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Das p53 Onkoprotein als immunologische Zielstruktur bei Plattenepithelkarzinomen des Kopf-Hals-Bereichs

Habilitationsschrift der Hohen Medizinischen Fakultät
der Heinrich-Heine-Universität Düsseldorf zur Erlangung der Venia legendi
im Fach Hals-, Nasen- und Ohrenheilkunde
vorgelegt von

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2003

INHALTSVERZEICHNIS

1. THEMA

Das p53 Onkoprotein als immunologische Zielstruktur bei Plattenepithelkarzinomen des Kopf-Hals-Bereichs

2. EINFÜHRUNG IN DIE THEMATIK

Weltweit werden pro Jahr – bei ansteigender Tendenz – mehr als 500.000 bösartige Neuerkrankungen im Kopf-Hals-Bereich erwartet (Boring et al. 1992). Für die Bundesrepublik Deutschland ergibt sich aus den Daten der Krebsregister eine geschätzte jährliche Neuerkrankungsrate von wenigstens 10.000 bösartigen Kopf-Halstumoren (Muir et al. 1987). Histologisch handelt es sich hierbei in über 90% der Fälle um Plattenepithelkarzinome.

Für kleinere Karzinome der Stadien I und II ist die Operation und/oder Bestrahlung mit guten bis sehr guten Heilungserfolgen die Therapie der Wahl. Die wesentlich häufigeren fortgeschrittenen Kopf-Halskarzinome der Stadien III und IV besitzen aber trotz aggressiver lokoregionärer Behandlungsmaßnahmen – in aller Regel eine Kombination aus Operation und Bestrahlung – eine recht unbefriedigende Prognose. So entwickeln 50-60% dieser Patienten nach der Erstbehandlung ein Rezidiv, und in etwa 20-30% der Fälle treten Fernmetastasen auf. Die hierfür verantwortlichen, im Rahmen der Erstbehandlung nicht erfassten und oft mikroskopisch kleinen Tumorzellreste bieten sich in erster Linie für systemisch wirksame, adjuvante Behandlungsstrategien an, zum Beispiel eine Immuntherapie (Bier et al. 1994, Resser et al. 1998, Whiteside 2001, Hoffmann et al. im Druck).

Die Erwartungen, die an immunologische Konzepte der Tumorbehandlung geknüpft werden, beruhen in erster Linie auf der hohen Spezifität von Erkennung und Abwehr, durch die sich das Immunsystem auszeichnet. Dabei können prinzipiell zwei Arten der

Immuntherapie unterschieden werden: einerseits die passive und andererseits die den vorliegenden Arbeiten zugrundeliegende, aktive Immuntherapie; hierzu zählt u.a. das Konzept der sog. „Tumorimpfung“.

Im Gegensatz zur prophylaktischen Immunisierung gegen Infektionskrankheiten, bei der die neutralisierende humorale Immunität die Hauptrolle spielt, wird die Auslösung einer Antigen-spezifischen T-Zell Antwort als vorrangiges Ziel bei der Entwicklung von Tumorvakzinen angesehen (Pardoll 1998). In den letzten Jahren sind vor allem eine Reihe von Melanom-assoziierten Tumorantigenen isoliert, beschrieben und als immunogen erkannt worden. Die Suche nach geeigneten Antigenen bei bösartigen Tumoren epithelialen Ursprungs war bislang jedoch wenig erfolgreich. Als potentielle Tumorvakzine werden Mucin (MUC-1) und Her-2/Neu (ErbB-2) für Brust- bzw. Ovarialkarzinome sowie die E6/7 Proteine der sog. „high risk“ Humanen Papilloma Viren (HPV) für das Cervixkarzinom diskutiert. Bei Kopf-Hals-Karzinomen konnten bislang noch keine relevanten, für eine Tumorvakzine geeigneten Antigene identifiziert werden (Whiteside 2001, Hoffmann et al. im Druck).

