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Immunogenic peptides generated by frameshift mutations in DNA mismatch repair-deficient cancer cells

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About 15% of all human colorectal, gastric, and endometrial tumors, and the majority of tumors in patients suffering from hereditary nonpolyposis colorectal cancer syndrome, are caused by loss of DNA mismatch repair functions. In the affected cancer cells, this results in insertion or deletion mutations at short, repetitive DNA sequences referred to as microsatellites. Such mutations in coding microsatellites (cMS) cause translational frameshifts that may destroy gene function. These frameshift mutations could also cause the translation of immunogenic neopeptides at the carboxy terminus. Several such mutations have been identified recently. However, since none of the frameshift-induced neopeptides identified so far is generated in all cancer cells with microsatellite instability (MSI), we aim to define a broad but comprehensive set of frameshift peptides (FSPs) that might be combined in a multivalent vaccine for MSI+ cancers. Here, we characterize the immunogenic properties of five additional HLA-A0201-restricted frameshift-induced neopeptides derived from mutations in three cMS-containing genes (*Caspase-5, TAF-1b,* and *HT001*) that are frequently hit in MSI+ cancer cells. One Caspase-5-derived FSP, ^{67}-FLIIWQNTM (FSP26), was identified as a novel HLA-A0201-restricted CTL epitope. FSP26-specific CTLs efficiently lysed colon carcinoma cells expressing HLA-A0201 and the underlying (-1) mutation. This mutation in an A(10) cMS is observed in up to 66% of MSI+ colorectal cancers. Thus, this newly identified CTL epitope may be another essential component of a multivalent vaccine against cancers with MSI.

Introduction

MSI caused by functional loss of the cellular DNA mismatch repair system leads to the accumulation of insertion or deletion mutations at short, repetitive DNA sequences. These repeated sequences are commonly referred to as microsatellites (1, 2, 3). MSI occurs in about 90% of hereditary nonpolyposis colorectal cancers and in about 10-15% of sporadic colorectal, gastric, and endometrial carcinomas (4). Several genes encompassing cMS are frequently affected by MSI. Among these are genes that encode proteins involved in signal transduction (*TGF-betaRII, IGFIIR*), DNA repair (*MSH3, MSH6*), apoptosis (*BAX*), inflammation (*Caspase-5*), transcriptional regulation (TCF-4), immune surveillance (*beta2m*), and protein translocation and modification (*Sec-63, OGT*), as summarized in Woerner *et al.* (5). It is commonly accepted that MSI-induced frameshift mutations in some of these genes contribute to the neoplastic transformation of the cells involved (6, 7). These mutations usually result in premature translation termination of the mutant gene products. However, these proteins, in general, encompass frameshift-induced neopeptides at their carboxy terminus downstream of the mutant cMS. These represent a novel class of tumor-associated antigens that can be presented to the immune system. This concept fits well with the clinical observation that patients with sporadic, as well as hereditary, colorectal MSI+ tumors have a better prognosis than those with microsatellite stable tumors (8). Moreover, MSI+ colorectal tumors often exhibit a significantly higher percentage of apoptotic tumor cells and strong infiltration with activated CTLs (9, 10). These observations point to an ongoing, active antitumoral immune response toward specific antigens generated in MSI+ carcinomas.

Recently, two HLA-A0201-restricted CTL epitopes generated by (-1) frameshift mutations in the *TGF-betaRII* (11, 12) and OGT (13) genes were identified. These FSPs were capable of inducing the expansion of effector T cells and also conferred sensitivity to cytolysis by CD8+ T cells when endogenously expressed and presented on MSI+ colon carcinoma cells (11, 12, 13).

Although some of these antigens are generated with high frequency in MSI+ cancer cells, none of them is present in all of the cells. In order to develop a potential vaccine for the prevention or treatment of MSI+ cancers, it seems reasonable to combine multiple epitopes derived from several such antigens. This combinatorial approach should cover at least one or two FSPs presented by a given MSI+ cancer cell.

Therefore, we aim to further characterize a comprehensive set of FSPs generated in MSI+ cancer cells. In the present study, we analyzed the immunological properties of five HLA-A0201-restricted FSPs derived from cMS mutations in the *Caspase-5, TAF-1b,* and *HT001* genes. All show high cMS mutation frequencies in MSI+ colorectal carcinomas, are predicted to be Real Common Target genes (5, 14), and have cMS mutations that lead to long FSP tails. Using *in vitro* T cell priming in combination with ELISPOT analysis and functional cytotoxicity assays, we identified a new HLA-A0201-restricted CTL epitope (^{67}-FLIIWQNTM; FSP26) originating from *Caspase-5(-1)*. These data further confirm the idea that FSPs generated by predictable mutations in cMS-containing genes are an attractive source of novel tumor-specific antigens expressed and presented by cancer cells with deficient mismatch repair systems.

Table 1
Frameshift and control peptides used in this study

Protein		Peptide		Theoretical Score[c]		Fluorescence
Name	Accession no.[a]	Name	Sequence[b]	Ken Parker	SYFPEITHI	index[d]
Growth regulated protein P68	226021	P68	^{128}-YLLPAIVHI	551	30	2.96
Caspase-5(-1)	U28015	FSP25	^{61}-KMFFMVFLI	1301	20	1.29
		FSP26	^{67}-FLIIWQNTM	22.8	21	3.24
TAF-1b(-1)	L39061	FSP27	^{108}-GMCVKVSSI	17	24	0.15
		FSP29	^{105}-AGIGMCVKV	1	20	0.01
HT001(-1)	NP 054784	FSP30	^{281}-VLRTEGEPL	n.d.	21	0.01

[a] Protein or nuleotide accession numbers are indicated.
[b] Position of the start amino acid in the protein is indicated.
[c] Predicted binding score to HLA-A0201 using computer-assisted analysis.
[d] Index = (mean fluorescence with peptide - mean fluorescence without peptide) / (mean fluorescence without peptide)

Results

Identification of HLA-A0201-restricted peptides

In our previous work we performed a genome-wide screen for potential target genes in MSI+ cancer cells (14) and developed a mathematical model to classify potential target sequences that are subject to a high mutation frequency in MSI+ cancers (5). These analyses revealed that, among others, the *Caspase-5, TAF-1b,* and *HT001* genes are true mutational targets of MSI+ carcinogenesis. Neopeptides generated by the respective frameshift mutations within these genes might therefore be attractive, novel tumor-associated antigens. To prove whether these FSPs are potentially immunogenic, we selected HLA-A0201-restricted peptides on the basis of two peptide-motif scoring systems that are described in detail in the Material and Methods (Table 1). For further analysis we included five FSPs with a predicted binding score of >100 for the Parker algorithm (15) and >19 for the SYFPEITHI algorithm (16).

