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CD8⁺ T cell Recognition of Polymorphic Wild Type Sequence p53₆₅₋₇₃ Peptides in Squamous Cell Carcinoma of the Head and Neck

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Abstract

The TP53 tumor suppressor gene contains a well-studied polymorphism that encodes either proline (P) or arginine (R) at codon 72, and over half of the world population is homozygous for R at this codon. The wild type sequence (wt) p53 peptide, p53₆₅₋₇₃, has been identified as a CD8⁺ T cell-defined tumor antigen for use in broadly applicable cancer vaccines. However, depending on the TP53 codon 72 polymorphism of the recipient, the induced responses to the peptides incorporating R (72R) or P (72P) can be “self” or “non-self.” Thus, we sought to determine which wt p53₆₅₋₇₃ peptide should be used in wt p53-based cancer vaccines. Despite similar predicted HLA-A2 binding affinities, the 72P peptide was more efficient than the 72R peptide in HLA-A2 stabilization assays. *In vitro* stimulation (IVS) of CD8⁺ T cells obtained from healthy HLA-A2⁺ donors with these two peptides led to the generation of CD8⁺ T cell effectors in one-third of the samples tested, a similar frequency as responsiveness to other wt p53 peptides. Interestingly, regardless of their p53 codon 72 genotype, CD8⁺ T cells stimulated with either 72P or 72R peptide were cross-reactive against T2 cells pulsed with either peptide, as well as HLA A2⁺ head and neck cancer (HNC) cell lines presenting 72P and/or 72R peptides for T cell recognition. Therefore, the cross-reactivity of CD8⁺ T cells for the polymorphic wt p53₆₅₋₇₃ peptides, irrespective of their p53 codon 72 polymorphism, suggests that employing either peptide in wt p53-based vaccines can result in efficient targeting of this epitope.

Keywords

Cytotoxic T lymphocytes; antigen presentation; tumor immunity

Introduction

Loss of function of the p53 gene is the most common event associated with human cancer, and is usually due to a single missense mutation of one allele, while the remainder of the accumulated p53 protein is wild-type (wt) in sequence. Although considered to occur more

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frequently in human cancers accumulating mutated p53 molecules, the processing and presentation of wt p53 peptides by tumors can lead to elevated presentation of wt p53 peptides that can stimulate cytotoxic T lymphocytes (CTL) (1-3). In this regard, a number of CTL-defined, HLA class I-restricted human wt p53 peptides have been identified for use in p53-based immunotherapy (2, 4-10). An obstacle to the success of wt p53 immunotherapy, however, is the weak immunogenicity of these “self” tumor peptides and the need to circumvent the tolerance imposed on these antigens. CTL responses to a range of “self” human tumor peptides, including wt p53 peptides, have been improved by amino acid substitution, or optimization, of the parental sequence. Indeed, variant peptides have been shown *in vitro* to reverse “nonresponsiveness” in PBMC of some HLA-A*0201 (HLA-A2)⁺ donors to the wt p53₂₆₄₋₂₇₂ and₁₄₉₋₁₅₇ peptides, thereby expanding the pool of donors likely to respond to vaccines targeting these epitopes (11, 12).

The most common polymorphism in TP53 has been identified at codon 72 and encodes proline (P) or arginine (R). The biological and clinical significance of this polymorphism has been reported (13), and the nonconservative amino acid change at codon 72 is associated with altered electrophoretic mobility of the two polymorphic variants, thus suggesting structural modifications of the p53 protein (14). One of the HLA-A2-restricted, CTL-defined wt p53 peptides identified for use in cancer immunotherapy is the wt p53₆₅₋₇₃ peptide. Consequently, two HLA-A2-restricted, wt p53₆₅₋₇₃ peptides, 72P and 72R, which express either proline or arginine at residue 8, could be potentially recognized by CTL. The initial studies involving the wt p53₆₅₋₇₃ epitope, however, did not take into account the p53 codon 72 polymorphism expressed by the responding CD8⁺ T cells and/or stimulating wt p53₆₅₋₇₃ peptide, which was 72P. Therefore, it was unclear whether the immune responses generated against this peptide were “self” or “non-self.” As a result, the immunogenicity of the wt p53₆₅₋₇₃ peptide was open to further investigation. Because over half of the world population is homozygous (R/R) at codon 72, determining whether the 72R and/or 72P induce the expansion of CTL equally cross-reactive against both peptides is important to the targeting of this epitope by p53-based cancer vaccines. Furthermore, enhanced binding of one of these polymorphic peptides to HLA A2 molecules compared to that of the other might make it a more immunogenic wt p53₆₅₋₇₃ peptide for use in vaccination. Consequently, we studied whether the 72P and/or 72R peptide might be employed as enhanced variant peptides to obviate self-tolerance for individuals without germ line expression of the particular wt p53₆₅₋₇₃ polymorphic peptide used in a vaccine. In this report, we investigated the HLA-A2- binding of the 72P and 72R peptides, characterized the CTL raised against each of these peptides, and studied their cross-reactivity against human HLA A2⁺ head and neck cancer (HNC) cell lines, which were either homozygous (R/R or P/P) or heterozygous (R/P) at TP53 codon 72.

