p53 Specific (auto)immunity in mice

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Chapter 3

Prediction of the immunogenic potential of frameshift -mutated antigens in microsatellite instable cancer

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3

Abstract

Microsatellite instable (MSI) cancers express frameshift-mutated antigens, the C-terminal polypeptides of which are foreign to the immune system. Consequently, these antigens constitute a unique pool of tumor-specific antigens that can be exploited for patient diagnosis and selective, immune-mediated targeting of cancers. However, other than their sequence, very little is known about the characteristics of the majority of these proteins. We therefore developed a methodology for predicting their immunogenic behavior that is based on a gene expression system in which each of the proteins was fused to a short C-terminal polypeptide comprising two epitopes that can be readily detected by T cells and antibodies respectively. In this manner, accumulation of the antigens, and processing of peptides derived thereof into MHC, can be monitored systematically. The antigens that accumulate in the cells in which they are synthesized are of primary interest for cancer immunotherapy, because peptide epitopes derived thereof can be presented by dendritic cells in addition to the tumor cells themselves. As a result, these antigens constitute the best targets for a coordinated immune response by both CD8+ and CD4+ T cells, which increases the likelihood that tumor-induced immunity would be detectable against these antigens in cancer patients, as well as the potential value of these antigens as components of anti-cancer vaccines. Our data indicate that, of fifteen frameshift-mutated antigens examined in our present study, four (TGFβR2-1; MARCKS-1; -MARCKS-2; CDX2-2) are of primary interest and four additional antigens (TAF1B-1; PCNXL2-2; TCF7L2-2; Baxα+1) of moderate interest for further tumor immunological research.

Introduction

A high frequency of microsatellite instability (MSI-H) is a molecular feature of tumors associated with the Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome (1;2). Furthermore approximately 15% of sporadic colorectal, gastric and endometrial cancers, as well as lower frequencies of various other sporadic cancers, are characterized by widespread MSI (3-7). Microsatellites are repetitive nucleotide sequences of different length, distributed throughout the human genome that are prone to small insertion/deletion mutations, caused by DNA polymerase slippage during DNA replication (8). Usually, these errors are corrected by the inherent proofreading capacity of a group of proteins involved in mismatch repair (9;10). Defects in mismatch repair, like in MSI-H tumors, allow the accumulation of errors in microsatellites. Deletion or insertion of one or two base pairs in a coding gene results in a shift of the reading frame downstream of the mutation and thereby translation of an abnormal protein product.

These frameshift-encoded products constitute 'foreign' antigens for the immune system and therefore represent an unique pool of tumor specific antigens (11). Specific T-cell and antibody responses have indeed been found against a limited number of frameshift products (12-14). Furthermore, MSI-H tumors are associated with several treats that point at immune surveillance, such as increased lymphocyte infiltrate, increased incidence of MHC class I loss, and better patient survival prognosis as compared to microsatellite stable tumors. (15-17). Therefore, these frameshift-mutated proteins, in particular the non-self segment encoded by sequences downstream of the mutation, are considered promising candidates for preventive vaccination of subjects with HNPCC, or as adjuvant therapy in combination of surgery for patients with sporadic MSI-H tumors. The potential of these antigens for development of anti-cancer vaccines is further supported by the notion that mutations in several genes are found at high (>50%) frequencies in MSI-H cancers (18-22).

Unfortunately, little is known about the immunogenic properties of most proteins encoded by these commonly mutated genes. In order to predict their potential for inclusion in vaccines, it is of particularly interest to know whether they accumulate in tumor cells or are rapidly degraded. Accumulation of stable frameshift-mutated antigens in tumor cells can result in cross-presentation of antigen-derived peptides by "professional" antigen presenting cells (APCs), in particular dendiritic cells (DCs), in the tumor-draining lymphoid tissues and, thereby, in pre-existing antigen-specific T-cell responses in cancer patients. Furthermore, vaccination-induced CD4+ T cells are in this case expected to provide 'help' to the anti-tumor response, because they will encounter their cognate antigen on DCs in the peritumoral area. In contrast, instable antigens that do not reach significant steady-state levels are unlikely to become cross-presented (23-25). Consequently, neither 'spontaneous' priming of anti-tumor T-cell immunity, nor efficacy of vaccine-induced CD4+ T helper cells against such antigens are to be expected. Nevertheless, break down of these instable antigens through the ubiquitin-proteasome pathway can result in peptides that may be presented in the context of MHC class I at the tumor cell surface. In that case, vaccine-induced CD8+ T cells recognizing these peptides could be used to target the tumor.

In the present study, we made use of an expression system to systematically analyze the characteristics and immunogenic properties of proteins encoded by a selection of frame-shift-mutated genes that are commonly found in MSI-H cancers. This inventory provided important information on the manner in which these antigens should be used for further studies concerning patient diagnosis and cancer immunotherapy.