In order to confirm the predicted HLA-A0201 binding properties, we performed a T2 stabilization assay (17) with the five selected FSPs and included the growth-regulated protein P68 as a positive control (Table 1). FSP26, one of the *Caspase-5(-1)*-derived peptides we investigated, was generated by a frameshift mutation in a coding A(10) repeat. It gave rise to optimal stabilization of HLA-A0201 and thus qualified as a strong binder. The second *Caspase-5(-1)*-derived peptide, FSP25, exhibited moderate HLA-A0201 stabilization. Analysis of the peptides originating from a (-1) frameshift mutation in a coding A(11) repeat of *TAF-1b* revealed that FSP27 stabilized HLA-A0201 molecules weakly. In contrast, the remaining two peptides, FSP29 (from *TAF-1b*) and FSP30, arose from a (-1) frameshift mutation in a coding A(11) repeat of *HT001* and did not confer HLA-A0201 stabilization.

Induction of FSP-specific cytotoxic T cells

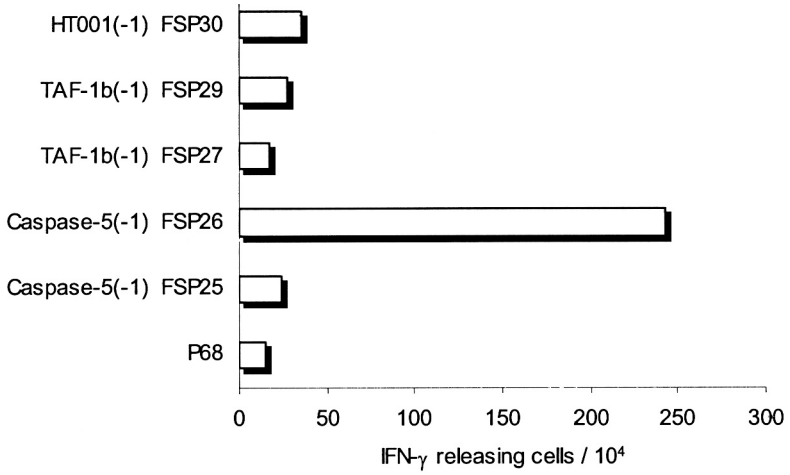
Published data suggest a direct relationship between the MHC class I binding affinity and the immunogenicity of potential cytotoxic T cell epitopes (18, 19). However, we recently identified another frameshift-derived T cell epitope originating from a (-1) mutation in the OGT gene that displays only intermediate MHC class I binding affinity (13). In order not to miss a potential CTL epitope, all five FSPs, which had been selected according to their theoretical HLA-A0201 binding scores (Table 1) were tested for their ability to induce FSP-specific cytotoxic T cells *in vitro*. To this end, we generated CD40-activated B cells (CD40Bs) from the enriched B cells of a healthy HLA-A0201+ donor, using an *in vitro* CD40L culture system (11, 20), to be used as highly efficient antigen-presenting cells. These CD40Bs were loaded with the five FSPs selected and used for the repetitive stimulation of autologous CD3+ T cells. After 35 d in culture, T cell numbers had increased 17-fold. Stimulation for more than 3 mo resulted in a 3 x 10^4-fold T cell expansion. Detailed characterization of proliferating T cells was performed by flow cytometry analysis of cell surface markers, as well as of intracellular molecules (Table 2). Almost all T cells expressed CD2 and CD3, and consisted of 27.8% CD4+ and 67.5% CD8+ cells at day 35 of culture. Further restimulations reduced the CD4+ population to less than 1%, while the CD8+ fraction increased up to 97.9% at day 98 of culture. The majority of T cells (>80%) expressed the costimulatory molecule CD28, the activation markers CD25, CD69, and CD71, the T cell integrin CD49d, and the intercellular adhesion molecule CD50. They also displayed a memory phenotype (CD45RO+). Intracellular analysis of cytokines revealed that a significant part of the T cell population expressed IFN-gamma (94.4%) and more than 40% were IL-2+, but that only a minor fraction of cells showed TNF-alpha expression (9.9%). Almost all T cells produced granzyme B (98.1%), and about 16% produced perforin. These data demonstrate that highly activated CD8+ T cells were generated.

Table 2
FACS® analysis of the T cell bulk culture

Antigen	Positive cells (%)
CD2[a]	98.9
CD3[a]	99.0
CD4[a/c]	27.8 / 0.9
CD8[a/c]	67.5 / 97.9
CD28[a]	82.0
CD45RO[a]	93.5
CD49d[a]	85.1
CD50[a]	99.1
CD25[a/c]	18.6 / 90.8
CD69[a/c]	31.1 / 89.1
CD71[a/c]	92.9 / 97.8
IFN-g[b]	93.4
TNF-a[b]	9.9
IL-2[b]	44.0
Perforin[b]	15.9
Granzyme B[b]	98.1

[a] Day 35 of T cell culture, extracellular staining.
[b] Day 84 of T cell culture, intracelluar staining.
[c] Day 98 of T cell culture, extracelluar staining.

Figure 1



ELISPOT analysis of FSP-specific T cells. T cells (1 x 10^4 cells/well) were stimulated with 3.5 x 10^4 T2 cells pulsed either with the FSP or the control peptide P68 as targets. The number of IFN-gamma-releasing activated T cells (spots) for the total number of cells analyzed (10^4) is given. Reactivity against P68 is shown as a measure of background IFN-gamma release.