Materials and Methods

Cells and Cell Lines

The HLA-A2⁺ SCCHN cell lines: SCC-4, PCI-13 and PCI-30 (*Lin, C Head and Neck, 2007*) and UD-SCC-6 (gift of Dr. Henning Bier, University of Dusseldorf) were used; their characteristics and derivation have been published elsewhere (15), and were cultured in

DMEM supplemented with 8% FCS and 1% penicillin/streptomycin solution, as 37°C and 5% CO₂. Naturally HPV-16 transformed UPCI:SCC090 (referred to as SCC90) cells were isolated, characterized and described recently (16). SaOS-2 p53 null cells or the stable mutant p53 cDNA transfected-SaOS-2 cell lines were previously described by Balz et al (Cancer Res 2003; 63).

PBMC were isolated from HLA-A2⁺ healthy normal controls (NCs) by centrifugation over Ficoll-Hypaque gradients (Amersham Pharmacia Biotech, Piscataway, NJ). Healthy control lymphocytes were obtained from leukapheresis products. HLA-A2 expression was determined first by flow-cytometry using the anti-HLA-A2 antigen-specific mAb BB7.2 (American Type Culture Collection, Manassas, VA), and an IgG isotype Ab as a negative control, with verification by PCR (17). Fresh or cryopreserved PBMC were used at a concentration of 5-10 × 10⁶ cells/ml in freezing medium consisting of 90% fetal calf serum (FCS - Life Technologies) + 10% DMSO (Fisher Scientific, Pittsburgh, PA), until further use.

Determination of p53 codon 72 genotype of SCCHN cells and healthy donor PBMC

To characterize the distribution of codon 72 genotype, we selected PBMC from 54 HLA-A2⁺ healthy donors and 55 SCCHN patients. Isolation of genomic DNA from SCCHN patients and PBMC from healthy donors was performed using the QIAGEN DNA Mini Kit (QIAGEN Inc., Valencia, CA), according to the manufacturer's instructions. PCR-based restriction fragment length polymorphism analysis was used to identify the p53 polymorphism in codon 72 with the primers 5'-ATC TCA AGT CCC CCT TGC CG-3' and 5'GCA ACT GAC CGT GCA AGT CA-3'. PCR amplification involved an initial denaturation step at 95°C for 5 minutes; 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. Then, PCR product (296-bp fragment) was digested by BstUI (New England Biolabs, Inc., Beverly, ME) overnight at 60°C, which reveals the presence two bands of an arginine encoding allele (BstUI sensitive, (18)) or a proline-encoding allele (BstUI insensitive), as visualized on ethidium bromide stained agarose gel electrophoresis (Figure 1A). Direct fluorogenic sequencing analysis (Figure 1B) of both coding and non-coding strands, with subsequent BLAST alignment was used to determine the genotype of TP53 in exons 2 through 11, with particular attention to the sequence of codon 72 of the TP53 exon 4. Using these techniques, we identified homozygous (R/R – two bands; and P/P – one band) and heterozygous (R/P – three bands) tumor cell lines and healthy donors PBMC.

Peptides

The 72P (RMPEAAPPV) and 72R (RMPEAAPRV) peptides were synthesized using f-MOC chemistry by the University of Pittsburgh Peptide Facility and provided at > 90% purity as determined by HPLC and confirmed by mass spectrometry. Peptides were stored until use in stock solutions at 1mg/ml in DMSO.

Tetramers

Lyophilized peptide was used by the NIH Tetramer Core Facility at Emory University, to produce the HLA-A2/72P and 72R tetramer linked to phycoerythrin (PE) and allophycocyanin (APC), respectively.