Material and methods

Tumor cell lines. The cell lines B3Z, Hela and Hela-Kb were used in experiments. Hela-Kb is a stable transfectant of the human Hela cell line, expressing the mouse H-2Kb MHC class I molecule (26). All cells were cultured at 37°C and 5% CO_2, in cell culture flasks (Corning, NY, USA) containing culture medium, composed of IMDM (BioWhittaker), supplemented with 10% heat-inactivated FCS, 100 µg/ml streptomycin, 100 IU/ml penicillin and 4 mM L-glutamine (all from Gibco).

Selection of frameshift products. Twelve genes, containing a microsatellite, were selected. Selection was based on known high mutation frequency in MSI-H colon cancer (18;19;22), previously described immunogenicity (12-14) or combinations of these factors. Insertion of 1 base pair or a deletion of two base pairs (-2/+1) result in the same reading frame but differ one amino difference in length. The same is valid for an insertions of 2 base pairs or a deletion of one base pair (-1/+2). Therefore only one type of a -2 or +1 and -1 or +2 mutation was used and only if the mutation resulted in a stretch of more than 4 new amino acids behind the microsatellite. Table 1 summarizes selected genes, mutation frequency in different types of cancer, whether -2/+1, -1/+2 or both sequences were constructed (15 in total), function and other characteristics.

Construction of frameshift products and transfection. Sequences of selected frameshift products were amplified by routine PCR using cDNA derived from several human colorectal MSI-H cell lines or were synthetically synthesized. Primers, containing a restriction site, were designed for start and end of the sequence. A KOZAK sequence was added before the initial ATG code of each fragment. PCR products were directly cloned with the pGEM-T-Easy cloning kit (Promega) and sequenced, using standard procedures. Expression plasmids for each of the selected frameshift were obtained, through unidirectional cloning of the sequences concerned into the mammalian expression vector, pcDNA3-OVA/Flag containing an in-frame OVA/Flag tag. The tag was located downstream of the sequence direct behind the restriction site containing the H-2Kb restricted CD8+ T-cell epitope of chicken ovalbumine (OVA: SIINFEKL) and the Flag epitope (DYKDDDDK), which were spaced by a triple alanine sequence to allow efficient processing of the OVA T-cell epitope. These plasmids were transiently transfected into cells using Lipofectamine 2000 (Invitrogen) in 6 wells plate. Per well 2 µg DNA was added. In some experiments 0.5 µg eGFP-pcDNA3 vector was added to the transfection medium to determine transfection efficiency. Cells were harvested 48 hours after transfection and used in experiments.

Detection of localization of frameshift products. Immunofluorescence was used to detect the localization of the different frameshift products. Cells were grown on Laboratory-Tech 8-well culture slides (Nalge-Nunc) pre coated with poly-L-lysine (Sigma-Aldrich). Two days after transfection, cells were fixed with 1% paraformaldehyde for 5 minutes, permeabilized with methanol at –20°C for 10 minutes and incubated with a mouse monoclonal anti-Flag antibody M2 (F3165, Sigma-Aldrich) for 1 hour at room temperature followed by incubation for 1 hour with Alexa-546-conjugated goat-anti-mouse secondary antibodies (Invitrogen). Stained cells were examined using a laser scanning confocal microscope (LSM510, Zeiss).

Expression of frameshift products. The expression of frameshift products was determined by flow cytometry with the PE-labeled anti-Flag antibody M2 (F3165, Sigma-Aldrich). Briefly, cells were harvested 48 hours after transfection with one of the sequences and in addition of some eGFP to detect transfection efficiency. Cells were fixed in 1% paraformaldehyde for 5 minutes on ice, permeabilized by incubation in methanol for 10 minutes at -20°C and stained with monoclonal anti-Flag antibody M2 (Sigma-Aldrich) followed by PE-conjugated goat-anti-mouse IgG1 polyclonal antibody (PickCell Laboratories BV). Expression was measured on a flowcytometer LSRII (BD Biosciences) equipped with the FACSDIVA software (BD Biosciences). Approximately 10,000 single cell events (as predicted by size) were analyzed per sample. The green (eGFP) and the red (PE) fluorescence were measured using a 530/30 nm and a 575/26 nm band pass filter respectively. Compensation was set using single positive stained controls. Analysis was performed using Winlist 5.2 software (Verity Software House). Expression was found positive when fluorescent signal was shifted to the right side. Experiments were performed in duplicate.

3

Gene	Locus	Mutation incidence (%)			Microsatellite repeat	Function	Mutations studied (length)
		A	B	C			
FTO	U79260	82	6.7	42	14T	Unknown	**-1 (51)**
TGFßRII	M85079	75	63	12	10A	Signal transducer	**-1 (34)**
TAF1B	L39061	75	87	58	11A	Transcription factor	**-1 (25)**
MARCKS	D10522	73	60	25	11A	Motility, phagocytosis, membrane trafficking and mitogenesis	**-1 (11) & -2 (27)**
PCNXL2 / FLJ11383	AK021445	66.0	---	---	10A	Unknown	**-2 (18)**
ACVR2	M93415	62	44	---	8A	Growth factor receptor	**-2 (18)**
C14orf106 / FLJ11186	AK002048	49	---	---	11A	Unknown	**-1 (23) & -2 (7)**
Caspase 5	U28015	46	47	14	10A	Apoptosis	**-1 (25)**
TCF7L2 / TCF-4	Y11306	45	12	3.8	9A	Transcription factor	**-1 (22) & -2 (7)**
Baxα	L22473	43	36	24	8G	Apoptosis	**-1 (18) & +1 (31)**
CDX2	Y13709	1.8	---	---	7G	Proliferation & differentiation	**-2 (29)**

Table 1. Characteristics of frameshift-mutated antigens included in study.

