium containing 10% FCS and supplemented with 10 μg/ml PHA-P (Sigma-Aldrich). At day 3, IL-2 (20 IU/ml) and IL-7 (10 ng/ml) were added to the culture. At day 6, PHA-activated cells were fixed with 1% PFA for 30 min at 4°C, washed three times with PBS, and pulsed for 2 h at 37°C with the various peptides at 20 μg/ml in serum-free medium (AIM V medium). Ninety-six-well polyvinylidene difluoride plates (Millipore) were coated with 100 μl capture anti-human IFN-γ mAb (Diaclone) and incubated overnight at 4°C. The plates were then saturated with 2% skimmed milk and incubated for 2 h at room temperature. Effector cells (10^5) and PHA-activated T cells (5×10^4) pulsed with the peptides were added to triplicate wells at 10^5 cells/well in AIM V medium for 20 h at 37°C in 5% CO_2. At the end of incubation, cells were washed and the second biotinylated anti-IFN-γ mAb (Diaclone) was added to the plate for 90 min at 37°C, followed by streptavidin-alkaline phosphatase conjugate (Diaclone) for 1 h at 37°C and by NBT/5-bromo-4-chloro-3-indolylphosphate toluidine mix (Diaclone) as substrate. Spots were counted using an automated stereomicroscope (Zeiss). The number of specific T cells expressed as spot-forming cells/10^5 cells was calculated after subtracting negative control values (background). Cells incubated with medium alone or PMA (100 ng/ml) (Sigma-Aldrich) and ionomycin (10 μM) (Sigma-Aldrich) were used as negative and positive controls, respectively.

Immunofluorescence staining

Formalin-fixed paraffin-embedded surgical specimens of two clear cell renal carcinomas were studied. Samples were sectioned at 4–6 μm with a cryostat, deparaffinized in xylene, rehydrated in graded alcohol, and equilibrated in PBS. Slides were treated with avidin/biotin blocker (Vector Laboratories) and 3% hydrogen peroxide and the Fc receptor was blocked by human serum (5%). Slides were then stained with a survivin-specific (Santa Cruz Biotechnology) or with a nonspecific rabbit polyclonal Ab and incubated overnight at 4°C. After washes, the slides were incubated with biotinylated donkey anti-rabbit (Vector Laboratories) and streptavidin-horseradish peroxidase. The 3,3′-diaminobenzidine was used as a chromogen (DakoCytomation). The various Abs were diluted in PBS.

Results

Survivin peptides bind to multiple HLA-DR and HLA-DP4 molecules

A set of 27 peptides encompassing the whole sequence of the tumor-shared Ag Survivin were synthesized and submitted to binding assays specific for preponderant HLA-DR and HLA-DP4 molecules (Table I). These assays included the seven most commonly expressed alleles encoded by the HLA-DRB1 genes (i.e., DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, and DRB1*1501). Eighty-five percent of people in the United States and Europe possess at least one of these molecules. They also included the main second HLA-DR molecules (DRB3*0101, DRB4*0101, and DRB5*0101) and the two HLA-DP4 molecules, which are carried by ~76% percent of Caucasian individuals. To optimize the binding capacity of the peptides, the overlaps were selected so that the peptides contained an aliphatic or aromatic residue in positions 1–5 in agreement with the binding specificity of HLA-DR and HLA-DP4 molecules (24, 28, 29). The data were presented as relative affinities to easily compare their binding properties to high-binder peptides that we used as references. An upper threshold of relative activity of 100 indicated that active peptides were at most 100× less efficient at binding to HLA II molecules than were high-binder peptides. As shown in Table I, some peptides are allele-specific, as exemplified by the peptides 1–15 and 4–18 that bound to DR11 or the peptide 41–55 that bound to DRB4. More interestingly, three regions in the survivin sequence (17–34, 84–113, and 122–142) contained peptides with a broad binding specificity for HLA-DR and HLA-DP4 molecules.

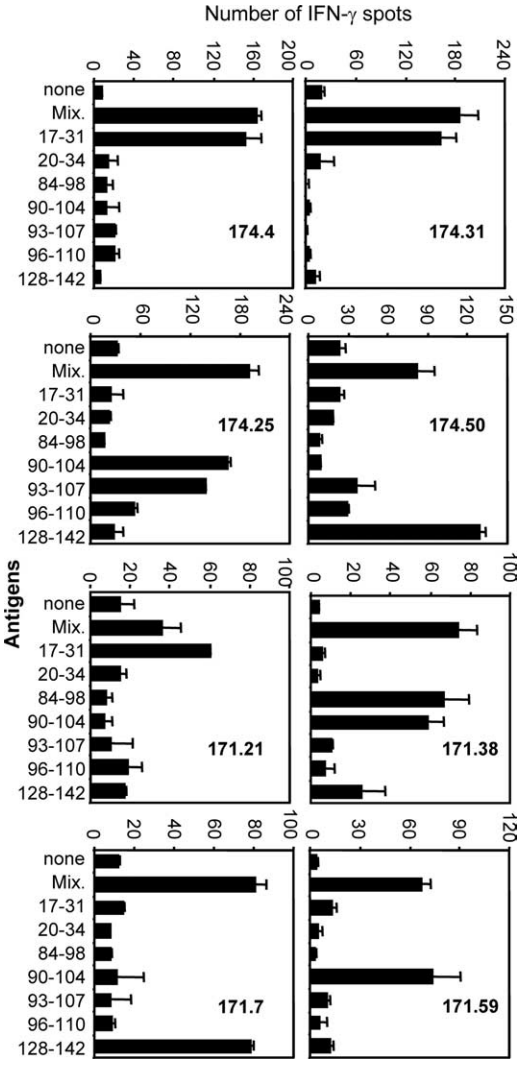

FIGURE 1. Peptide specificity of T cell lines obtained from healthy donors 171 and 174. $CD4^+$ T cell lines from two healthy donors (patients 171 and 174) were obtained after 3 weekly stimulations by autologous mature DC loaded with a mixture of seven selected peptides (17–31, 20–34, 84–98, 90–104, 93–107, 96–110, and 128–142). The specificity of the T cell lines was assessed by IFN-γ ELISPOT. T lymphocytes (10^4) were incubated in duplicate with 10^5 autologous PBMC in the presence or absence of peptides. Spot staining was done after a 24-h incubation.