HLA-A2 Stabilization Assay

For determination of peptide-induced surface HLA-A2 upregulation, T2 cells (19) were incubated for 72 hours, then divided and incubated with the indicated HLA-A2 binding peptides for 18 hours in AIM-V medium, at a final concentration of 10 μ M to 10 nM. Cells were then stained with mAbs against HLA class I monomorphic determinant (W6/32, (20)) or against HLA A2 (BB7.2, (21)) with similar results. Appropriate fluorescently labeled secondary Abs were added after washing twice. Flow cytometric analysis of at least 50,000 gated events was performed in at least three separate experiments for each indicated curve.

Generation and Maturation of DC

DC were generated from plastic adherent human PBMCs (Romani 1994), and cultured in medium supplemented with IL-4 (1×10^3 U/mL) and GM-CSF (1×10^3 U/mL). On day 6, DC maturation was induced using AIM-V plus 10 ng/ml TNF- α , 10 ng/ml IL-1 β and 10 ng/ml IL-6 for 18 hr. Both immature DC and mature DC were phenotyped by flow cytometry, staining for CD80, CD14, CD86, HLA-DR, CD40, and CD83 (4). DC were incubated for 4 h in the presence of the wt p53₆₅₋₇₃ peptides prior to addition of CTL lines.

IFN- γ ELISPOT assays

ELISPOT assay were performed as described (12). HLA class I antigen-specific mAb (W6/32) and as a control, HLA class II antigen-specific mAb (L243) were used for blocking recognition of target HLA-peptide complexes and defining the active T cell subset(s) in the assay system. ELISPOT analyses used IVS cultures of PBMC or enriched populations of CD8⁺ T-cells (CTL lines) obtained from HLA-A2⁺ healthy donors or SCCHN patients. Briefly, 96 well flat-bottomed plates are coated overnight at 4°C with anti-human IFN- γ mAb (1-D1K: Mabtech) in PBS. The responder cells (PBMC or enriched populations of CD8⁺ T-cells) are added. Controls consisted of unstimulated responder cells, responder cells in the presence of unpulsed APC, or APC cells alone. After incubation for 20 h at 37°C, the plates were washed with PBS/0.05% Tween 20, and supplemented with the biotinylated anti-IFN- γ mAb (7-B6-1: Mabtech). After 2hr incubation at 37°C, plates were washed with PBS/0.05% Tween 20, and developed with avidin-peroxidase complex (Vectastain Elite kit). Spots were counted by computer-assisted image analysis (Cellular Technologies).

In vitro stimulation (IVS) of CD8⁺ T cells with 72P and 72R peptides

IVS cultures for stimulation and propagation of CD8⁺ T cell lines specific for wt p53₆₅₋₇₃ were established as described, using autologous DC for the first 2 stimulations from PBMC, then peptide-loaded T2 cells thereafter (17). Briefly, DC were pulsed with 10 μ g/ml of wt p53_{65-72P-73} and p53_{65-72R-73} for 4 h at 37°C, and subsequently irradiated (50 Gy), washed, and resuspended in AIM-V medium containing 5% (v/v) human AB serum. CD4⁺ T-cells were negatively isolated from non-adherent PBMC using anti-CD4 microbeads (DAKO).

On day 0, 2×10^6 responder cells and 2×10^5 peptide-loaded DC were co-cultured. For expansion of CD8⁺ T-cells, the medium was further supplemented with 20 IU/ml of IL-2 (R&D) and 10 ng/ml of IL-7 (R&D), and on day 7, the responder cells were harvested and assayed.

Data Analysis

T cell reactivity as measured by the ELISPOT assay was considered positive if test well had significantly greater spots in triplicate than background wells when using a one-tailed permutation test at $\alpha = 0.05$. Analysis was performed using Statview statistical software (Abacus Concepts, Inc.).

Results

Frequency of codon 72 polymorphism in the PBMC donor population studied

We first determined the frequency of codon 72 polymorphism in our population of healthy donors to indicate the potential clinical impact of differential immunogenicity of the 72P or 72R peptides. Our population frequency of codon 72 genotypes in Western Pennsylvania, identified 37 (68%) with R/R genotype, 16 (30%) with R/P genotype and 1 with P/P genotype in 54 healthy donors, and 41 (74.5%), 8 (14.5%), and 6 (11%) respectively, in 55 HNC patients.