The majority of frameshift-mutated antigens included in this study were selected on basis of high incidence (>40%) in at least one type of cancer and a minimal length of the non-self amino acid stretch, C-terminal of the mutation, of more than 4 residues. Antigen CDX2 was included despite its low mutation incidence, because antibody responses were found against this antigen in a cancer patient (14), indicating that it could be employed as a positive control for a stable, accumulating antigen in our assays.

Names of genes and loci are based on GenBank nomenclature. Incidence of frameshift mutations in colorectal cancer (A), gastric cancer (B) and endometrial cancer (C), and (putative) function are based on previously published data (18;19;22) . The mutations studied are indicated with the length of the foreign amino acid sequences between parentheses. The -1 and -2 mutations were chosen as representatives of the two types of frameshift mutations that can occur.

Direct class I MHC presentation. Hela-Kb cells were plated in a 96-well flat-bottom plate and after overnight incubation transfected. Two days after transfection, B3Z hybridomas were added to the transfected Hela-Kb cells in a final concentration of 50,000 B3Z cells/well in 200 µl. B3Z is a T-cell hybridoma that recognizes the SIINFEKL peptide in the context of H-2Kb and expresses ß-galactosidase (ß-Gal) upon activation (27). After 24h at 37°C, the medium was replaced with 100 µl lysis buffer (PBS, 100 mM 2-mercaptoethanol, 9 mM $MgCl_2$, 0.125% NP-40, and 0.15 mM chlorophenol red-ß-D-galactopyranoside (Calbiochem) per well. Following color change, the absorbance at 590 nm was read using a 96 well plate reader. Per frameshift product 4 wells were transfected and analyzed. Experiments were done in duplo.

Mice. C57BL/6 mice and OT-1 TCR-transgenic mice (specific for OVA257-264/H2-Kb on Rag -/-CD45.1+ background) were bred in our own animal facilities (Leiden, The Netherlands) but were originally obtained from the Jackson Laboratory (Maine, USA). The experiments were approved by the animal experimental committee (UDEC) of Leiden University.

Cross presentation of frameshift products studied in a mouse model. Transfected Hela cells were harvested, lethally irradiated (4000 rad) and washed twice in PBS. C57BL/6 mice (n=3-4) received an intra peritoneal injection of $2*10^6$ irradiated cells suspension or 50 µg OVA peptide. After three days T cells freshly isolated from spleen and lymph nodes of OT-1 TCR-transgenic mice were labelled with 5 µM CFSE (InVitrogen, California, USA) and injected intravenous via the tail vein at a final concentration of $2*10^6$ cells in PBS. After three days mice were sacrificed and mesenteric lymph nodes were isolated. Single cells suspensions of lymph nodes were prepared by mechanical disruption and prepared for flow cytometric detection CFSE intensity of OT-1 cells using PerCP conjugated anti-CD8α (53-6.7), APC conjugated anti-CD45.1 (A20) antibodies. FACS data were analyzed using CellQuest software. All antibodies and analysis software were purchased from BD Pharmingen (New Jersey, USA). Calculations of average fluorescent intensity of groups of mice (n=3-4) and Student's T-test statistical analyses were performed by comparing all groups to the 'mock transfected' group using GraphPad software (GraphPad software Inc.).