We next examined peptide specificity and quantified the reactive cells in the expanded T cell population by determining the number of IFN-gamma-secreting T cells after stimulation with the cognate FSP in ELISPOT assays. On day 35 of culture, we observed low reactivity of T cells against the *TAF-1b(-1)* FSP27 or *HT001(-1)* FSP30 peptide, but a very strong response directed against the *Caspase-5(-1)* FSP26 peptide (data not shown). There was no detectable recognition of *Caspase-5(-1)* FSP25 and *TAF-1b(-1)* FSP29, thus these T cells were excluded from further analysis. Five weeks later, the expanded T cells remained strongly reactive against FSP26, and consisted of about 2.5% IFN-gamma-releasing cells (Figure 1). These results demonstrate that FSP26 can induce a strong T cell response *in vitro* and thus correlate very well with its observed potential to stabilize HLA-A0201 molecules (Table 1).

FSP26-specific and HLA-A0201-restricted recognition of target cells

In line with these data, subsequent cytotoxicity assays using peptide-loaded T2 target cells revealed high specific reactivity of the established CTLs against the *Caspase-5(-1)* FSP26 peptide, whereas T2 cells presenting the control P68 peptide were rarely lysed (Figure 2A). However, we could not detect any specific lytic activity of T cells raised against *TAF-1b(-1)* FSP27 or *HT001(-1)* FSP30 toward T2 targets exogenously loaded with cognate peptides. Therefore, T cells raised against these peptides were not investigated further. The observed reactivity against FSP26 was not confined to peptide-loaded T2 cells. More importantly, the FSP26-specific CTL bulk culture specifically recognized the MSI+ colon carcinoma cell lines LoVo, Colo60H, and HCT116 which endogenously express this *Caspase-5(-1)* frameshift mutation (14). Accordingly, FSP26-specific CTLs were able to lyse the stably HLA-A0201-transfected LoVo cells LoVo-A2 (13), but not the parental LoVo cells (Figure 2B), demonstrating HLA-A0201-restricted target cell recognition. In addition, the established FSP26-specific CTL population could also effectively lyse the Caspase-5(1)-displaying cell line Colo60H, whereas the microsatellite stable cell lines SW707 and SW480, which express *Caspase-5(wt)*, remained unaffected. Unspecific recognition of HLA-A0201 by FSP26-stimulated CTLs can be excluded since both SW707 and SW480 exhibit a stronger HLA-A0201 expression than HCT116, Colo60H, and LoVo-A2 as determined by FACS® analysis (data not shown). The observed cytotoxicity was not mediated by NK cells because of the lack of reactivity against the erythroleukemia cell line K562 (Figure 2C).

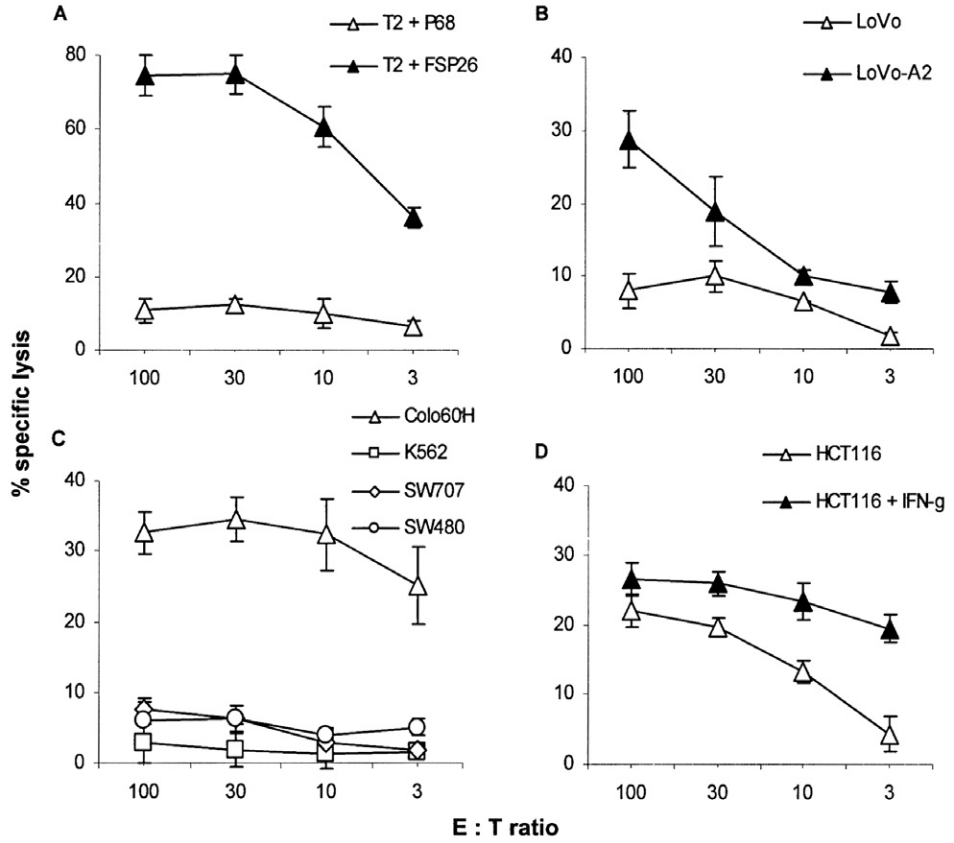
Since overnight treatment of HCT116 colon carcinoma cells with IFN-gamma results in a 3- to 6-fold upregulation of HLA-A2 expression and a 3-fold increase of MHCI expression as determined by flow cytometry (data not shown), we investigated whether IFN-gamma could enhance the susceptibility of HCT116 cells to recognition and lysis by peptide-specific CTLs. As shown in Figure 2D, the lytic potential of the expanded FSP26-specific CTL bulk culture was increased by IFN-gamma pretreatment of the HCT116 cells.