Mutationen des Tumorsuppressorgens p53 sind mit Abstand die häufigste genetische Veränderung bei Plattenepithelkarzinomen des Kopf-Hals-Bereichs (Balz et al. 2003). Das mutierte p53-Genprodukt, das seine Funktion als Tumorsuppressor in aller Regel verloren hat, stellt somit einen möglichen Angriffspunkt für antitumorale Vakzinierungsversuche dar (DeLeo 1998, Offringa et al. 2000). Da Mutationen des p53 in vielen Fällen zur Akkumulation des aberranten Proteins in der Zelle führen (p53-Überexpression), kommt es hierüber wahrscheinlich zu einer verstärkten „Präsentation“ von p53 an das Immunsystem (DeLeo 1998, Offringa et al. 2000). Dabei werden die p53-Moleküle nach ihrer intrazellulären Aufspaltung in 8-11 Aminosäuren lange Bruchstücke als Peptidfragmente an MHC-Klasse-I Moleküle gebunden und schließlich an der Zelloberfläche immunologisch wirksam exprimiert. MHC-Klasse-I Moleküle agieren in Verbindung mit den so präsentierten Peptiden

als Erkennungssystem für T-Lymphozyten, wodurch möglicherweise eine sog. „MHC-Klasse-I restringierte“ Immunantwort von T-Zellen gegenüber p53-Peptiden eingeleitet wird.

Eine Immuntherapie auf der Basis mutierter p53-Peptide hätte allerdings den Nachteil, dass sie die individuelle Berücksichtigung der jeweils vorliegenden p53-Mutation erfordern würde. Deshalb könnte anstelle mutierter p53-Peptide der Einsatz von Wildtyp (Wt) p53-Peptiden als Tumorvakzine sinnvoll sein: Da es sich bei p53-Mutationen in der überwiegenden Anzahl der Fälle um Punktmutationen handelt, weisen die übrigen Abschnitte des aberranten p53-Proteins Wt-Eigenschaften auf. Diese nicht mutierten Wt p53-Abschnitte können im Rahmen der p53-Überexpression ebenfalls als Wt p53-Peptide verstärkt durch MHC-Klasse-I Moleküle präsentiert werden und stehen dann für die Generierung einer T-Zell-Immunantwort zur Verfügung (DeLeo 1998, Offringa et al. 2000, Parmiani et al. 1993, Soussi 1996).

Unabdingbare Voraussetzung für eine MHC-Klasse-I restringierte Antigenerkennung durch T-Zellen ist die Identifikation derjenigen Wt p53-Sequenzen, die überhaupt eine Bindung mit MHC-Klasse-I Molekülen eingehen können. Ein solches Fragment wurde mit dem Peptid der Position 264-272 innerhalb der p53-Aminosäuresequenz gefunden (Wt $p53_{264\text{-}272}$). Es wird an das MHC-Klasse-I Molekül HLA-A2.1 gebunden (Houbiers et al. 1993, Zeh et al. 1994) und darüber hinaus in p53-akkumulierenden Karzinomen spontan an der Zelloberfläche exprimiert (Theobald et al. 1995, Soussi et al. 1996, Röpke et al. 1996, Chikamatsu et al. 1999, Hoffmann et al. 2000[a]). Wt $p53_{264\text{-}272}$ bietet sich somit als potentielles Zielantigen für die Erkennung durch entsprechend sensibilisierte Wt $p53_{264\text{-}272}$-spezifische T-Zellen an.

Die suffiziente Induktion einer anti-p53 T-Zell Abwehr erfordert die Kooperation mit Antigen-präsentierenden Zellen. Hierzu eignen sich in besonderer Weise sog. „Dendritische Zellen“. Sie können wegen ihrer Expression von MHC-Molekülen und der Produktion von

kostimulatorischen Molekülen in besonderer Weise die Antigenpräsentation und somit T-Zell-Stimulation vermitteln (Steinman 1991). So lassen sich beispielsweise tumorassoziierte Peptide direkt an MHC-Klasse-I Moleküle Dendritischer Zellen koppeln (zelluläre Vakzine) und der T-Zell Abwehr präsentieren (Gilboa et al. 1998). Im Mausmodell gelang hierdurch bereits die effektive Immunisierung mit Wt p53-Peptid, d.h. es konnte eine zytotoxische T-Zell-Antwort gegen verschiedene p53-überexprimierende Sarkome induziert werden (Mayordomo et al. 1995 und 1996).