Table II. *Capacity of the seven selected peptides to induce peptide-specific T cell lines*[a]

Donors	HLA II Typing			No. of Peptide-Specific T Cell Lines						
	DRB1	Second DR	DPB1	17–31	20–34	84–98	90–104	93–107	96–110	128–142
P169	1502	DRB5	0402	0	0	0	1	1	12	0
P171	0101, 0401	DRB4	0401	2	1	3	3	0	0	7
P174	0701, 1501	DRB4, DRB5	0401	10	0	0	3	2	1	1
P179	0701, 1301	DRB4, DRB3	—[b]	1	0	2	2	0	2	4
P187	0701, 0401	DRB4	—[b]	4	6	0	1	0	2	1
P188	1101	DRB3[c]	0401	1	2	3	7	3	1	0
P180	0101, 1101	DRB3[c]	0401	0	0	3	2	2	1	1
P241	1301, 16	DRB3, DRB5	—[b]	10	0	0	0	1	4	3
Total				28	9	11	19	9	23	17
Responder frequency				5/8	3/8	4/8	7/8	4/8	7/8	6/8

[a] T cell lines were derived from seven HLA II unrelated healthy donors. Their peptide specificity was evaluated by ELISPOT as described in Fig. 1.
[b] Not HLA-DP4.
[c] DRB3*0202, which is different from DRB3 (DRB3*0101).

Eight peptides located in these peptide regions bound to at least one of the HLA-DP4 molecules. Most of them bound to both molecules, in agreement with their very similar binding motifs (24). They also exhibited a good capacity to bind to HLA-DR molecules and bound to five to seven HLA-DR molecules. For example, the peptide 17–31 bound to six HLA-DR molecules (DR1, DR7, DR11, DR13, DR15, and DRB5) and the two DP4 molecules, while the peptide 84–98 is also a binder for six HLA-DR molecules (DR3, DR7, DR11, DR13, DR15, and DRB4) and the two HLA-DP4 molecules. Additionally, peptide 128–142 did not bind to HLA-DP4 but bound to seven different HLA-DR molecules (DR1, DR3, DR4, DR7, DR11, DR15, and DRB4). As a result, we retained seven peptides (17–31, 20–34, 84–98, 90–104, 93–107, 96–110, and 128–142) on the basis of their wide specificity for the HLA II molecules and the coverage of the sequence of the survivin Ag.

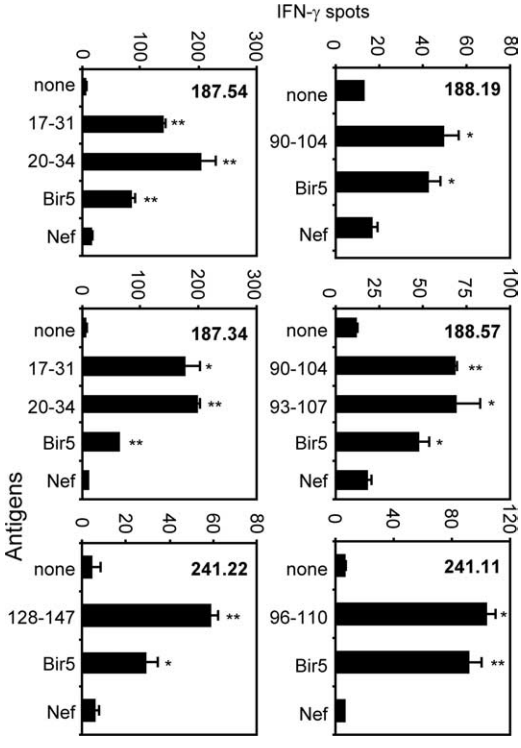

FIGURE 2. Presentation of the recombinant protein survivin to peptide-specific T cell lines. Peptide-specific T cell lines were obtained from two healthy donors (187 and 188). The protein survivin (Bir5) and HIV Nef protein were incubated at 1–3 μM for 4 h with immature autologous DC. After washings, DC (10^4) were incubated with T lymphocytes. T lymphocyte response was evaluated by IFN-γ ELISPOT. Each bar represents the mean spot number of duplicates ± SD. **, $p < 0.01$; *, $p < 0.05$.

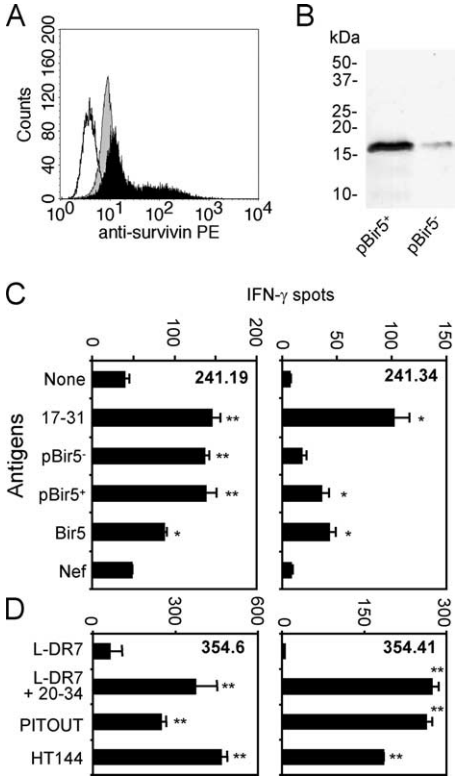

FIGURE 3. Stimulation of peptide-specific T cell lines by immature DC loaded with tumor lysates. *A*, HeLa cells were transfected with the survivin gene. Expression level of survivin was evaluated by flow cytometry after intracellular staining using a survivin-specific Ab. Black, surviving-transfected HeLa; gray, untransfected HeLa; open, isotype control of the transfected HeLa cells. *B*, Cell lysates were obtained by five rapid freeze-thaw cycles in AIM V medium. Survivin content was evaluated by Western blot using survivin-specific Abs. *C*, Immature DC (0.5 M/ml) were pulsed for 4 h with the tumor lysates (equivalent to 2.5 M/ml) of survivin-transfected HeLa (pBir5$^+$) or untransfected cells (pBir5$^-$) or pulsed with the recombinant proteins Bir5 and Nef at 3 μM. T cell lines (241.19 and 241.34) were incubated with 5000 pulsed DC for 24 h and subsequently submitted to IFN-γ ELISPOT assay. *D*, HLA-DR7-transfected murine L cells (L-DR7), HLA-DR7$^+$ EBV (PITOUT), and melanoma (HT144) cell lines were incubated (30,000 cells/well) with 20–34 specific T cell lines (354.6 and 354.41). T cell activation was revealed by IFN-γ ELISPOT assay. **, $p < 0.01$; *, $p < 0.05$.