Overall, these results indicate both the presence of the *Caspase-5(-1)* frameshift epitope FSP26 on MSI+ tumor target cells and the HLA-A0201-restriction of the established FSP26-specific CTL population.

Lysis of colorectal tumor cell lines by cytotoxic T cell clones

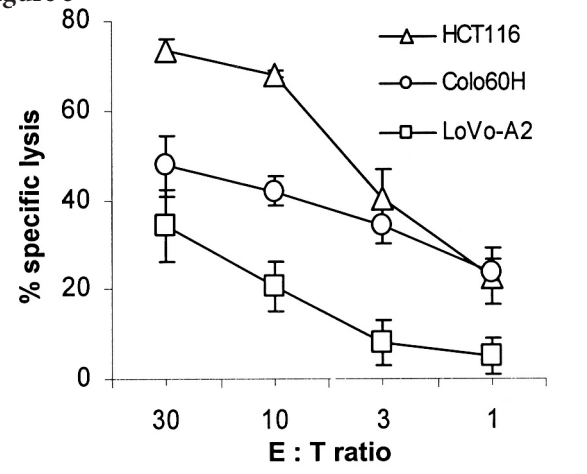
To examine the observed *Caspase-5(-1)* FSP-specific reactivity at the clonal level, CTL clones were generated by limiting dilution of the FSP26-specific CTL bulk culture. After 6 restimulations with FSP26-loaded irradiated autologous CD40Bs, more than 80 outgrowing T cell clones were analyzed for their lytic potential. In initial cytotoxicity experiments, 38 CTL clones were able to lyse FSP26-loaded T2 targets with up to 94% effectiveness (data not shown). Subsequently, we investigated the lytic activity of these FSP26-specific clones toward HLA-A0201+ colon carcinoma cell lines that express the *Caspase-5(-1)* frameshift mutation. In chromium release assays, 16 CTL clones specifically killed HCT116 cells. Of those, five clones were also reactive toward Colo60H targets, and three also lysed LoVo-A2 cells. The cytotoxic potential of one representative FSP26-specific CTL clone (clone 1) against these colon carcinoma cell lines is shown in Figure 3. Moreover, lysis of radiolabeled HCT116 targets was substantially inhibited by adding an excess of cold FSP26-loaded T2 cells, as compared to cytotoxic reactivity without cold target blocking. In contrast, cold T2 cells loaded with the control peptide P68 had only a minor effect on target cell killing (Figure 4)

Figure 2



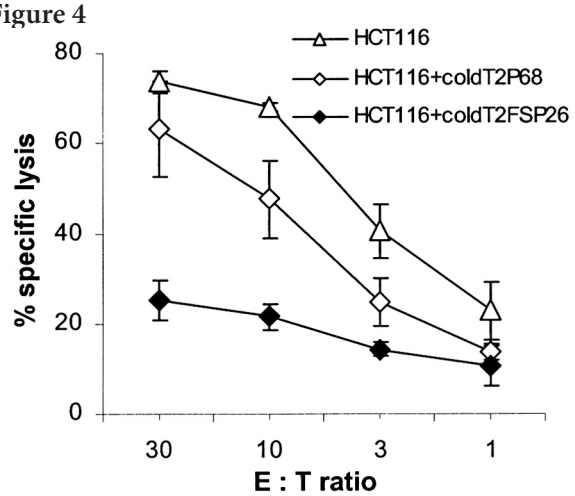
Cytotoxic reactivity of the FSP26-specific T cell bulk culture toward tumor cell targets. (A) Specific lytic activity against T2 cells loaded with FSP26, derived from the *Caspase-5(-1)* frameshift mutation, as compared to cells presenting the control peptide P68. (B) HLA-A0201-restricted killing of LoVo-A2 (HLA-A0201-transfected LoVo cells) endogenously expressing the *Caspase-5(-1)* FSP is compared to the HLA-A0201-negative parental cell line. (C) Specific lysis of the *Caspase-5(-1)*-expressing, HLA-A0201+ Colo60H cell line is shown in contrast to that of the *Caspase-5(wt)*-expressing, HLA-A0201+ control cell lines SW707 and SW480. The lack of reactivity against the NK sensitive cell line K562 excludes NK cell activity. (D) Killing efficiency against the HCT116 colon carcinoma cell line [HLA-A0201+, *Caspase-5(-1)*] in the presence or absence of IFN-gamma (200 U/ml, overnight). Target cells were labeled with ^{51}Cr-sodium chromate for 1 h. Different effector-to-target cell ratios (E:T ratios) are shown. All results are displayed as the mean and standard deviation from three replicate wells.

Figure 3



Lytic potential of FSP26-specific CTL clone 1. HCT116, Colo60H, and LoVo-A2 cells endogenously expressing *Caspase-5(-1)* were efficiently killed by FSP26-specific CTL clone 1. Killing of targets is shown at different effector-to-target cell ratios (E:T ratios). All results are displayed as the mean and standard deviation from three replicate wells. Lysis was measured after 12 h.

Figure 4



Peptide-restricted cytotoxic activity of FSP26-specific CTL clone 1. The FSP26 specificity of CTL clone 1 was tested in the presence of unlabeled cold targets, T2 cells pulsed either with peptide FSP26 (solid rhombus) or with control peptide P68 (open rhombus) at an inhibitor-to-target cell ratio of 30:1. Lysis of HCT116 cells without cold targets is shown as a control (open triangles). Killing of target cells is depicted at different effector-to-target cell ratios (E:T ratios). All results are displayed as the mean and standard deviation from three replicate wells.

To determine whether the susceptibility to CTL-mediated lysis could be enhanced by additional peptide loading of tumor cells, we compared the lysis of HCT116 cells in the presence or absence of exogenously added FSP26. Three highly reactive CTL clones (clones 2, 3, and 4) that grew sufficiently were chosen for this analysis. As shown in Figure 5, peptide loading had no significant effect on HCT116 killing, albeit the reactivity of CTL clone 3 toward HCT116 cells was moderately increased by the addition of FSP26. As previously demonstrated for the bulk culture, the killing efficiency of the CTL clones was also enhanced by preincubation of tumor cells with IFN-gamma (data not shown). FACS® analysis of the clones revealed a CTL phenotype (CD3+, CD8+, CD4-, data not shown).