Das HLA-A2.1 bindende Wt $p53_{264-272}$ weist die Aminosäuresequenz LLGRNSFEV auf. Innerhalb dieser Sequenz sind die Positionen 2, 3, 9 für die Bindung an das HLA-A2.1 Molekül verantwortlich, wohingegen die Positionen 4, 5, 8 für die Erkennung durch den T-Zell-Rezeptor benötigt werden (Houbiers et al. 1993). Der gezielte Austausch einzelner Aminosäuren innerhalb der originalen Peptidsequenz kann daher Auswirkungen auf seine Interaktion mit dem MHC-Klasse I Molekül und/oder T-Zell-Rezeptor haben, und so unterschiedliche Immunantworten auslösen (Boehnke et al. 1993, Berzofsky 1995, Loftus et al. 1996 und 1998).

3. ZIELSETZUNG DER UNTERSUCHUNGEN

In einer Serie von *in vitro* Experimenten sollte die Eignung von Wt $p53_{264-272}$ als antigene und vor allem immunogene Struktur in p53-akkumulierenden Plattenepithelkarzinomen der Kopf-Hals-Region untersucht werden (**Teil 1. Wt $p53_{264-272}$**). Um für die Charakterisierung der Interaktion zwischen Wt $p53_{264-272}$-spezifischen T-Zellen und korrespondierendem Tumor ein möglichst realistisches Bild der *in vivo* Situation zu erhalten, wurde eine neuartige Untersuchungs-Technologie zur Bestimmung der relevanten Lymphozytenpopulation herangezogen (**Teil 2. Tetramer Studie**). Schließlich sollte die Immunogenität des verwendeten Wt $p53_{264-272}$ durch den Austausch einzelner Aminosäuren innerhalb der originalen Peptidsequenz erhöht werden (**Teil 3. $p53_{264-272}$-Varianten**). Gemeinsam dienen diese Versuche der Vorbereitung einer möglicherweise klinisch einsetzbaren p53-Vakzine bei Patienten mit Plattenepithelkarzinomen der Kopf-Hals-Region.

4. MATERIAL UND METHODEN

4.1 Teil 1. Wt $p53_{264-272}$

4.1.1 Wt $p53_{264-272}$

Das HLA-A2.1-bindende Wt $p53_{264-272}$ wurde mit der Standard Fmoc Methode synthetisiert, mittels HPLC aufgereinigt und in seiner Sequenz durch Massenspektroskopie bestätigt.

4.1.2 HLA-A Subtypisierung

Die HLA-A Subtypisierung der Lymphozyten und Tumorzellen erfolgte durchflußzytometrisch mit einem anti-HLA-A2 Antikörper (BB7.2). Für die HLA-A2.1 Subtypisierung wurden die Exone 2-4 des HLA-A Antigens aus der genomischen DNA amplifiziert. Die Bestimmung der jeweiligen Allele erfolgte durch Exon-spezifische Primer und Cycle-Sequencing mit nachfolgender Sequenzanalyse.

4.1.3 Herstellung und Charakterisierung von Dendritischen Zellen

Für die Generierung HLA-$A2.1^+$ Dendritischer Zellen (DZ) wurden mononukleäre Zellen (Peripheral-Blut Mononukleäre Zellen, PBMZ) mit der Ficoll-Hypaque Dichtegradient-Zentrifugation aus venösem Blut oder Leukozytenkonzentraten von gesunden Probanden sowie Kopf-Hals-Karzinompatienten isoliert. DZ wurden zunächst durch die Zugabe von GM-CSF und IL-4 aus Monozyten differenziert (Sallusto et al. 1994, Hoffmann et al. 2000[b]) und anschließend durch eine gezielte Ausreifung (proinflammatorischer Zytokincocktail aus 10ng/ml IL-1 β, 1000 U/ml IL-6, 10 ng/ml TNF α, 1 µg/ml PGE_2) in ihrer Eigenschaft als professionelle Antigen-präsentierende Zellen optimiert (Hoffmann et al. 2001, Abb. 1).