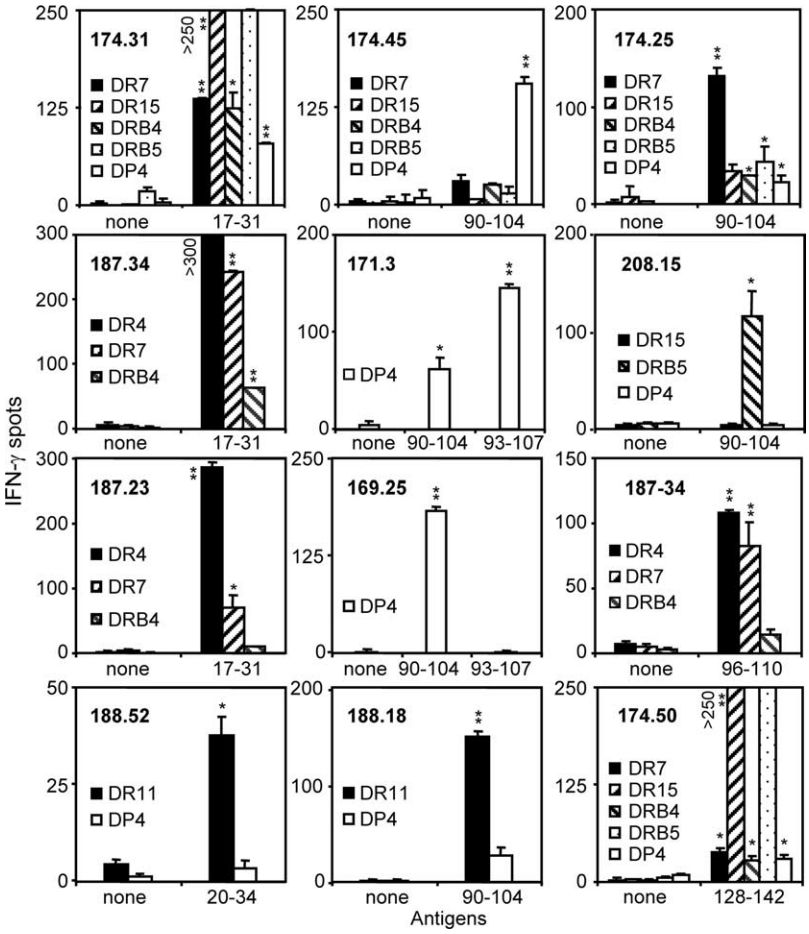

FIGURE 4. HLA-DR and HLA-DP4 restriction of peptide-specific T cell lines. T lymphocyte response was evaluated by IFN-γ ELISPOT. Approximately 10^4 T lymphocytes were incubated in duplicate with 3×10^4 L cells transfected with either an HLA-DR or HLA-DP4 molecules in the presence or absence of the appropriate peptide. Omission of the transfected L cells gave rise to a reduced number of spots (not shown). Each bar represents the mean spot number of duplicates ± SD. **, $p < 0.01$; *, $p < 0.05$.

The seven selected peptides induce peptide-specific CD4$^+$ T cell lines in HLA II unrelated healthy donors

The capacity of the seven peptides to prime CD4$^+$ T cells harvested from healthy donors was evaluated as previously described (25). CD4$^+$ T cells were seeded in 96-well plates and stimulated weekly by mature DC loaded with the mixture of the peptides. Their peptide specificity was evaluated by IFN-γ ELISPOT using autologous PBMC as APCs. Fig. 1 shows eight T cell lines derived for healthy donors 171 and 174. Among 60 seeded wells per donors, 12 and 13 peptide-specific T cell lines were discovered for donors 171 and 174, respectively, suggesting that they derived from few T cell precursors, which have been amplified by the weekly restimulation. Accordingly, the T cell lines were generally specific for either a unique peptide or for two overlapping peptides, suggesting that they are specific for the common part of the two peptides. A total of eight healthy donors were recruited and possessed the most frequently encountered HLA-DR alleles, except for DR3 (Table II). Five donors possessed the HLA-DP4 molecules, in agreement with the high frequency of this molecule in the Caucasian population. Although the panel of donors contains multiple HLA II haplotypes, all the peptides induced specific T cell lines in a minimum of three donors. Peptide 17–31 induced 28 T cell lines, 20–34, 9; 84–98, 11; 90–104, 19; 93–107, 9; 96–110, 23; and 128–142, 14 lines. Peptides 90–104 and 96–110 were stimulating in almost all of the donors, while peptides 17–31 and 128–142 induced T cell lines in five and six donors, respectively. Further characterization was performed depending on the quantity of T cells remaining from the microculture.