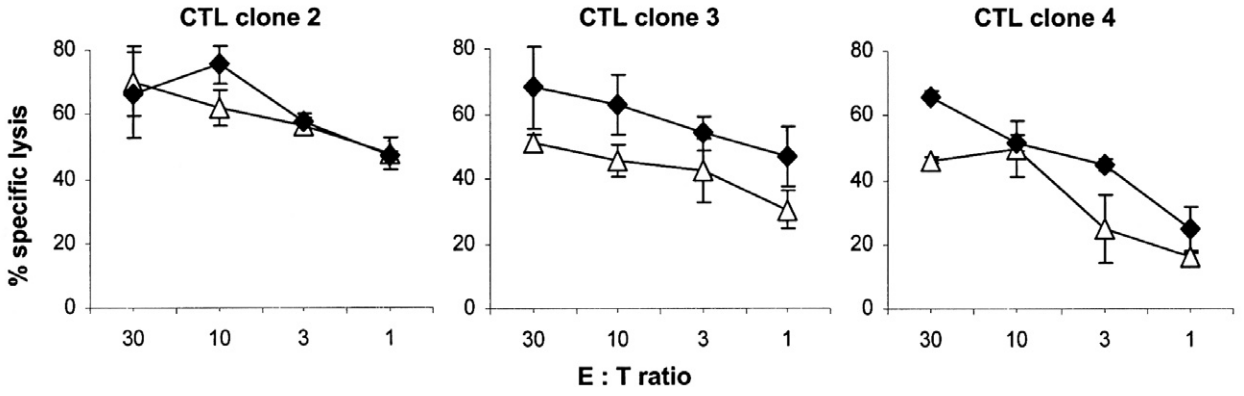
In another experiment, we aimed to confirm the HLA-A0201 restriction of FSP26-specific CTLs. In Ab-blocking ELISPOT experiments, preincubation of FSP26-pulsed T2 cells with the anti-HLA-A2 mAb BB7.2 blocked the recognition of FSP26-specific CTL clones substantially and resulted in approximately 50% inhibition of IFN-gamma release. The pan-anti-MHCI mAb W6/32 almost completely abolished recognition of FSP26-coated T2 cells by CTL clones 2, 3, and 4. In addition, targeting CD28 and CD3 molecules on T cell clones with blocking CD28.1 or OKT3 mAbs unequivocally reduced the number of spots, suggesting that the observed reactivity is indeed T cell mediated (Figure 6).

Discussion

MSI in coding genes might result in the expression of mutant proteins that encompass novel amino acid sequences downstream of the mutant microsatellite sequences. Thus, mutations in these coding repeats might lead to inactivation of gene function and, if the right combination of genes is hit, to neoplastic transformation. In line with this hypothesis, a consistent combination of specific critical genes has been identified in MSI+ cancers (5, 14, 21). Expression of these mutant genes, however, may also result in the translation of novel peptide sequences encoded by the mutant genes, which can be presented as potentially immunogenic and highly tumor-specific peptides on the surface of the cancer cells affected.

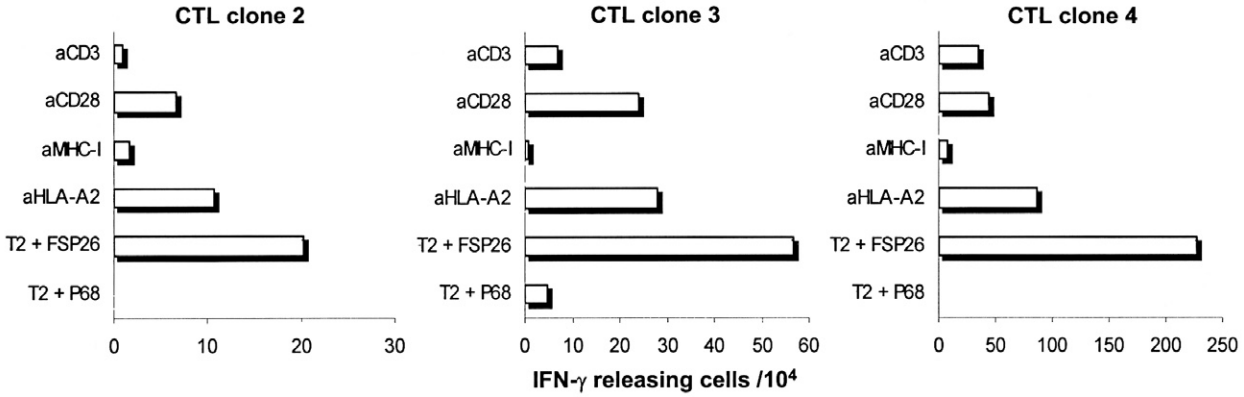
Recent reports suggest that at least some of these frameshift-associated neopeptides are potent tumor-rejection antigens that mediate the cytotoxic attack of peptide-specific lymphocytes against MSI+ tumor cells (11, 12, 13). Peptides encoded by

Figure 5



Cytotoxic activity of FSP26-specific CTL clones 2, 3, and 4. No significant increase in CTL reactivity upon addition of FSP26 is seen. Reactivity against HCT116 cells [HLA-A0201+, *Caspase-5(-1)*] with (solid rhombus) and without (open rhombus) exogenously added FSP26 is shown. Killing of targets is depicted at different effector-to-target cell ratios (E:T ratios). All results are displayed as the mean and standard deviation from three replicate wells. Lysis was measured after 12 h.