Stabilization of HLA-A2 molecules by wt p53₆₅₋₇₃ 72P and 72R peptides

The 72P and 72R peptides were predicted by two HLA class I binding-algorithms, <http://bimas-dcrt.nih.gov/molbio/hla-bind> “score >100” and www.syfpeithi.de “score >24”, ref. (22), to have similar binding affinities for HLA-A2 molecules. The binding of the 72P and 72R peptides HLA-A2 molecules was determined *in vitro* using the T2 stabilization assay (Filho, 2009 International J Cancer). The ability of these peptides to stabilize surface HLA-A2 expression varied significantly in T2 cells incubated with Flu₅₈₋₆₆, 72P or 72R peptides (listed in descending order of binding, Figure 2). Contrary to the predicted similar binding for each peptide, 72P displayed a consistently higher affinity for HLA-A2 molecules than the 72R peptide (need p value)

CD8⁺ T cells generated against either 72P or 72R are cross-reactive and recognize both peptides

Because the affinity of a given peptide for a HLA class I molecule does not necessarily correlate with its *in vitro* ability to induce the expansion of CTL (23), CD8⁺ T cells obtained from 21 HLA-A2⁺ healthy donors, categorized into 3 groups according to their genotype (10 R/R, 10 R/P and 1 P/P), were stimulated *in vitro* with autologous DC pulsed with either the 72P or 72R peptides. After three rounds of IVS, the outgrowing lymphocytes derived from CD8⁺ T cells from 7 of these 21 donors (33%) demonstrated p53-specific CTL reactivity, and were characterized for their ability to recognize 72P and 72R-pulsed T2 cells in IFN- γ ELISPOT assays using unpulsed T2 cells and HIV-POL₄₇₆₋₄₈₄ peptide-pulsed T2 cells were negative controls. Among the R/R and R/P donor PBMC tested, IVS of CD8⁺ T cells obtained from 3 donors yielded reactivity in the ELISPOT assay, with consistent cross-reactivity against the 72P/72R peptides for all three. The reactivity was blocked HLA class I

antigen-specific mAb but not with HLA class II antigen-specific mAb (data not shown). Furthermore, independent of the p53 codon 72 polymorphisms of the stimulating peptide and donor's PBMC, the induced CTL effectors showed greater recognition of 72R-pulsed T2 cells (Figure 3). The outgrowing lymphocytes also were analyzed by flow cytometry for 72P and 72R-specific CD8⁺ T cells using the appropriate HLA-A2/wt p53 72P and 72R peptide tetramers (Figure 4).

Only 2/54 healthy donors tested encoded the P/P genotype, a finding consistent with its frequency in the population (3-8%, (24-27)). Only one, however, was HLA-A2⁺ and suitable for this study. As noted before, IVS of CD8⁺ T cells obtained from this P/P donor with 72P and 72R peptides yielded CTL effectors that were cross-reactive against T2 cells loaded with either peptide (Figure 3).

CD8⁺ T cells generated against 72P and 72R recognize SCCHN cell lines expressing either peptide

We sought to determine whether CD8⁺ T cells induced by either 72P or 72R peptides recognized the naturally processed polymorphic wt p53₆₅₋₇₃ peptides presented by human HLA-A2⁺ tumor cells, a pre-requisite for clinical application in cancer vaccines. CD8⁺ T cells induced with either 72P or 72R were tested for recognition of 3 HLA-A2⁺ HNC cell lines, PCI-13 (R/R genotype), SCC-90 (R/P genotype), or SCC-4 (P/P genotype) in IFN- γ ELISPOT assays. The peptide-induced effectors, independent of the polymorphisms of the peptide and donor CD8⁺ T cells used to induce them, recognized all three HNC target cells (Figure 5A and 5B). Recognition was blocked by HLA class I antigen- but not HLA class II antigen-specific mAb. More importantly, the CTL showed appreciable cross-reactivity against HNC target cells that encode homozygous (R/R or P/P) alleles at TP53 codon 72, despite the lack of prior exposure of these CTL to the polymorphic peptide presented by the target cell. This observation was confirmed using Saos-2 cells (p53 null) stably transfected with mutant p53 cDNA encoding either the codon 72 P or R polymorphism (Figure 5C).