Peptide-specific T cell lines are stimulated by the survivin protein and tumor lysates presented by immature DC

We evaluated the capacity of eight peptide-specific T cell lines from two different donors to recognize the native protein. As shown in Fig. 2, two T cell lines (187.54 and 187.34) specific for the 17–31 and 20–34 peptides reacted specifically with autologous DC previously loaded with the recombinant survivin protein. They were not, however, stimulated by unloaded DC and by DC fed with the recombinant HIV Nef protein. Similarly, T cell lines 188.19, 188.57, 241.22, and 241.11, which were specific for peptides 90–104, 93–107, 128–142, and 96–110, respectively, specifically recognized the survivin protein. We also investigated the presentation of tumor cell lysates by immature DC to two peptide-specific T cell lines (Fig. 3). All the tumor cell lines we investigated expressed at least a low level of survivin, as shown for the HeLa cells by FACS (Fig. 3*A*) and Western blot (Fig. 3*B*). We therefore modulated the level of expression of survivin by using HeLa cells transfected with survivin gene or untransfected. As shown Fig. 3*A*, survivin is ~10-fold more expressed in the transfected cells as compared with untransfected ones. The 17–31-specific T cell line 214.34 was specifically stimulated by immature DC loaded with the protein survivin and by tumor lysates of transfected HeLa cells (Fig. 3*C*). It was not, however, stimulated by the HIV Nef protein or by the untransfected cells, demonstrating the specificity of the stimulation and that the level of endogenously expressed survivin was insufficient to stimulate the T cell line. In contrast, the T cell line 214.19 was stimulated by the immature DC loaded with the survivin protein and with tumor lysates produced by transfected and untransfected HeLa cells, but not by the DC

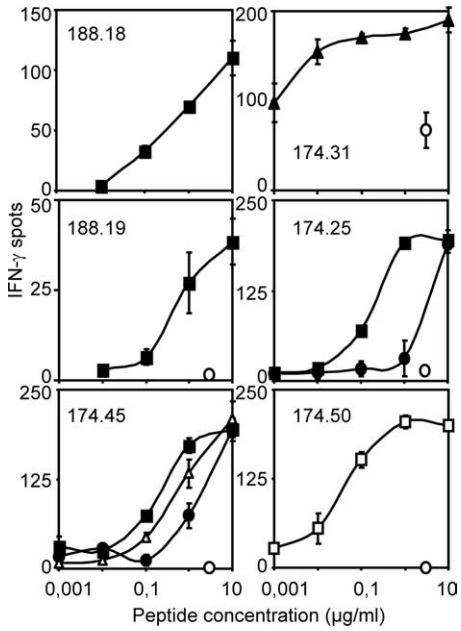

FIGURE 5. Dose response of peptide-specific T cell lines. CD4+ T cell lines (188.18, 188.19, 174.45, 174.31, 174.25, and 174.50) were incubated in duplicate with 10^5 autologous PBMC and a dose range of appropriate peptides for 24 h. The specificity of the T cell lines was assessed by IFN-γ ELISPOT: 17–31 (▲), 90–104 (■), 93–107 (●), 96–110 (△), 128–142 (□), no peptide (○).

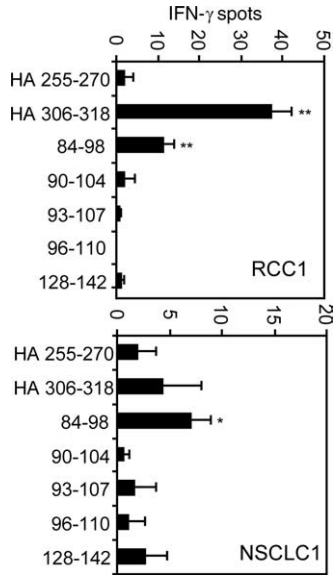

FIGURE 6. Peptide specificity of T lymphocytes collected in cancer patients. Donors RCC1 and NSCLC1 were RCC and NSLC patients, respectively. Peptide reactivity of the T cells was evaluated by IFN-γ ELISPOT after 1 wk in vitro stimulation with the peptides as described in *Materials and Methods*. Each bar represents the mean spot number of triplicates ± SD. **, $p < 0.01$; *, $p < 0.05$.

alone or by the DC loaded with the HIV Nef protein. Finally, two HLA-DR7-restricted T cell lines specific for the peptide 20–34 were stimulated by the HLA-DR-matched EBV cell line PITOUT and melanoma cells HT144, although the murine L cells transfected by HLA-DR7 were not recognized unless they were loaded with the peptide 20–34 (Fig. 3*D*). We concluded from these experiments that the peptides 17–31, 90–104, 93–107, 96–110, and 128–142 are appropriately processed by immature DC loaded with the native protein, while at least the peptide 20–34 is naturally presented by tumor cells. We also observed that the endogenous level of survivin expression could be sufficient to stimulate CD4+ T lymphocytes.

Survivin peptides are presented to CD4+ T cell lines by multiple HLA-DR and HLA-DP4 molecules

As the seven selected peptides bound to multiple HLA II molecules, HLA II restriction molecules supporting the peptide-specific T cell stimulation was evaluated for 19 T cell lines using L cells transfected by HLA-DR or DP4 molecules. Data presented in Fig. 4 are from 12 of them. T cell lines were mostly stimulated by their corresponding peptide presented by only one HLA II molecule, but degenerate recognition was also observed as shown for the 174.31 and 187.50 T cell lines. In both cases, DR15 and DRB5 molecules emerged as the most stimulating, suggesting that these molecules have contributed to T cell priming. However, significant presentation by other molecules (DR7, DRB4, and DP4) was also observed. A given donor can develop a peptide-specific T cell response using two different restriction elements, as exemplified by the T cell lines 174.45 and 174.25, which recognized the peptide 90–104 presented by HLA-DP4 and HLA-DR7, respectively. As a result, we observed the presentation to T cells of the peptide 17–31 by HLA-DR7, DR15, DRB4, DRB5, and DP4; 20–34 by DR4, DR7, DR11, and DP4; 90–104 by DR7, DR11, DR15, DRB4, DRB5, and DP4; 93–107 by DR11 and DP4; 96–110 by DR4, DR7 and DP4; and 128–142 mainly by DR15 and DRB5. Finally, peptide concentration was evaluated for eight different T cell lines specific for 17–31, 90–104, 93–107, 96–110, or 128–142 (Fig. 5). Midstimulation was reached at a concentration <0.1 μg/ml for all the peptides, except for peptide 93–107. This indicated an efficient avidity of specific T cell lines against survivin-derived peptides.