Results

Validation of a fusion gene expression system for assessment of immunogenic properties of potential tumor antigens
For many of the genes that are frequently mutated in MSI-H cancers no information is available with respect to the immunogenic properties of these proteins. Because no knowledge on T cell epitopes, nor specific T cell clones for *in vitro* tests were at hand to enable analysis of the expression and immunogenicity of the individual antigens, we designed a gene expression system in which each of these proteins can be fused to a short C-terminal polypeptide comprising sequences that can readily be detected by T cells and antibodies respectively: the H-2Kb-restricted CD8+ T-cell epitope of chicken ovalbumin (OVA; SIINFEKL, 28) and the Flag tag epitope (DYKDDDDK, Figure 1A, 29). To validate this screening methodology, we chose to insert the coding sequences of wild-type (wt) and mutated (V143A) p53 into the expression vector, as these constitute prime examples of proteins (in fact, versions of the same protein) with very different expression characteristics (30). Whereas wt.p53 has a very short half-life, resulting in very low nuclear expression levels, mutations in p53 result
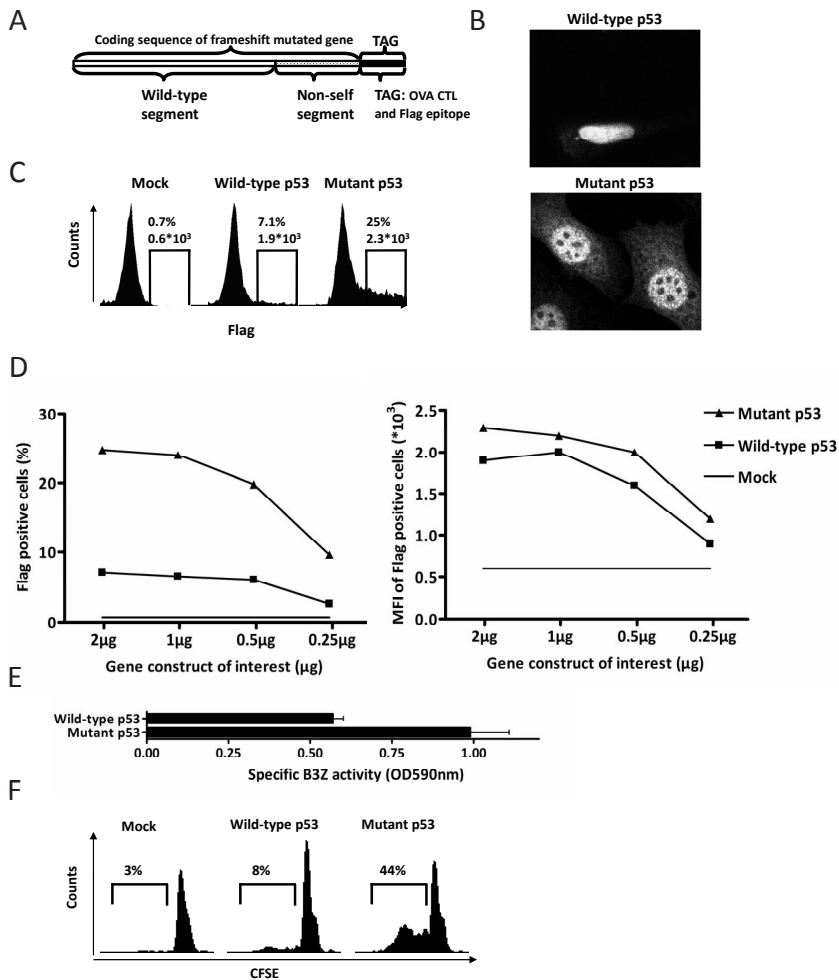
Figure 1. Design of screening methodology and validation of assays on basis of characteristics wild-type and mutant p53.

A) Full-length coding sequences of frameshift-mutated genes that lack stop codons were fused in frame to a sequence encoding the H-2Kb-restricted CD8+ T-cell epitope of chicken ovalbumin (OVA; SIINFEKL, 28) and the FLAG tag epitope (DYKDDDDK, 29), which were spaced by a triple alanine sequence to allow efficient processing of the OVA T-cell epitope (40). B) Detection of tagged fusion gene products comprising wild-type (wt) and mutant (V143A) human p53 by means of immunofluorescence in paraformaldehyde-fixed Hela cells that were transfected with the indicated constructs 48 hrs. prior to analysis. C) Detection of p53 antigen by flow cytometry in wt.p53, V143A p53 and mock transfected Hela cells. Percentage of cells with FLAG-tag-specific staining above background and mean fluorescence intensity (MFI) of positive fraction are indicated. D) Percentage of positive cells of positive fraction in relation to amount of DNA transfected is plotted. Comparable transfection efficiency between samples was ensured by co-transfection of DNA encoding eGFP. E) Direct MHC class I-restricted presentation of the OVA (SIINFEKL) epitope in fusion-gene transfected Hela-Kb cells, as measured by reactivity of B3Z T-cell hybridoma cells. Magnitude of response is measured on base of β-galactisodase activity in stimulated B3Z cells and expressed as absorbance (OD 590nm) of converted substrate in quadruplicate samples. F) *In vivo* cross-presentation of the OVA epitope derived from fusion-gene transfected Hela cells (lacking H-2Kb expression) cells, as determined by proliferation of SIINFEKL-specific OT-1 TCR-transgenic CD8+ T cells. Hela cells transfected with wt.p53, V143A p53 or control DNA were injected in mice that received naïve, CFSE-labeled OT-1 cells. Proliferation of OT-1 on basis of dilution of CSFE was assessed three days later. Percentage of divided CFSE cells is indicated.

in accumulation of this protein in both nucleus and cytoplasm of the cell. Furthermore, the degradation of p53 through the ubiquitin/proteasome pathway can result in efficient presentation of peptides derived thereof into MHC class I, even at the surface of cells that do not exhibit detectable intracellular levels of this protein (31). In accordance with the above, cells transiently transfected with the mutated p53 gene construct displayed high levels of p53 that are detected in nucleus and cytoplasm, while wt.p53-transfected cells displayed exclusive nuclear localization of this antigen (Figure 1B). Flow cytometry, used to further quantify the level of expression, confirmed that mutated p53 accumulated in a major fraction of the transfected cells. Transfection of wt.p53 resulted in positive staining in a considerably smaller fraction of cells, even though transfection efficiencies as determined by co-transfection of an eGFP-encoding gene construct were comparable (Figure 1C). The high levels of antigen detected in a minor fraction of the wt.p53 transfected cells (Figure 1B, C) is probably due to the uptake of massive amounts of DNA by the cells concerned and the very high synthesis of the protein resulting from this. As shown in figure 1D, optimal distinction between accumulation of wt and mutated p53 is obtained when cells are transfected with 1-2 μg of p53-encoding DNA.