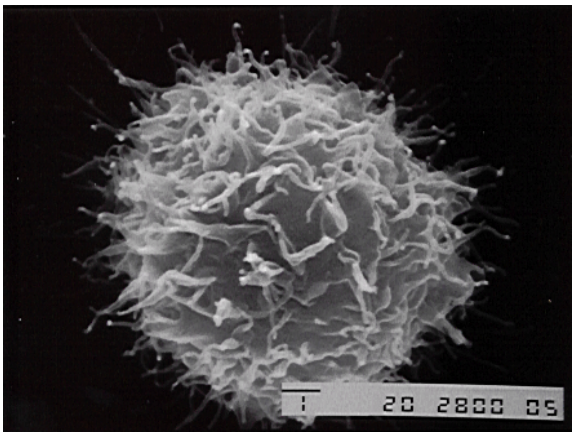

Abbildung 1. Dendritische Zelle in elektronenmikroskopischer Vergrößerung

Die Phänotypisierung erfolgte durchflußzytometrisch unter Verwendung von Fluoreszenzfarbstoff-konjugierten Antikörpern gegen HLA-Klasse I, HLA-DR sowie CD-14, CD-25, CD-40, CD-80, CD-83 und CD-86.

4.1.4 In vitro-Stimulation von T-Zellen mit Wt p53$_{264-272}$-beladenen DZ

Etwa 2·10^6 DZ wurden in PBS mit 10µg Wt p53$_{264-272}$/ml resuspendiert und für 4 h bei 37°C inkubiert (Tag 0). Danach wurden die Peptid-beladenen DZ mit autologen PBMZ (beinhaltet T-Zellen) im Verhältnis 1:10 in AIM-V Medium mit 10% humanem AB-Serum und 25 ng/ml IL-7 inkubiert. An Tag 3 wurden 20 IU/ml IL-2 zugegeben und die Zellen an Tag 7 sowie wöchentlich danach mit Peptid-beladenen DZ restimuliert und expandiert, um eine antitumorale, Wt p53$_{264-272}$-induzierte T-Zell Antwort hervorzurufen (Abb. 2).

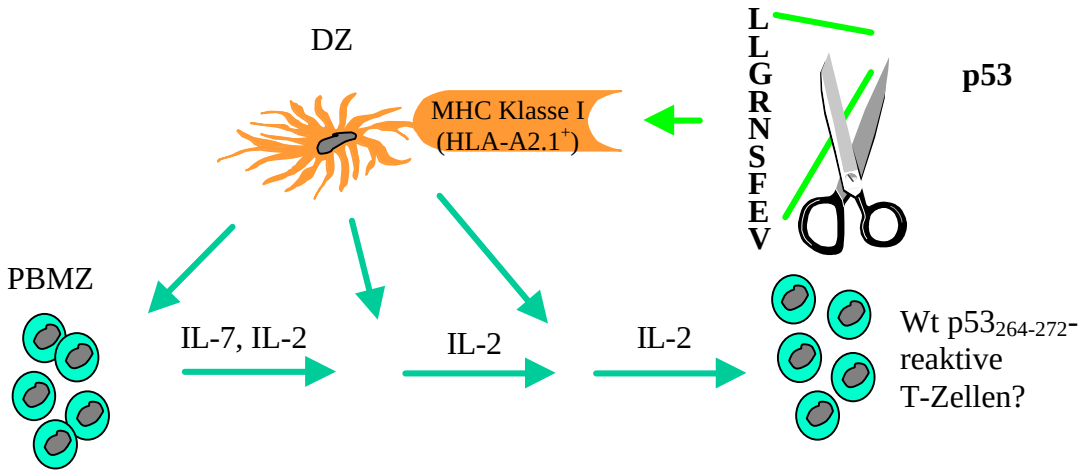

Abbildung 2. Schematische Darstellung der Stimulation von PBMZ durch DZ, die Wt p53$_{264-272}$ präsentieren (Hoffmann et al. 2000).

4.1.5 Auswertung der Immunantwort

Die Untersuchungen zur Spezifität und Reaktivität der stimulierten T-Zellen erfolgte mit verschiedenen Kopf-Hals-Karzinomzellinien (*endogene* Peptidexpression), der Peptid-beladenen, TAP (Transporter in Antigen Processing mit freien HLA-A2.1^{+} Bindungsstellen) – defizienten Zellinie T2 (*exogene* Peptidexpression) und Kontrollzellen (z.B. HLA-A2.1^{-} oder p53 normal exprimierende Tumorzellinien).

Die *Spezifität* der T-Zellen wurde durch Fluorochrom-konjugierte Wt $p53_{264-272}$-MHC-Klasse I Komplexe (sog. Tetramere, siehe unten) nachgewiesen. Die Zellen wurden hierfür mit Phycoerythrin (PE)-markiertem Tetramer, Fluorescein Isothiocyanate (FITC)-konjugiertem anti-CD8 Antikörper sowie Peridinin Chlorophyll-a Protein (PerCP)-konjugiertem anti-CD3 Antikörper markiert und der 3-Farben Durchflußzytometrie zugeführt.