Figure 6



Recognition of FSP26-pulsed T2 cells is blocked by mAbs. *In vitro*-primed CTLs (3,000 cells/well) were stimulated with 35,000 T2 cells pulsed either with FSP26 or with the control peptide P68 as targets. HLA specificity was determined by blocking HLA-A2 on T2 target cells with the anti-HLA-A2 mAb BB7.2 or the anti-MHCI mAb W6/32. The influence of blocking CD28 and CD3 on the release of IFN-gamma by T cells was tested using the CD28-2.1 and OKT3 mAbs, respectively. Cells were incubated 1 h with hybridoma supernatant before being seeded in 96-well plates. The number of IFN-gamma-releasing activated T cells (spots) for the total number of cells analyzed (10^4) is given. As a measure of background IFN-gamma release, reactivity against control peptide P68 is shown.

mutant microsatellites of genes that are commonly hit by MSI in cancer cells provide us with an opportunity to establish a vaccine that might prevent outgrowth of MSI+ cancer cells in an immunized individual. This could become the basis for what would eventually be a protective vaccine against the most prevalent hereditary form of colon cancer, hereditary nonpolyposis colorectal cancer. Since none of the frameshift-induced neopeptides investigated so far is expressed in all MSI+ tumors, and some of these peptides might not be sufficiently immunogenic, we extended our earlier analysis to five HLA-A0201-restricted peptides derived from three potential target genes, *Caspase-5, TAF-1b,* and *HT001*.

T cells from a normal, healthy HLA-A0201+ donor were sensitized with FSPs *in vitro*, and sustained reactivity was detected against one nonamer peptide derived from *Caspase-5(-1),* ^{67}-FLIIWQNTM (FSP26). We failed to generate T cells persistently reactive toward the *Caspase-5(-1)*-derived FSP25, the *TAF-1b(-1)*-derived FSP27 and FSP29, or FSP30 from *HT001(-1)*. This can be explained by the weaker HLA-A0201 binding of these FSPs, as the MHC binding affinity of a peptide has a major impact on its immunogenic potential. Whereas high affinity MHC-peptide epitope interactions were conducive to immunogenicity, less avid interactions were associated with immunogenicity in only approximately 60% of the cases (18, 22). However, successful *in vitro* priming with peptides of moderate affinity has been demonstrated, as well as the induction of specific CD8+ T cells against such epitopes (13, 22, 23). This observation could be caused by a low dissociation rate of these peptides from the MHC class I molecule and consequently by the generation of stable MHC peptide complexes (24).

To generate peptide-specific CTLs, we used CD40Bs as highly efficient APCs. The activation of B cells by CD40L modulates a number of processes involved in antigen presentation, such as the increased expression of costimulatory molecules (25) and the secretion of immunomodulatory cytokines. Together these lead to enhanced antigen presentation and T cell stimulatory capacity, thus ensuring proper presentation of peptides and effective T cell priming by CD40Bs (20).

The FSP26-specific bulk culture and the CTL clones derived therefrom efficiently lysed peptide-sensitized T2 target cells at low effector-to-target cell ratios. Most importantly, these CTLs also revealed specific reactivity toward HLA-A0201+ colon carcinoma cell lines endogenously expressing the *Caspase-5(-1)* frameshift mutation. The lysis was HLA-A0201-restricted and peptide-specific because no reactivity was found against HLA-A0201-negative and *Caspase-5(-1)* expressing cells, nor against HLA-A0201+ and *Caspase-5(wt)*-expressing cell lines. Peptide specificity and HLA-A0201 restriction were further corroborated by cold target inhibition and antibody-blocking experiments. However, some of the CTL clones that were generated did not recognize endogenously processed antigen, possibly due to low affinity T cell receptors (12). IFN-gamma treatment of MSI+ colon carcinoma cells induced enhanced lysis of these cells by FSP26-specific CTLs, possibly by improving FSP26 processing and presentation. Thus, besides the standard proteasome, FSP26 must be processed by the immunoproteasome.

Phenotypic characterization of the T cells that had been FSP26-sensitized *in vitro* revealed that predominantly highly activated memory CD8+ T lymphocytes could be expanded. Upon antigenic stimulus, these produced granzyme B, IFN-gamma, and IL-2, all of which are considered to be primary effector molecules for CD8+ T cell-mediated tumor regression (26, 27, 28). Together, these results indicate that FSP26 represents a prominent T cell epitope, and thus the (-1) frameshift mutation of the *Caspase-5* gene is indeed immunogenic.

The A(10) repeat affected by frameshift mutations in the coding sequence of *Caspase-5* shows a high incidence of mutations in MSI+ tumor cell lines (82%) and in MSI+ tumors of the colon (up to 66%), endometrium (up to 28%), and stomach (44%) (14, 29). Recently, mutations in the same A(10) repeat were described in two new tumor entities. Hosomi *et al.* found frameshift mutations in 2 of 30 unselected primary lung cancers and speculated that *Caspase-5* might be a suppressor gene of highly metastatic potential in lung cancer (30), while Scott *et al.* identified microsatellite mutations in 4 of 16 human T cell lymphoblastic lymphomas and acute lymphoblastic leukemias (31). This strengthens the association with defective mismatch repair and suggests a possible role for this gene as an important tumor suppressor in hematopoietic cancers (31). Altogether these data suggest that *Caspase-5* is a common target gene in the microsatellite mutator pathway of MSI+ cancers. Mutations in the *Caspase-5* gene appear to be positively selected in MSI+ cancer cells, irrespective of the tissue of origin. But even if the *Caspase-5(-1)* frameshift mutations do not play a role in tumorigenesis by altering cell growth or survival capabilities, they could still have an indirect impact on tumor progression by altering the elicited immunoresponse.

The present study identified a novel CTL epitope (^{67}-FLIIWQNTM; FSP26) derived from a frameshift mutation affecting an A(10) coding microsatellite of the *Caspase-5* gene and demonstrated the immunogenic potential of this frameshift-derived peptide. Overall, these data agree very well with our earlier finding that the immunogenicity of FSPs originated from (-1) mutations in coding repeats of the *TGF-betaRII* (11) and *OGT* (13) genes. This further corroborates the notion that frameshift-derived peptides are a special class of MSI+ specific potential tumor-rejection antigens that are ideally suited to be part of an anti-MSI+ tumor vaccine.