To evaluate processing and presentation of p53 degradation products into MHC class I, the recognition of the transfected cells by OVA-specific T-cell hybridoma cells (B3Z) was evaluated. Because the octamer peptide recognized by this T-cell constitutes only a very small part of the fusion proteins tested, we deem it highly conceivable that the behavior of the OVA T-cell epitope in the context of the fusion protein reflects that of putative T-cell epitopes within the sequences of the wt and mutated p53 sequences. In concordance with its accumulation in a large fraction of transfected cells, expression of the fusion protein comprising mutated p53 resulted in efficient presentation of the OVA T-cell epitope in MHC class I (Figure 1E). However, also transfection of the fusion gene comprising wt.p53 resulted in clearly detectable epitope presentation. This is in line with our previous observation that accumulation of p53 is not required for direct MHC-restricted presentation of CTL epitopes derived from this antigen (31).

Cross-presentation of cell-derived antigens by professional APC does depend on the availability of these antigens in sufficient amounts (23-25). To test whether our fusion gene expression system can also be used to address the behavior of antigens in cross-presentation, we transfected human cells with genes comprising wt or mutated p53 and injected these xenogeneic cells into mice that were infused with naive, CSFE-labeled T cells (OT-1) that recognize the OVA CTL epitope. The use of this xenogeneic system is justified by the notion that the antigen processing machinery is highly conserved between mouse and human (32). Figure 1F shows that *in vivo* challenge of OT-1 through injection of cells transfected with a fusion gene comprising mutated p53 elicited strong proliferation of OT-1, while injection of cells transfected with the wt.p53 gene construct induced only modest OT-1 proliferation.

In conclusion, the wt.p53 OVA/FLAG fusion construct encoded a protein with short half-life that failed to accumulate in the majority of transfected cells and was therefore not available for efficient uptake and cross-processing by professional APC, while the turn over of this protein nevertheless resulted in direct MHC class I-restricted presentation by the antigen-expressing cell. In contrast, the OVA/FLAG fusion gene comprising mutated p53 encoded a stable protein that accumulated in transfected cells, and that could serve as a basis for both direct and cross-presentation. Taken together these results demonstrate that the behavior of these fusion proteins properly reflects that of the antigens under examination, and that our expression system is a suitable tool for evaluating the characteristics of
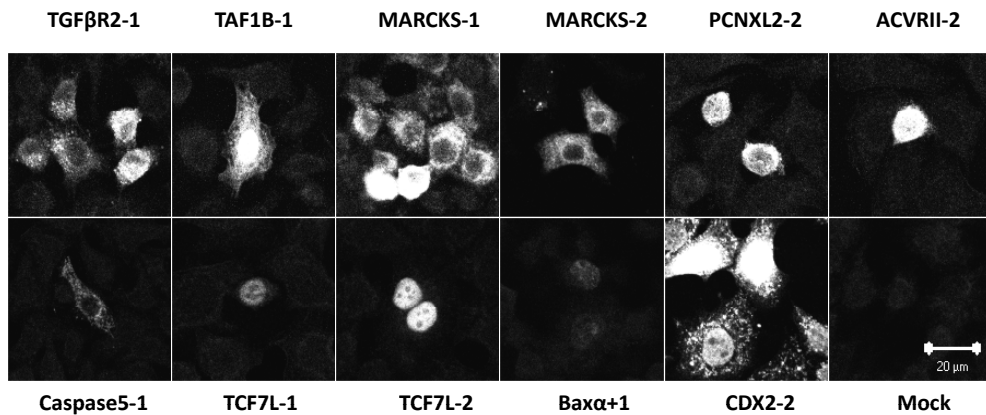
Figure 2. Steady state expression of frameshift antigens.

Detection of tagged fusion gene products by means of immunocytochemistry in fixed Hela cells, transfected with the indicated constructs 48 hrs. prior to analysis. Cells shown in pictures are representative for positive cells obtained after transfection with indicated gene construct. Frequency of positively staining cells depends on gene construct used (see Figure 3C). Images were made under standardized conditions, using 40x magnification and the same exposure.

additional antigens, such as those encoded by the frameshift mutated genes commonly found in MSI-H cancers.

Steady-state expression levels of frameshift-mutated antigens

After validation of our expression system, we cloned multiple frameshift-mutated genes into the OVA/FLAG gene cassette. All but one of genes to be examined in our proof of concept study were selected on basis of two criteria. First the high prevalence of a given frameshift mutation in MSI-H cancers as reported in previous studies, in particular a reported frequency of at least 40% in at least one type of MSI-H cancer. Second, we focused on frameshift mutations that gave rise to foreign sequences, downstream of the frameshift mutation, of at least 5 amino acids in length, because shorter sequences are unlikely to render immunogenic T-cell epitopes. An overview of the selected frameshift-mutated genes is shown in Table 1. The mutated CDX2 gene was included in our studies despite its low incidence, because antibody responses were found against its gene product in a cancer patient (14), arguing that this antigen could be used in our screening as a positive control for a stable, accumulating antigen.