Die *Reaktivität* der T-Zellen wurde nach Abschluss der Stimulation in zwei verschiedenen Testsystemen überprüft: Einerseits mit dem Interferon (IFN)-γ-ELISPOT zur Darstellung der IFN-γ Produktion der T-Zellen auf Einzelzellniveau und andererseits mit dem ^{51}Cr-Freisetzungs-Test zur Bestimmung der T-Zell-Zytotoxizität. Als Zielzellen der T-Zell-Reaktivität wurden T2-Zellen (*exogene* Peptidexpression) und verschiedene Tumorzellinien (*endogene* Wt $p53_{264-272}$) eingesetzt. Unter den letztgenannten befanden sich HLA-A2.1 positive, $p53_{264-272}$-präsentierende Kopf-Hals-Karzinomlinien (PCI-13, SCC-9), eine mit Wt p53 transfizierte Sarkomzellinie (SaOS-2Cl3), und Zellinien, die das Zielpeptid entweder nicht präsentieren können oder einen anderen HLA-A Subtyp aufweisen (SCC-4, PCI-4B, SaOS-2).

4.1.6 Bestimmung des p53-Status

Die p53-Proteinexpression wurde immunhistochemisch bestimmt, wobei der Tumor als p53-positiv bewertet wurde, wenn mehr als 25% der Tumorzellen eine Färbeintensität von mehr als 2 auf einer Skalierung von 0 bis 4 aufwiesen.

Für die p53-Sequenzanalyse wurde Tumorzell-RNA mit der Reverse-Transkriptase Polymerase-Kettenreaktion (RT-PCR) in cDNA umgeschrieben und die Exone 5 bis 8 des p53-Gens unter Verwendung entsprechender Primer amplifiziert. Die Analyse der PCR-Produkte erfolgte in einer zyklischen Sequenzier-Reaktion, elektrophoretischen Auftrennung der Sequenzierprodukte und abschließenden Auswertung mit einer entsprechenden Software.

4.2 Teil 2. Tetramer Studie

4.2.1 Patienten und Kontrollen

Peripheralblut von 30 HLA-A2.1$^+$ Kopf-Hals-Karzinompatienten und 31 HLA-A2.1$^+$ gesunden Probanden wurde mit der Ficoll-Hypaque Dichtegradient-Zentrifugation aufgearbeitet, um mononukleäre Zellen (PBMZ) zu gewinnen. Diese Präparationen wurden unmittelbar (ohne weitere *in vitro* Manipulation) der weiteren Untersuchung zugeführt.

4.2.2 Vier-Farben–Durchflußzytometrie

Für die Vier-Farben–Durchflußzytometrie wurden als Antikörper anti-CD14-FITC, anti-CD3-Phycoerythrin-Texas Red (ECD) und anti-CD8-Phycoerythrin Cyanin-5.1 (PC5) verwendet. Das vierte Fluorochrom (PE) wurde für die Markierung des Tetramers reserviert. Tetramerisch angeordnete, mit einem Fluorochrom markierte Peptid-MHC-Klasse I Moleküle (sog. Tetramere, Abb. 3) erlauben den Nachweis der Bindung spezifischer CD8$^+$ T-Zellen am

korrespondierenden T-Zell-Rezeptor (TZR) mit Hilfe der Durchflußzytometrie (Altman et al. 1996, Meidenbauer et al. 2003).

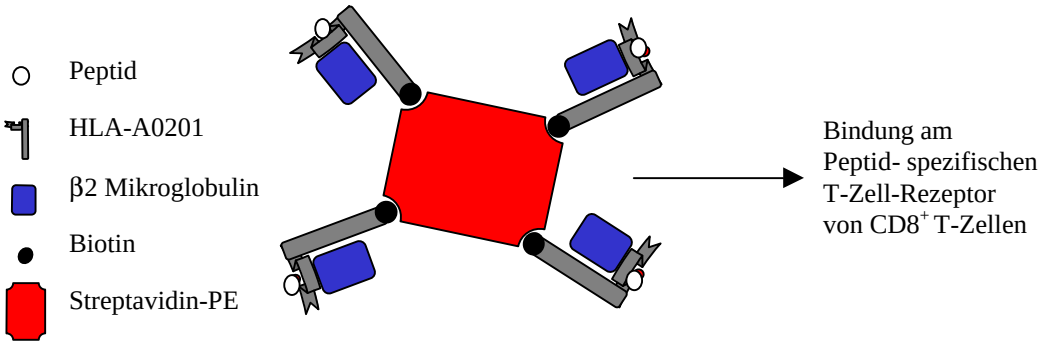

Abbildung 3. Modell eines tetramerischen Peptid-MHC-Klasse I Komplexes (Tetramer).