Discussion

Since the HLA-A2-restricted, CTL-defined wt p53₆₅₋₇₃ peptide (REF) incorporates the common p53 codon 72 polymorphism, two polymorphic peptides, 72P and 72R, are available for use in cancer vaccines. To clarify their utility in human cancer vaccines, we sought to determine whether a differential *in vitro* immunogenicities for these peptides could be observed when the polymorphism of the peptide and the induced donor CD8⁺ T cells were taken into account. Namely, to what extent were the donor T-cell responses to the two polymorphic wt p53₆₅₋₇₃ peptides, 72P and 72R, dependent on them being "self" or "non-self." Interestingly, despite algorithms that predict similar binding to HLA-A2 by either peptide, we observed significantly greater HLA-A2 stabilization by the 72P peptide.

Using the 72P and 72R peptides to stimulate CD8⁺ T cell cultures, we observed that the CTL were able to cross-react against T2 cells loaded with either peptide. In addition, these T cells also recognized HLA-A2⁺ SCCHN cell lines, which are either homozygous (R/R or P/P) or heterozygous (R/P) at TP53 codon 72. This was confirmed using Saos-2 (p53 null) cells stably transfected with mutant p53 cDNA that encode either R or P at codon 72.

Moreover, we found that IVS of healthy donor HLA-A2⁺ PBMC lead to the generation of CTL that were reactive against the same polymorphic peptide sequence endogenously encoded by the donor, as well as against the peptide sequence not encoded. Obviously, the important aspect of our findings for clinical utility is the cross reactivity of the CD8⁺ T cells recognizing the 72R epitope when generated by IVS using the 72P peptide. We confirmed this phenomenon by demonstrating the ability of T cells to recognize both the 72P and 72R peptides either pulsed on T2 cells or naturally presented by HLA A2⁺ SCCHN cells. IVS cultures using autologous DC as antigen presenting cells loaded with the peptide of interest were specifically designed to ensure that the alternate, polymorphic peptide was not introduced into the culture either through peptide loading or endogenous antigen processing and presentation. Taken together, these data suggest that in a greater proportion of patients, the 72P peptide would be more useful than the 72R peptide in for broadly applicable, T cell-mediated immunotherapy of human cancer.

The basis for the cross-reactivity of wt p53₆₅₋₇₃ effectors for the 72P and 72R peptides requires further investigation, such as exploring the T cell receptor (TCR) $\nu\beta$ usage in various CTL populations, which may be heterogeneous in these IVS cultures after stimulation or vaccination using either polymorphic peptide. It is possible that CTL generated against the exogenous polymorphic wt p53₆₅₋₇₃ peptide react against and eliminate expanding high affinity, T cells specific for this peptide in the IVS cultures. This phenomenon could potentially skew the outgrowth of low-moderate affinity, cross-reactive CTL, which were then detected in our ELISPOT assays after 3-4 rounds of IVS. Nonetheless, our data clearly provide evidence that either (or both) peptides may be used *in vivo* in clinical vaccine trials for p53 overexpressing malignancies. If one peptide were to be selected for cancer vaccines, it may be desirable to choose the 72P peptide, based on improved HLA-A2 binding and stabilization. The enhanced stabilization of HLA-A2 antigens by this peptide may provide a stronger stimulus *in vivo*, which may not translate *in vitro* into a quantitatively detectable difference in CTL induction efficiency or ELISPOT reactivity, since these assays lack ideal quantitative features necessary to detect such an effect in culture. This information, if confirmed in expanded studies, would have potential implications for peptide selection in wt p53-based immunotherapy.

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Figure 1. Genotype analysis in the three different tumor cell lines. DNA were generated from SCCHN cell lines

A. Three different tumor cell lines were cultured and harvested as described in material and methods for PCR amplification with the primers 5'-ATC TCA AGT CCC CCT TGC CG-3' and 5'-GCA ACT GAC CGT GCA AGT CA-3'. An initial denaturation step were used at 95°C for 5 minutes; 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute. PCR product (296-bp fragment) was digested by BstUI (New England Biolabs, Inc., Beverly, ME) overnight at 60°C, which reveals the presence two bands of an arginine (visible fragment 169 bp and 127 bp) encoding allele (BstU1 sensitive, (18)) or a proline encoding allele (BstU1 insensitive), as visualized on ethidium bromide stained agarose gel electrophoresis. **B.** These three SCCHN cell lines (SCC-4 not shown) were sent for direct fluorogenic sequencing analysis of both coding and non-coding strands, with subsequent BLAST alignment was used to determine the genotype of the sequence of codon 72 of the TP53 exon 4 and confirmed the homozygous of PCI-13 (R/R) and heterozygous of SCC-90 (R/P).