T cell antigenicity of survivin peptides in cancer patients

Spontaneous response to survivin peptides was investigated in eight different cancer patients and six healthy donors. None of the healthy donors gave rise to peptide-specific stimulation (data not shown). In contrast, a specific T cell response was detected for the 84–98 peptide in two patients, one with renal cancer and the other with NSLC (Fig. 6). This response appears relatively weak but is in the order of magnitude of that seen for the Flu peptides (HA

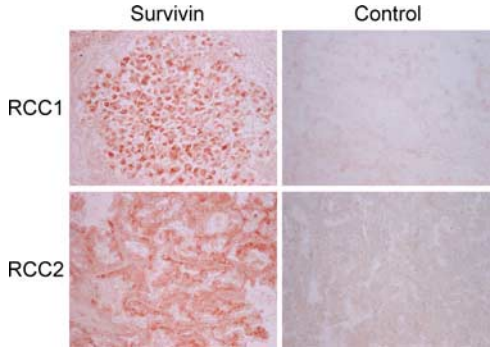

FIGURE 7. Survivin expression in RCC tumors from two patients tested for the presence of anti-survivin CD4+ T cells. RCC tumors were stained with anti-survivin Ab (*left*) or negative control Ab (*right*). RCC1 corresponds to the one patient with detection of anti-survivin CD4+ T cells, whereas RCC2 corresponds to one patient with no anti-survivin CD4+ T cell detection.

255–270 and HA 306–318). At least in these two donors, we therefore observed a spontaneous CD4 T cell response to one survivin peptide, namely 84–98. We had access to the tumor biopsy of one of the responding RCC patients and of another non-responding RCC patients. High levels of survivin expression were observed in both cases (Fig. 7).

Discussion

Although survivin is a small and overexpressed tumor Ag (1), we show herein that it is able to generate a $CD4^+$ T cell response in various healthy individuals and cancer patients. This response involves $CD4^+$ T cell epitopes restricted to multiple HLA II alleles and is mainly supported by HLA-DR molecules.

Survivin is one of the rare tumor Ags expressed in a large number of tumors of various types (1). It contributes to the resistance of tumor cells to apoptosis (2) and is involved in their proliferative capacity (3). It is not, however, completely absent from normal cells and hence could lead to T cell tolerance. Because the survivin-specific $CD4^+$ T cell response, which is required to sustain the tumor-specific cytotoxic response, is poorly documented (17), we characterized this response in multiple donors. Seven peptides were able to prime $CD4^+$ T lymphocytes in a specific manner and were clustered in three different sequence regions, namely 17–34, 84–110, and 128–142. Three main properties characterize T cell epitopes for vaccine purposes, namely immunodominance, immunoprevalence, and hierarchy. As defined for protein immunization (30) and viral infection (31), immunodominant T cell epitopes are sensu stricto recognized in the context of priming with the native Ag and hence are generated by the processing of the native Ags. As T cell lines had been primed with peptides, we only assessed in vitro the appropriate presentation of the native Ag. Peptides 17–31, 90–104, 93–107, 96–110, and 128–142 appeared appropriately processed by immature DC loaded with the native protein. Two T cell lines specific for the peptide 17–31 were stimulated by immature DC loaded with the lysate of survivin-transfected cells, while one was stimulated by the lysate of untransfected cells, suggesting that endogenous expression of survivin could be sufficient for $CD4^+$ T cell presentation. Two peptide 20–34-specific T cell lines directly recognized HLA-DR matched tumor cells, suggesting that at least the peptide 20–34 is presented by the tumor cell lines. Finally, a spontaneous response in two cancer patients involved the peptide 84–98, confirming its in vivo presentation.

We then investigated the immunoprevalence of the T cell epitopes. Immunoprevalence corresponds to the frequency of responders and was evaluated in eight different donors. As shown in Table II, all the peptides exhibited a good immunoprevalence, as they were all stimulating in at least half of the donors. Two peptides (90–104 and 96–110) primed specific T cell lines in almost all of the individuals. Relatively few peptides with such high immunoprevalence have been described in tumor Ags (26, 32, 33) and in viral or bacterial Ags (25, 34–36). This emphasizes the interest of these peptides for vaccine purposes. Finally, we observed a different hierarchy between the defined epitopes for each healthy donor. Epitope hierarchy is documented by the proportion of the total response produced by one epitope and defined major (>30% of the response) and minor epitopes. For instance, for donor P169, the response was dominated by peptide 96–110, which gave rise to 85% of the generated T cell lines, while peptides 90–104 and 93–107 were minor epitopes for this donor. In contrast, peptide 96–110 became a minor epitope for donor P174, and the major epitope was peptide 17–34. Major epitopes are therefore carried by different peptides and, as suggested by recent investigations in mice, may result from size variation of the naive precursor repertoire (37). Variation in epitope hierarchy was also reported in vaccinated donors (38). Altogether, we suggest introducing in a peptide vaccine at least the peptides 17–31, 90–104, 96–110, and 128–142, which are major epitopes and hence are expected to elicit a strong response in each donor. We do not propose to limit the composition of the vaccine to the most immunoprevalent peptides (90–104 and 96–110). These peptides induce a response in most donors, but may recruit a relatively low number of T cells in some donors. Their combination with the peptides 17–31 and 128–142 is expected to induce a better T cell response.

We also analyzed the restriction of peptide-specific T cell lines to identify the main HLA II molecules implicated in these responses. High immunoprevalence of T cell epitopes may result from a wide specificity for HLA-DR molecules or from the capacity to bind a preponderant molecule as HLA-DP4 (24). HLA-DP4 is present in ~76% of Caucasian individuals and covers approximately the same proportion of the population as six HLA-DR molecules. Four peptides in the survivin Ag bound to HLA-DP4 molecules, while seven peptides including them were retained because of their promiscuity for HLA-DR molecules. In contrast to our previous observations with HIV Ags (39) and the MAGE-A1 tumor Ag (40), survivin-specific T cell epitopes restricted to HLA-DP4 induce few T cell lines and do not appear strongly immunogenic. $CD4^+$ T cell response to survivin is essentially HLA-DR restricted, as similarly observed for the NY-ESO-1 Ag (26, 32, 33), although this Ag also contains HLA-DP4-restricted epitopes (32, 41). As a result, immunoprevalence of the survivin-specific $CD4^+$ T cell response mainly relies on HLA-DR promiscuity as compared with HLA-DP4. One interesting issue would be to evaluate the role of HLA-DQ molecules, especially as these molecules exhibited different peptide specificity with respect to HLA-DR and HLA-DP4 molecules (42, 43).