Several lines of evidence support this concept. MSI patients often harbor mutations in more than one gene that carries nucleotide repeat sequences (14), and the risk of tumor escape by immune selection after immunization *in vivo* can be substantially reduced if distinct frameshift-derived peptides representing several T cell epitopes are used. Moreover, MHC class II restricted T cell epitopes have been described in the case of *TGF-betaRII(-1)* (12, 32), and recently Ishikawa et al. described antibodies specific for frameshift-mutated *CDX2* in a cancer patient suffering from an MSI+ colon tumor (33), highlighting the potential of the human immune system to recognize and react to mutated proteins arising from frameshift mutations.

It is therefore reasonable to design a vaccine against MSI+ colorectal tumors based on a combination of peptides, generated by frameshifts in common target genes of cMSI and known immunogenic properties, such as *TGF-betaRII, OGT,* and *Caspase-5*, and ideally containing both MHC class I and class II T cell epitopes to increase its potential therapeutic efficiency.

Abbreviations

CD40B, CD40-activated B cell; cMS, coding microsatellite; FSP, frameshift peptide; MSI, microsatellite instability; OGT, O-GlcNAc transferase; TGF-betaRII, transforming growth factor beta receptor type II

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Materials and methods

Cell lines and generation of CD40-activated human B cells

All tumor cell lines were obtained from the ATCC, CLS (Heidelberg, Germany) or from the German Cancer Research Center (DKFZ) tumor bank and grown in RPMI 1640 medium supplemented with 10% FCS and antibiotics. The following colon carcinoma cell lines were used for cytotoxicity analysis: HCT116 [HLA-A0201+, *Caspase-5(-1)*, MSI+]; Colo60H [HLA-A0201+, *Caspase-5(-1)*, MSI+]; LoVo [HLA-A0201-, *Caspase-5(-1)*; MSI+]; the HLA-A0201-transfected LoVo cell line LoVo-A2 described by Ripberger *et al.* (13); SW707 [HLA-A0201+, *Caspase-5(wt)*, MSI-], SW480 [HLA-A0201+, *Caspase-5(wt)*, MSI-]. In some assays T2 cells (174xCEM.T2 hybridoma; HLA-A0201+, TAP1 and TAP2 deficient) were used. The erythroleukemia cell line K562 (HLA-A0201-) was used as a target cell in cytotoxicity assays to test for NK activity. All tissue culture media and supplements were purchased from the Invitrogen Corporation (Karlsruhe, Germany) unless indicated otherwise. IL-4, IL-7, and IFN-gamma were obtained from R&D (Wiesbaden, Germany); IL-2 was obtained from PromoCell (Heidelberg, Germany).

We generated CD40-activated B cells (CD40Bs) from a healthy HLA-A0201+ donor via NIH/3T3-CD154 feeder cells as described previously (11, 20). Analysis of the phenotype of these CD40Bs revealed high expression of MHCs classes I and II, costimulators (CD40, CD80, and CD86), intercellular adhesion molecules (CD50, CD54, CD58, and CD102), activation markers (CD69 and CD71), as well as B cell specific antigens (CD19 and CD23), corresponding to the properties of professional APCs.

Peptides and HLA-A0201 binding assay

Peptides displaying HLA-A0201 binding motifs were selected by computer algorithms (34, 35). Peptides were synthesized by the Peptide Synthesis Facility at the German Cancer Research Center. Stock solutions (5 mg/ml in DMSO) were stored at -70°C and diluted to 500 µg/ml in PBS before use. HLA-A0201 binding was measured using T2 cells, which are deficient in antigen processing and express high numbers of empty and temperature-sensitive HLA-A0201 molecules (36). HLA-A0201 stabilization by the exogenous addition of peptides was analyzed by determining cell surface HLA-A0201 staining, which reflects the relative HLA-A0201 binding affinity of cognate peptides. T2 cells were pulsed with 50 µg/ml peptide and 5 µg/ml beta-2-microglobulin (Sigma, Deisenhofen, Germany) overnight at 37°C. HLA-A0201 expression was analyzed by flow cytometry using mAb BB7.2 (hybridoma culture supernatant) followed by staining with FITC-labeled goat antimouse IgGs (Dianova, Hamburg, Germany).

T cell purification

Peripheral blood was obtained from a healthy HLA-A0201+ donor and collected in heparinized tubes. PBMCs were isolated by Ficoll-density gradient centrifugation. Whole CD3+ T cells were obtained from PBMCs by magnetic depletion of non-T cells using the MACS Pan T Cell Isolation Kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Preparations contained at least 98% CD3+ cells, as assessed by flow cytometry.

Generation of peptide-specific cytotoxic T lymphocytes

CD40Bs of a healthy HLA-A0201+ donor were incubated with peptide (10 µg/ml) in serum-free Iscove's DMEM medium for 1 h at 37°C, washed to remove excess peptide, irradiated (30 Gy), and then added to purified CD3+ autologous T cells at a ratio of 4:1 (Tc:CD40Bs) in Iscove's DMEM containing 10% human AB-serum, supplements (1:100), and hIL-7 (10 ng/ml, R&D, Wiesbaden, Germany). Cells were plated at a density of 2 x 10^6 T cells/ml. For restimulation of T cells, this treatment was repeated weekly. At days 21 and 24, hIL-2 (10 IU/ml, R&D, Wiesbaden, Germany) was given together with hIL-7. From day 28 onwards, hIL-2 was used instead of hIL7.

Cloning of FSP26-specific cytotoxic T lymphocytes

Cloning of peptide FSP26 (FLIIWQNTM)-specific T cells was performed by limiting dilution of T cell bulk culture. Briefly, T cells were seeded under limiting dilution conditions (0.7 cells/well) in V-bottom, 96-well plates containing 3 x 10^5/well lethally irradiated (30 Gy), autologous CD40Bs loaded with peptide FSP26 (10 µg/ml) in a final volume of 200 µl Iscove's DMEM with 10% human AB-serum, supplements (1:100), and IL-2 (10 IU/ml). This treatment was repeated weekly to restimulate T cells. After 6 wk, outgrowing clones were screened for peptide-specific cytotoxic activity in a standard chromium release assay (37, 38).