Steady-state expression levels and intracellular localization of the selected frameshift product were initially determined by performing immunocytochemistry on transiently transfected cells, using the FLAG tag-specific antibody. The resulting data showed that only four of the gene constructs (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2) gave rise to high protein levels in a large fraction of the cells (Figure 2). Expression of the different frameshift proteins was further quantified by means of flow cytometry. These experiments confirmed that only 4 of the gene constructs tested (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2) encoded antigens that accumulated in a large fraction of the transfected cells (Figure 3A, B). Five additional gene products (TAF1B-1; PCNXL2-2; TCF7L2-1; -2; Baxα+1) were found expressed in a modest fraction of transfected cells, while expression of the remaining genes
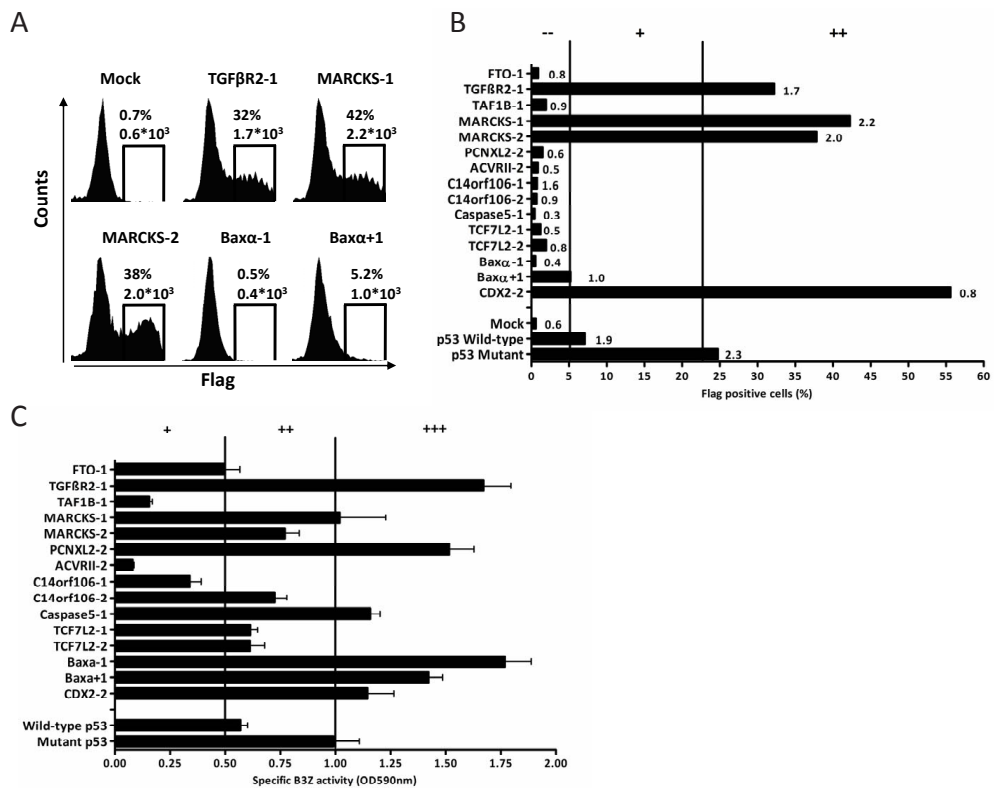
Figure 3. Quantitative evaluation of steady state expression and direct processing into MHC class I.

A) Flow cytometric analyses of Hela cells transfected with 2 μg gene constructs encoding the indicated frameshift proteins. Results for several of the proteins tested are shown. Percentages of cells with FLAG-tag-specific staining and MFI of positive fraction are indicated. B) Summary of the flow cytometric analyses for all antigens tested. The bars represent percentages of Flag positive cells. Number behind each bar indicates the MFI of the Flag-positive cells. These analyses were performed twice with very similar outcome; data from one experiment are shown. Expression of frameshift products is categorized as strong (+++, > mutant p53), moderate (++, > wt.p53 - < mutant p53), low (+, > Mock - < wt.p53) and negative (-, - < Mock). Comparable transfection efficiency between samples was ensured by co-transfection of DNA encoding eGFP (not shown). C) Overview of direct MHC class I-restricted presentation of the OVA SIINFEKL epitope in fusion-gene transfected Hela-Kb cells, as measured by reactivity of B3Z T-cell hybridoma cells. B3Z responses against transfected antigens are categorized as strong (+++, >1.0), moderate (++, 0.5-1.0) and low (+, <0.5). Error bars indicate SD.

did not surpass that of background levels. Transfection efficiencies were comparable for all assays, as determined by co-transfection of an eGFP-encoding gene construct.