Figure 2. HLA-A2 stabilization assay comparing p53 peptides and Influenza

Stabilization of cell surface HLA-A2 molecules was determined after incubation of T2 cells with p53_{72P} and p53_{72R}, or Flu peptides at various concentrations (ranging from 10 μg/ml - 0.01 μg/ml) as described in Materials and Methods. Flow cytometry staining was performed by incubating 5×10^5 loaded cells with anti-HLA class I mAb W6/32, then FITC labeled secondary mAb for 30 minutes each at 4°C. Mean fluorescence intensity detected by flow cytometry is shown, reflecting results representative of three similar experiments.

Figure 3. Cross-reactivity of CTL derived by IVS using wt p5372P and p5372R-specific T cells against both allelic peptide

CD8⁺ T cells from HLA-A2⁺ healthy donors (named as 1-7) were stimulated using IVS with DC loaded either with wt p5372P and p5372R. Reactivity of CTL against peptide-pulsed target cells was tested using IFN- γ ELISPOT assays. This recognition was blocked by HLA class I and HLA-A specific mAb, but not an HLA class II specific mAb (not shown). Cross-reactivity of p5372P and p5372R peptide-specific CTL was observed and to our surprise, better recognition of T2 cells loaded with wt p53_{65-72R-73}, independent of the peptide used during IVS or the donor genotype. Each bar represents the mean spot number of triplicate experiments $\pm$ SD with 5×10^4 CTL per well. Background IFN- γ secretion was measured in response to T2 cells pulsed with HIV peptide or T2 cells alone. Background IFN- γ release was quantified using T2 plus HIV-1 derived peptide. Each data point represents the mean spot number of triplicate determinations $\pm$ SD with 10^4 CTL per well.

Figure 4. Frequency of wt p53_{72P} and wt p53_{72R} specific tetramer⁺ cells in PBMC from healthy donors (HD) after 3-week in vitro stimulation (IVS)

IVS was performed using autologous DC from HLA-A2⁺ HD loaded with wt p53_{72P} and p53_{72R} peptides (10 µg/ml at 37°C for 4 hrs). Autologous CD8⁺ T cells were negatively isolated from PBMC with immunomagnetic beads (Miltenyi Biotech, Germany) and added at 1×10^6 cells/ml to 1×10^5 peptide-pulsed DC in a final volume of 2 ml of culture medium (24-well tissue culture plate). The cells were cultured for 7 days at 37°C with IL-2 (20 units/ml) and IL-7 (5 ng/ml). On day 7 lymphocytes were harvested and restimulated until completed 21 days. Two different genotypes (R/R and R/P) were tested for the specificity of PE-labeled HLA-A2- wt p53_{72P} and APC-labeled HLA-A2-wt p53_{72R} tetramer. The PE-labeled HLA-A2- wt p53_{72P} and APC-labeled HLA-A2-wt p53_{72R} tetramer were obtained from the Tetramer Facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA). Specificity was confirmed by staining of the CTL line specific for each peptide after IVS and the lack of staining of irrelevant (MAGE-3₂₇₁₋₂₇₉ specific or HIV Pol 642A) CTL or HLA-A2⁻ PBMC obtained from normal donors (not shown). Three-color flow cytometry assays (FACScan, BD Biosciences) were performed with fluorescent labeled anti-CD3 and FITC-anti-CD8 Abs (Beckman Coulter) and PE-tetramer or APC-tetramer. Flow cytometry was performed on a CyAnTM flow cytometer (Dako, Ft. Collins, CO) machine, and data analyzed using Summit V4.3 software. Generally, 100,000 events per sample were collected after gating on lymphocytes by forward- and side-scatter.

A

B **C**

Figure 5. CTL recognition and cross-reactivity of wt p53_{72P} and p53_{72R} on SCCHN cells

Wt p53_{72P} and p53_{72R} specific CTL recognition of R/P donor (5A) and R/R donor (5B) of naturally processed peptide expressed by SCCHN cell lines SCC-4 (P/P), PCI-13 (R/R) and SCC-90 (R/P) using IFN- γ ELISPOT assays. Recognition was confirmed using stable transfectants of p53 null Saos-2 cells, which express only one p53 allele (72R or 72P, 5C) in the P/P donor. IFN- γ release was blocked by an anti-HLA class I mAb, but not an HLA class II specific mAb (not shown). No reactivity was observed using HLA-A2⁻ SCCHN cells (not shown). SCCHN cells were treated with IFN- γ (100 IU/ml for 72 hr at 37°C).