Als Peptide wurden für die Experimentalgruppe LLGRNSFEV (Wt $p53_{264-272}$), als Positivkontrolle GILGFVFTL (Influenza Matrix Peptid 58-66) und als Negativkontrolle ILKEPVHGV (HIV-1 Reverse Transkriptase pol 476-484) verwendet. In Vorversuchen fanden Titrationsreihen sowie Kontrollen mit HLA-$A2.1^+$ und HLA-$A2.1^-$ Lymphozyten statt, darüber hinaus wurden auch Peptid-spezifische T-Zellinien getestet. Nach Inkubation der PBMZ mit den jeweils relevanten Tetrameren und Antikörpern wurden unter Verwendung eines Coulter Epics XL Durchflußzytometers mindestens $1x10^6$ Zellen/Experiment gemessen, und die Datenanalyse erfolgte mit der WinList Software (Verity SW House, Topsham, USA).

4.3 Teil 3. $p53_{264-272}$-Varianten

4.3.1 $p53_{264-272}$-Varianten Vorauswahl

Um die Immunogenität des verwendeten Wt $p53_{264-272}$-Antigens zu erhöhen, wurden über ein Computer-Simulationsprogramm einzelne Aminosäuren innerhalb der originalen Peptidsequenz ausgetauscht. Unter Aussparung der Ankerpositionen 2 und 9 entstand hierbei

eine Serie von 19 Peptid-Varianten: 264E; 264F; 264V; 266L; 266F; 266W; 267K; 267L; 268K; 268L; 269G; 269T; 269Y; 270L; 270P; 270W; 270Y, 271A; 271Y. Auch diese Peptid-Varianten wurden wie oben beschrieben synthetisiert, aufgereinigt und in ihrer Sequenz bestätigt.

Von diesen Peptid-Derivaten wurden dann in einer Vorauswahl besonders immunogene Varianten identifiziert, die für weitere Untersuchungen bzw. *in vitro* Stimulationen Verwendung finden sollten. Hierzu diente eine etablierte, $p53_{264-272}$-spezifische T-Zellinie (Chikamatsu et al. 1999), deren Zytotoxizität gegen die mit den genannten Peptid-Varianten beladene Zellinie T2 bestimmt wurde. Schließlich konnten drei Peptid-Varianten - 269T, 270W und 270P - identifiziert werden, die eine besonders ausgeprägte, zytotoxische T-Zell-Antwort auslösten.

4.3.2 T-Zell-Rezeptor Repertoire

Von Wt $p53_{264-272}$-spezifischen T-Zellinien, die mit dem originalen $p53_{264-272}$-Peptid oder der immunogenen 270W-Variante stimuliert worden waren, wurde RNA extrahiert und in cDNA umgeschrieben. Es folgten ein Screening für die Expression der variablen Ketten des TZR durch Primer für die TZR Vβ Amplifikation nach Puisieux et al. (1994) sowie eine Run-off Reaktion mit Fluorophore-markierten, für die konstante Region der TZRβ-Kette spezifischen Primern. Die markierten Run-off Produkte wurden einer DNA-Fragment Analyse und die amplifizierten Produkte direkt einer DNA-Sequenz-Analyse zugeführt.

Die weiteren Schritte sind identisch mit denen von 4.1.2. - 4.1.6.