Direct antigen processing and presentation into MHC class I

As shown for wt.p53, failure of antigens to accumulate does not necessarily preclude processing of peptides derived thereof into MHC class I (Figure 1E). Accordingly, recognition of the OVA CTL epitope by the B3Z hybridoma on transfected cells was not limited to the 4 gene constructs that encode stable antigens (Figure 3B). In addition, efficient CTL epitope recognition was observed for cells transfected with gene constructs encoding PCNXL2-2, Caspase5-1, Baxα-1 and Baxα+1, while transfection of four other constructs (FTO-1,
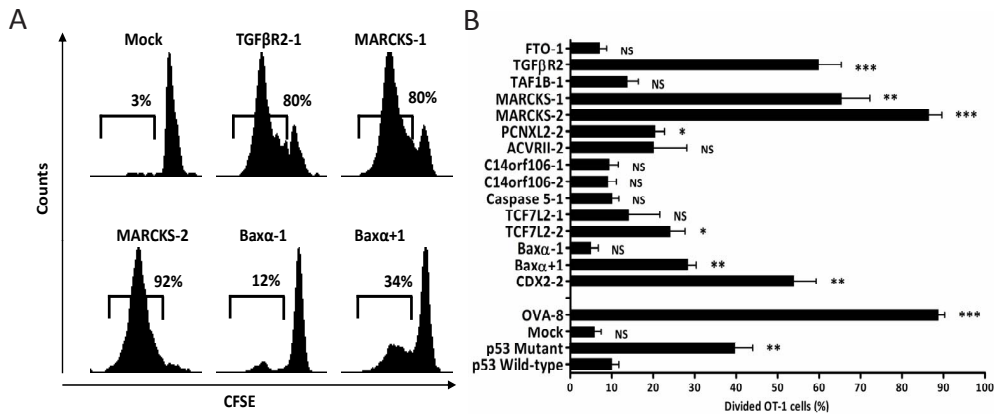
Figure 4. *In vivo* processing of frameshift mutated antigens.

A) Hela cells transfected with indicated frameshift gene constructs were injected in mice that received naïve, CFSE-labeled OT-1 cells. Proliferation of OT-1 on basis of dilution of CSFE was assessed three days later. Percentage of divided CFSE cells is indicated in each graph (examples shown). B) Summary of the outcome of *in vivo* cross-presentation experiments for all gene constructs studied. Dashed line indicates mean percentage of CFSE-low OT-1 cells in mice injected with mock-transfected Hela cells plus the SEM of these values. T-test was used to calculate significant difference from mock transfected cells. Samples are categorized as strong (***, p<0.001), moderate (**, p<0.01-p>0.001), low (*, p<0.05-p>0.01) and not significant different from mock (NS, p>0.05).

C14orfl06-2, TCF7L2-1 and TCF7L2-2) resulted in moderate T-cell recognition (Figure 3C). These results confirm that accumulation of protein is not a prerequisite for direct processing and presentation of epitopes into MHC class I.

In vivo cross-presentation of antigen-derived epitopes
Whereas T-cell action at the effector level requires direct presentation of epitopes by the target cell, the prevailing view on priming of T-cell responses is that successful activation of T-cell immunity requires uptake, processing and presentation of antigen by dendritic cells. We therefore analyzed the capacity of the frameshift-mutated antigens to give rise to cross-presented T-cell epitopes by immunizing mice with transiently transfected xenogeneic (human) cells. Presentation of the OVA CTL epitope was monitored by analyzing the antigen-specific proliferation of CSFE-loaded OT-1 cells that were infused into the mice. The capacity of gene constructs to give rise to *in vivo* cross-presentation of the OVA epitope correlated with the accumulation of the corresponding antigen in transfected cells (Figure 4A,B). Most efficient cross-presentation was restricted to the four gene constructs that gave rise to accumulating antigen in a large fraction of transfected cells (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2). Four of the five gene constructs that gave rise to protein accumulation in a modest fraction of transfected cells (Figure 3B), resulted in low but detectable levels of cross-presentation, (BAXα+1, PCNXL2-2, TCF7L2-2 and TAF1B-1). No *in vivo* cross-presentation above background was found for the fifth of this set of genes (TCF7L2-1). This can most likely be explained by our finding that protein accumulation in transfected cells for this gene was lower than for the other four genes (Figure 3B).

	Coding sequence and type of mutation	Experimental data			Predicted immunogenic profile	
		Accumulation	Presentation in MHC class I	Cross-presentation	Tumor induced immunity	Vaccine component
1	**TGFßR2-1**	+++	+++	+++		
2	**MARCKS-1**	+++	+++	+++	CD4 & CD8	CD4 & CD8
3	**MARCKS-2**	+++	++	+++		
4	**CDX2-2**	+++	+++	+++		
5	**Baxα+1**	+	+++	+++		
6	**TCF7L2-2**	+	++	+++	Possibly	
7	**PCNXL2-2**	+	+++	++	CD4 & CD8	(CD4 &) CD8
8	**TAF1B-1**	+	+	+		
9	Caspase5-1	-	+++	-		
10	**Baxα-1**	-	+++	-		
11	**FTO-1**	-	++	-		
12	**C14orf106-2**	-	++	-	-	CD8
13	**TCF7L2-1**	+	++	-		
14	**ACVR2-2**	-	+	-		
15	**C14orf106-1**	-	+	-		

Table 2. Summary of expression characteristics of frameshift-mutated antigens and their expected relevance for evaluation of spontaneous T-cell immunity and vaccine design.