Because of its expression during the cell cycle of normal cells, survivin is not exclusively expressed in tumor cells. This low level of expression may induce tolerance by deletion of the efficient Tcr clonotype (44), as shown for P53 Ag (45). However, our data do not support the existence of tolerance induction against survivin in normal donors and cancer patients. Indeed, we observed that all of the healthy donors responded to at least one of the identified peptides, although they exhibited a variety of HLA II molecules. Moreover, the dose response of the T cell lines revealed a good efficiency in recognizing the peptides and thus suggested that clonotypes of high affinity for survivin were not negatively selected. In two of eight cancer patients, we detected a spontaneous $CD4^+$ T cell response specific for one survivin peptide, while this spontaneous response was absent in healthy donors. At least in these two cancer patients, survivin produced by the tumor seems to elicit a response and not a tolerance status. Accordingly, spontaneous humoral (18, 19) and cytotoxic responses (46) raised against survivin have been reported. These observations therefore suggest that the basal level of survivin in normal cells is not enough to provoke tolerance, while its overexpression in tumor cells could lead to specific T cell priming, as has been previously shown for cancer germ line Ags such as NY-ESO-1 (33) and TRAG (47).

CD8 T cell response specific for survivin have been investigated in several studies, especially for HLA-A2 patients. Two HLA-A2-restricted T cell epitopes have been delineated, namely 5–14 and 96–104 (6, 7), while peptide 80–88 and peptides 46–54 and 51–59 have been identified as $CD8^+$ T cell epitopes restricted by HLA-24 (8) and HLA-B35 (9), respectively. Of note is the proximity of these epitopes to the $CD4^+$

T cell epitopes we report in this study. Peptide 96–104 is encompassed by 90–104 and 96–110 immunoprevalent $CD4^+$ T cell epitopes, which are therefore able to elicit both $CD4^+$ and $CD8^+$ T cell responses, as shown for other combinations of T cell epitopes (41). We have also confirmed the priming capacity of peptide 5–14 and have derived six T cell lines from three independent healthy donors (data not shown). We demonstrated their capacity to recognize HLA-$A2^+$ tumor cell lines, although this has not been previously shown (6). Besides the 90–104 and 90–110 sequences, we therefore propose as vaccine candidates the 5–31 and 5–34 peptides, as they also advantageously combine $CD8^+$ and $CD4^+$ T cell epitopes.

In conclusion, our work demonstrates the efficiency of peptides in eliciting a $CD4^+$ T cell response specific for survivin, showing their immunodominance, immunoprevalence, and proximity to $CD8^+$ T cell epitopes. We also show that HLA-DR promiscuity rather than specificity for HLA-DP4 molecules supports their priming capacity in multiple donors. Considering the wide distribution of survivin in tumor cells, this work contributes to the design of new peptide vaccines against various cancers.