ELISPOT assay

ELISPOT assays were performed using nitrocellulose-lined 96-well plates (Multiscreen, Millipore, Bedford, MA, USA) that were covered with mouse antihuman IFN-gamma mAb (Mabtech, Stockholm, Sweden) and blocked with serum-containing medium. T effector cells (1 x 10^4) were plated in triplicate with 3.5 x 10^4 peptide-loaded T2 cells as targets. After incubation for 16 h at 37°C, the plates were washed, incubated with biotinylated rabbit antihuman IFN-gamma mAb for 4 h, washed again, incubated with streptavidin-alkaline phosphatase for 2 h, and washed again. Spots were detected by incubation with NBT/BCIP (Sigma-Aldrich, Steinheim, Germany) for up to 1 h. The reaction was stopped with water, and after drying, the spots were counted using an AID-ELISPOT reader (AID, Strassberg, Germany).

For Ab-blocking experiments, T2 target cells were preincubated for 1 h with either BB7.2 (anti-HLA-A2) or W6/32 (anti-MHCI, both hybridoma culture supernatants). The influence of CD3, CD8, and CD28 blocking on T cells was analyzed by preincubating T cells with OKT3, OKT8, and CD28-2.1 (all hybridoma culture supernatants), respectively, for 1 h before using them as effectors in the ELISPOT assay (22).

Analysis of extracellular staining by FACS®

HLA-A02 expression on tumor cell lines was analyzed using primary mAb BB7.2 followed by FITC-labeled goat antimouse IgGs (Dianova, Hamburg, Germany). Cells treated without primary antibody were used as negative control.

CD40Bs and T cells were examined for the expression of the following surface markers: MHCI (W6/32), HLA-A2 (BB7.2), MHCII (12G6), CD3 (OKT3), CD28 (CD28.2), CD40 (5C3), CD49d (L25) with unlabeled primary antibodies and FITC-labeled goat antimouse IgGs as second antibody, CD2 (MT910, DAKO, Denmark), CD4 (RPA-T4), CD8 (RPA-T8), CD11a (38, Cymbus Biotechnology, Hampshire, UK), CD19 (HIB19), CD23 (M-L233), CD45RO (UCHL1), CD50 (101-1D2, Cymbus Biotechnology, Hampshire, UK), CD54 (HA58), CD58 (1C3), CD80 (BB1/B7-1), CD86 (B70/B7-2), and CD102 (B-T1, Serotec, Oxford, UK) were directly FITC-conjugated, whereas CD25 (M-A251), CD69 (FN50), CD71 (SOM4D10, Diatec, Oslo, Norway) were PE-conjugated. Isotype-matched mAbs were used as a negative control. Antibodies and signal detection reagents were obtained from BD Biosciences (Heidelberg, Germany), unless stated otherwise.

Analysis of intracellular staining by FACS®

For intracellular staining, T cells were incubated with the protein transport inhibitor brefeldin A (2 µg/ml, Serva, Heidelberg, Germany) for 15 h at 37°C. After two washes with PBS/1% FCS, cells were fixed with cold 4% paraformaldehyde (PFA, Serva, Heidelberg, Germany) in PBS for 10 min at 4°C, washed twice with PBS/1% FCS, and permeabilized with saponin buffer (PBS, 0.1% saponin, 1% FCS, and 1 M Hepes) for 10 min at room temperature. T cells were subsequently stained for intracellular cytokines with PE-labeled anti-IFN-gamma (4SB3), anti-TNF-alpha (Mab11), anti-IL-2 (MQ1-17H12) mAb, or IgG1 isotype control mAb (all BD Biosciences, Heidelberg, Germany) in saponin buffer for 20 min at 4°C. Cells were washed twice with saponin buffer and resuspended in PBS/1% FCS before measurement. Intracellular perforin was detected with PE-labeled anti-perforin (dG9) mAb. For the analysis of intracellular granzyme B, cells were incubated with the antigranzyme B mAb 2C5/F5 (Serotec, Oxford, UK), followed by FITC-labeled goat antimouse IgGs.

Cells treated without primary antibody were used as negative control. Cell surface and intracellular immunofluorescence were obtained on a FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA) using CellQuest software. Typically, 20,000 cells were acquired from each sample.

Cytotoxicity assay

Standard chromium release assays were performed as described (37, 38). Tumor target cells were labeled with 100 µCi [^{51}Cr]-sodium chromate for 1 h at 37°C. For peptide recognition, T2 cells were incubated with 5 µg/ml peptide for 1 h at 37°C in FCS-free medium, washed, and subsequently labeled. For each experimental condition, cells were plated in triplicate in V-bottomed, 96-well plates with 10^3 target cells/well. Varying numbers of CTLs were added for a final volume of 200 µl and incubated for 4 h (or 12 h in selected experiments) at 37°C. Spontaneous and maximal release was determined in the presence of target cells in medium alone, or in target cells in medium supplemented with 1% NP-40. Supernatants (100 µl/well) were harvested and counted in a gamma-counter (1282 Compugamma, LKB-Wallac, Stockholm, Sweden). The percentage of specific lysis was calculated as follows: 100% x (experimental release - spontaneous release) / (maximal release - spontaneous release). In selected experiments, colorectal cancer cell lines were incubated overnight with IFN-gamma (200 U/ml) before being used in cytotoxicity assays.

Cold target inhibition experiments used nonradiolabeled T2 cells loaded with 5 μg/ml cognate peptides for 1 h at 37°C or the HLA-A0201-binding P68 peptide as a negative control to compete for recognition of ^{51}Cr-labeled colon carcinoma cells. The ratio of cold (inhibitor) to hot (radiolabeled) targets was 30:1.

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