The results of the experiments concerning steady state protein expression (Figure 3B), direct presentation in MHC class I (Figure 3C) and *in vivo* cross-presentation (Figure 4B) have been quantitated as described in the figures. On basis of these data, the top 8 antigens, in particular (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2), are of interest with respect to evaluation of anti-tumor immunity in non-vaccinated patients, as well as for inclusion into vaccines that elicit potentially effective anti-tumor immunity through both CD8+ and CD4+ T-cell subsets.

Discussion

We performed a series of experiments aimed at predicting the immunogenic behavior of proteins encoded by a selection of frameshift-mutated genes that are frequently found in MSI-H cancers. Our data demonstrate that fusion proteins comprising eight of the antigens examined (TGFβR2-1; MARCKS-1; -MARCKS-2; CDX2-2; BAXα+1, PCNXL2-2, TCF7L2-2 and TAF1B-1) gave rise to direct epitope presentation by the cell expressing the antigen, as well as to cross-presentation by DCs. (Table 2). Our proof of concept study provides important guidelines for further research concerning this antigen family in the context of cancer immunotherapy. The antigens that, in view of their capacity to accumulate in tumor cells, are cross-presented by DCs, can become visible to naïve T cells in the tumor-draining lymphoid tissues. Consequently, one can expect cancer patients to display 'tumor-induced' T-cell or IgG-type immunity against these antigens. Indeed, such responses have been found against the frameshift-mutated TGFβR2-1 and CDX2-2 proteins (13;14;33). Our results argue that further evaluation of spontaneous immunity against frameshift-mutated antigens should be focused on the eight antigens mentioned above and on defining additional antigens with similar characteristics. Moreover, our data indicate that antigens belonging to this subclass are most valuable as components of vaccines that are intended to raise anti-tumor immunity by both CD4+ and CD8+ T-cell subsets. Vaccination-induced CD4+ T cells can only provide efficient 'help' to the immune response if they encounter their cognate antigen on cross-presenting DCs in the tumor microenvironment and/or draining lymphoid tissue. In addition, they may contribute to the effector response by recognizing their antigen on MHC class II-expressing colorectal tumors (34-36). On basis of our experiments with fusion

gene constructs, such class II MHC-restricted presentation by professional APCs or by tumor cells, the latter of which lack efficient MHC class II processing, is less likely to occur for the seven remaining, non-accumulating antigens (Table 2). Therefore, the impact of CD4+ T cells raised by vaccination against these latter antigens will be limited to the vaccination phase. Due to the lack of their cognate antigen in the peritumoral area, these CD4+ T cells will fail to contribute to the effector phase. For the latter antigens (FTO-1; ACVR2-2; C14orf106-1; -2; Caspase5-1; TCF7L2-1; Baxα-1), breakdown did result in class I MHC-restricted epitope presentation by the antigen-expressing cells, indicating that the CD8+ T-cell arm of a vaccine-induced response against these antigens could be used to target tumors (Table 2). Whether MHC class I-restricted epitope presentation by the tumor truly occurs in a given human subject will, of course, depend on the compatibility between the proteolytic fragments generated and the repertoire of class I molecules comprised within the subject's HLA-type. The available HLA-specific peptide-binding motifs can be used for further prediction of such epitopes within the antigens concerned.

The degree at which the fusion proteins comprising frameshift-mutated antigens accumulate in transfected cells correlates well with the efficiency by which these antigens give rise to *in vivo* cross-presentation after injection of transfected cells (compare Figures 3B and 4B). Protein accumulation and cross-presentation are high for fusion proteins containing TGFβR2-1, MARCKS-1, -MARCKS-2 or CDX2-2, while being modest for fusion proteins comprising BAXα+1, PCNXL2-2, TCF7L2-2 or TAF1B-1. Notably, the fusion protein comprising wt.p53, although accumulating at considerably higher efficiency than the latter four fusion constructs, did not give rise to detectable cross-presentation (Figure 4B). This suggests that factors other than steady-state levels, such as efficiency of antigen uptake or intracellular routing after uptake, may impact on the handling of antigens by cross-presenting DCs. We would like to emphasize that the methodology described in our present paper should be regarded as part of a multifaceted selection procedure that also involves evaluation of the immunogenicity of these antigens by means of *in vitro* human T-cell cultures using lymphocytes from MSI-H cancer patients and healthy subjects. Furthermore, the potential impact of frameshift mutations on mRNA stability through nonsense-mediated RNA decay (NMD) should be taken into account, as this might preclude efficient synthesis of gene products (37). NMD was reported not to impact on 7 of the 8 frameshift mutations that we found to encode accumulating gene products (38;39). Whether NMD affects expression of the remaining gene product (PCNXL2-2) still needs to be determined.

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3