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Disclosures

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References

1. Li, F. 2003. Survivin study: what is the next wave? *J. Cell. Physiol.* 197: 8–29.
2. Ambrosini, G., C. Adida, and D. C. Altieri. 1997. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med.* 3: 917–921.
3. Li, F., G. Ambrosini, E. Y. Chu, J. Plescia, S. Tognin, P. C. Marchisio, and D. C. Altieri. 1998. Control of apoptosis and mitotic spindle checkpoint by survivin. *Nature* 396: 580–584.
4. Adida, C., C. Haioun, P. Gaulard, E. Lepage, P. Morel, J. Briere, H. Dombret, F. Reyes, J. Diebold, C. Gisselbrecht, et al. 2000. Prognostic significance of survivin expression in diffuse large B-cell lymphomas. *Blood* 96: 1921–1925.
5. Olie, R. A., A. P. Simoes-Wust, B. Baumann, S. H. Leech, D. Fabbro, R. A. Stahel, and U. Zangemeister-Wittke. 2000. A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy. *Cancer Res.* 60: 2805–2809.
6. Schmitz, M., P. Diestelkoetter, B. Weigle, F. Schmachtenberg, S. Stevanovic, D. Ockert, H. G. Rammensee, and E. P. Rieber. 2000. Generation of survivin-specific $CD8^+$ T effector cells by dendritic cells pulsed with protein or selected peptides. *Cancer Res.* 60: 4845–4849.
7. Andersen, M. H., L. O. Pedersen, J. C. Becker, and P. T. Straten. 2001. Identification of a cytotoxic T lymphocyte response to the apoptosis inhibitor protein survivin in cancer patients. *Cancer Res.* 61: 869–872.
8. Hirohashi, Y., T. Torigoe, A. Maeda, Y. Nabeta, K. Kamiguchi, T. Sato, J. Yoda, H. Ikeda, K. Hirata, N. Yamanaka, and N. Sato. 2002. An HLA-A24-restricted cytotoxic T lymphocyte epitope of a tumor-associated protein, survivin. *Clin. Cancer Res.* 8: 1731–1739.
9. Reker, S., J. C. Becker, I. M. Svane, E. Ralfkiaer, P. T. Straten, and M. H. Andersen. 2004. HLA-B35-restricted immune responses against survivin in cancer patients. *Int. J. Cancer* 108: 937–941.
10. Casati, C., P. Dalerba, L. Rivoltini, G. Gallino, P. Deho, F. Rini, F. Belli, D. Mezzanzanica, A. Costa, S. Andreola, et al. 2003. The apoptosis inhibitor protein survivin induces tumor-specific $CD8^+$ and $CD4^+$ T cells in colorectal cancer patients. *Cancer Res.* 63: 4507–4515.
11. Schmidt, S. M., K. Schag, M. R. Muller, M. M. Weck, S. Appel, L. Kanz, F. Grunebach, and P. Brossart. 2003. Survivin is a shared tumor-associated antigen expressed in a broad variety of malignancies and recognized by specific cytotoxic T cells. *Blood* 102: 571–576.
12. Fuessel, S., A. Meye, M. Schmitz, S. Zastrow, C. Linne, K. Richter, B. Lobel, O. W. Hakenberg, K. Hoelig, E. P. Rieber, and M. P. Wirth. 2006. Vaccination of hormone-refractory prostate cancer patients with peptide cocktail-loaded dendritic cells: results of a phase I clinical trial. *Prostate* 66: 811–821.
13. Nagaraj, S., V. Pisarev, L. Kinarsky, S. Sherman, C. Muro-Cacho, D. C. Altieri, and D. I. Gabrilovich. 2007. Dendritic cell-based full-length survivin vaccine in treatment of experimental tumors. *J. Immunother.* 30: 169–179.
14. Otto, K., M. H. Andersen, A. Eggert, P. Keikavoussi, L. O. Pedersen, J. C. Rath, M. Bock, E. B. Brocker, P. T. Straten, E. Kampgen, and J. C. Becker. 2005. Lack of toxicity of therapy-induced T cell responses against the universal tumour antigen survivin. *Vaccine* 23: 884–889.
15. Wobser, M., P. Keikavoussi, V. Kunzmann, M. Weininger, M. H. Andersen, and J. C. Becker. 2006. Complete remission of liver metastasis of pancreatic cancer under vaccination with a HLA-A2 restricted peptide derived from the universal tumor antigen survivin. *Cancer Immunol. Immunother.* 55: 1294–1298.
16. Toes, R. E., F. Ossendorp, R. Offringa, and C. J. Melief. 1999. CD4 T cells and their role in antitumor immune responses. *J. Exp. Med.* 189: 753–756.
17. Piesche, M., Y. Hildebrandt, F. Zettl, B. Chapuy, M. Schmitz, G. Wulf, L. Trumper, and R. Schroers. 2007. Identification of a promiscuous HLA DR-restricted T-cell epitope derived from the inhibitor of apoptosis protein survivin. *Hum. Immunol.* 68: 572–576.
18. Megliorino, R., F. D. Shi, X. X. Peng, X. Wang, E. K. Chan, E. M. Tan, and J. Y. Zhang. 2005. Autoimmune response to anti-apoptotic protein survivin and its association with antibodies to p53 and c-myc in cancer detection. *Cancer Detect. Prev.* 29: 241–248.
19. Rohayem, J., P. Diestelkoetter, B. Weigle, A. Oehmichen, M. Schmitz, J. Mehlhorn, K. Conrad, and E. P. Rieber. 2000. Antibody response to the tumor-associated inhibitor of apoptosis protein survivin in cancer patients. *Cancer Res.* 60: 1815–1817.
20. Charalambous, A., M. Oks, G. Nchinda, S. Yamazaki, and R. M. Steinman. 2006. Dendritic cell targeting of survivin protein in a xenogeneic form elicits strong $CD4^+$ T cell immunity to mouse survivin. *J. Immunol.* 177: 8410–8421.
21. Tu, S. P., X. H. Jiang, M. C. Lin, J. T. Cui, Y. Yang, C. T. Lum, B. Zou, Y. B. Zhu, S. H. Jiang, W. M. Wong, et al. 2003. Suppression of survivin expression inhibits in vivo tumorigenicity and angiogenesis in gastric cancer. *Cancer Res.* 63: 7724–7732.
22. Texier, C., S. Pouvelle, M. Busson, M. Herve, D. Charron, A. Menez, and B. Maillere. 2000. HLA-DR restricted peptide candidates for bee venom immunotherapy. *J. Immunol.* 164: 3177–3184.
23. Texier, C., S. Pouvelle-Moratille, M. Busson, D. Charron, A. Menez, and B. Maillere. 2001. Complementarity and redundancy of the binding specificity of HLA-DRB1, -DRB3, -DRB4 and -DRB5 molecules. *Eur. J. Immunol.* 31: 1837–1846.
24. Castelli, F. A., C. Buhot, A. Sanson, H. Zarour, S. Pouvelle-Moratille, C. Nonn, H. Gahery-Segard, J. G. Guillet, A. Menez, B. Georges, and B. Maillere. 2002. HLA-DP4, the most frequent HLA II molecule, defines a new supertype of peptide-binding specificity. *J. Immunol.* 169: 6928–6934.
25. Castelli, F. A., M. Leleu, S. Pouvelle-Moratille, S. Farci, H. M. Zarour, M. Andrieu, C. Auriault, A. Menez, B. Georges, and B. Maillere. 2007. Differential capacity of T cell priming in naive donors of promiscuous $CD4^+$ T cell epitopes of HCV NS3 and core proteins. *Eur. J. Immunol.* 37: 1513–1523.
26. Zarour, H. M., B. Maillere, V. Brusic, K. Coval, E. Williams, S. Pouvelle-Moratille, F. Castelli, S. Land, J. Bennouna, T. Logan, and J. M. Kirkwood. 2002. NY-ESO-1 119–143 is a promiscuous major histocompatibility complex class II T-helper epitope recognized by Th1- and Th2-type tumor-reactive $CD4^+$ T cells. *Cancer Res.* 62: 213–218.
27. Godard, B., A. Gazagne, A. Gey, M. Baptiste, B. Vingert, B. Pegaz-Fiornet, L. Strompf, W. H. Fridman, D. Glotz, and E. Tartour. 2004. Optimization of an elispot assay to detect cytomegalovirus-specific $CD8^+$ T lymphocytes. *Hum. Immunol.* 65: 1307–1318.
28. Rammensee, H. G., T. Friede, and S. Stevanoviic. 1995. MHC ligands and peptide motifs: first listing. *Immunogenetics* 41: 178–228.
29. Sturniolo, T., E. Bono, J. Ding, L. Raddrizzani, O. Tuereci, U. Sahin, M. Braxenthaler, F. Gallazzi, M. P. Protti, F. Sinigaglia, and J. Hammer. 1999. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. *Nat. Biotechnol.* 17: 555–561.
30. Gammon, G., N. Shastri, J. Cogswell, S. Wilbur, S. Sadegh-Nasseri, U. Krzych, A. Miller, and E. Sercarz. 1987. The choice of T-cell epitopes utilized on a protein antigen depends on multiple factors distant from, as well as at the determinant site. *Immunol. Rev.* 98: 53–73.
31. Assarsson, E., J. Sidney, C. Oseroff, V. Pasquetto, H. H. Bui, N. Frahm, C. Brander, B. Peters, H. Grey, and A. Sette. 2007. A quantitative analysis of the variables affecting the repertoire of T cell specificities recognized after vaccinia virus infection. *J. Immunol.* 178: 7890–7901.
32. Mandic, M., F. Castelli, B. Janjic, C. Almunia, P. Andrade, D. Gillet, V. Brusic, J. M. Kirkwood, B. Maillere, and H. M. Zarour. 2005. One NY-ESO-1-derived epitope that promiscuously binds to multiple HLA-DR and HLA-DP4 molecules and stimulates autologous $CD4^+$ T cells from patients with NY-ESO-1-expressing melanoma. *J. Immunol.* 174: 1633–1640.
33. Gnjatic, S., D. Atanackovic, E. Jager, M. Matsuo, A. Selvakumar, N. K. Altorki, R. G. Maki, B. Dupont, G. Ritter, Y. T. Chen, et al. 2003. Survey of naturally occurring $CD4^+$ T cell responses against NY-ESO-1 in cancer patients: correlation with antibody responses. *Proc. Natl. Acad. Sci. USA* 100: 8862–8867.
34. Panina-Bordignon, P., A. Tan, A. Termijtelen, S. Demotz, G. Corradin, and A. Lanzavecchia. 1989. Universally immunogenic T cell epitopes: promiscuous binding to human MHC class II and promiscuous recognition by T cells. *Eur. J. Immunol.* 19: 2237–2242.
35. Gelder, C. M., J. R. Lamb, and B. A. Askonas. 1996. Human $CD4^+$ T-cell recognition of influenza A virus hemagglutinin after subunit vaccination. *J. Virol.* 70: 4787–4790.
36. Diepolder, H. M., J. T. Gerlach, R. Zachoval, R. M. Hoffmann, M. C. Jung, E. A. Wierenga, S. Scholz, T. Santantonio, M. Houghton, S. Southwood, et al. 1997. Immunodominant $CD4^+$ T-cell epitope within nonstructural protein 3 in acute hepatitis C virus infection. *J. Virol.* 71: 6011–6019.
37. Moon, J. J., H. H. Chu, M. Pepper, S. J. McSorley, S. C. Jameson, R. M. Kedl, and M. K. Jenkins. 2007. Naive $CD4^+$ T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity* 27: 203–213.
38. Jing, L., T. M. Chong, B. Byrd, C. L. McClurkan, J. Huang, B. T. Story, K. M. Dunkley, L. Aldaz-Carroll, R. J. Eisenberg, G. H. Cohen, et al. 2007.

Dominance and diversity in the primary human CD4 T cell response to replication-competent vaccinia virus. *J. Immunol.* 178: 6374–6386.

39. Cohen, W. M., S. Pouvelle-Moratille, X. F. Wang, S. Farci, G. Munier, D. Charron, A. Menez, M. Busson, and B. Maillere. 2006. Scanning the HIV genome for $CD4^+$ T cell epitopes restricted to HLA-DP4, the most prevalent HLA class II molecule. *J. Immunol.* 176: 5401–5408.
40. Wang, X. F., W. M. Cohen, F. A. Castelli, C. Almunia, B. Lethe, S. Pouvelle-Moratille, G. Munier, D. Charron, A. Menez, H. M. Zarour, et al. 2007. Selective identification of HLA-DP4 binding T cell epitopes encoded by the MAGE-A gene family. *Cancer Immunol. Immunother.* 56: 807–818.
41. Zeng, G., Y. Li, M. El-Gamil, J. Sidney, A. Sette, R. F. Wang, S. A. Rosenberg, and P. F. Robbins. 2002. Generation of NY-ESO-1-specific $CD4^+$ and $CD8^+$ T cells by a single peptide with dual MHC class I and class II specificities: a new strategy for vaccine design. *Cancer Res.* 62: 3630–3635.
42. Raddrizzani, L., T. Sturniolo, J. Guenot, E. Bono, F. Gallazzi, Z. A. Nagy, F. Sinigaglia, and J. Hammer. 1997. Different modes of peptide interaction enable HLA-DQ and HLA-DR molecules to bind diverse peptide repertoires. *J. Immunol.* 159: 703–711.
43. Sidney, J., C. Oseroff, S. Southwood, M. Wall, G. Ishioka, F. Koning, and A. Sette. 1992. DRB1*0301 molecules recognize a structural motif distinct from the one recognized by most DR beta 1 alleles. *J. Immunol.* 149: 2634–2640.
44. Staveley-O'Carroll, K., E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll, and H. Levitsky. 1998. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc. Natl. Acad. Sci. USA* 95: 1178–1183.
45. Theobald, M., J. Biggs, J. Hernandez, J. Lustgarten, C. Labadie, and L. A. Sherman. 1997. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J. Exp. Med.* 185: 833–841.
46. Andersen, M. H., L. O. Pedersen, B. Capeller, E. B. Brocker, J. C. Becker, and P. thor Straten. 2001. Spontaneous cytotoxic T-cell responses against survivin-derived MHC class I-restricted T-cell epitopes in situ as well as ex vivo in cancer patients. *Cancer Res.* 61: 5964–5968.
47. Janjic, B., P. Andrade, X. F. Wang, J. Fourcade, C. Almunia, P. Kudela, A. Brufsky, S. Jacobs, D. Friedland, R. Stoller, et al. 2006. Spontaneous $CD4^+$ T cell responses against TRAG-3 in patients with melanoma and breast cancers. *J. Immunol.* 177: 2717–2727.