5. ERGEBNISSE UND DISKUSSION

5.1 Teil 1. Wt $p53_{264-272}$

Ursprünglich waren wir davon ausgegangen, dass nur bei solchen Patienten eine anti-p53 Immunantwort ausgelöst werden kann, deren Tumore eine p53-Akkumulation aufweisen. Um so überraschender waren die in der nachfolgenden Tabelle zusammengefassten Ergebnisse:

Patient	*in vitro* Stimulation: anti-p53-Immunantwort	Tumorstatus: p53-Mutation	Tumorstatus: $p53_{264-272}$-Präsentation
1	-	E7-248 R-W	+
2	-	E5-157 V-F	+
3	-	E8-286 E-K	+
4	-	E6-213 stop	-
5	+	Wt	-
6	+	Wt	-
7	+	E8-273 R-H	-

Tab. 1. Beziehung zwischen anti-p53 T-Zell-Immunantwort und p53-Tumorstatus bei sieben Patienten mit Plattenepithelkarzinomen des Kopf-Hals-Bereichs (Hoffmann et al. 2000[a]).

Demnach konnte *in vitro* bei 3 von 7 Patienten eine Immunantwort gegen das Wt-Peptid ausgelöst werden. Bei 4 der 7 Patienten war allerdings keine anti-p53 Immunantwort induzierbar, obwohl die hier vorliegenden p53-Mutationen in 3 der 4 Fälle zu einer p53-Akkumulation führen und somit eine (eigentlich) wichtige Voraussetzung für die Präsentation

des Wt $p53_{264-272}$ erfüllen. Demgegenüber hatten die 3 Patienten, bei denen eine anti-p53 Immunantwort auslösbar war, Tumore, die entweder einen p53-Wildtyp aufwiesen (hier erfolgt keine Protein-Akkumulation) oder eine p53-Mutation trugen (E8-273 R-H), die keine Präsentation von Wt $p53_{264-272}$ erlaubt (Theobald et al. 1998). Somit „fehlte“ in allen 3 Fällen, in denen nachweislich eine anti-p53 Immunantwort ausgelöst wurde, die eigentlich relevante Zielstruktur (Hoffmann et al. 2000[a]).

Hieraus leiteten wir die Hypothese ab, dass unter dem immunologischen Druck von Wt $p53_{264-272}$-spezifischen T-Zellen eine Immunselektion von Tumorvarianten stattfindet, die eben dieses Wt $p53_{264-272}$-Epitop verloren haben und sich hierdurch dem Angriff durch Wt $p53_{264-272}$-spezifische T-Zellen entziehen (Abb. 4).

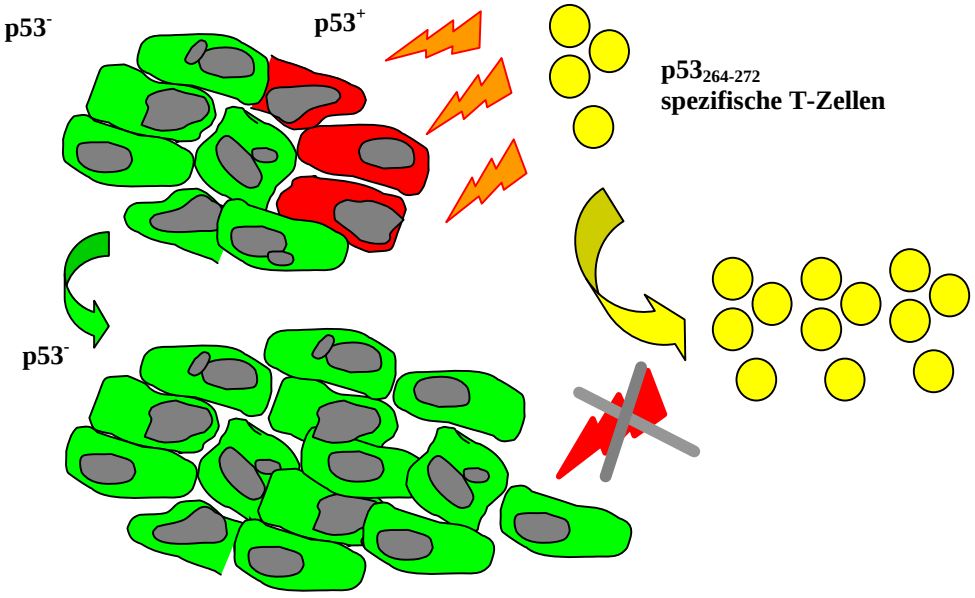

Abbildung 4. Immunselektions-Hypothese: Wt $p53_{264-272}$-spezifische T-Zellen greifen einen p53-heterogenen Tumor an und p53-akkumulierende Tumoranteile ($p53^+$) werden eliminiert, wodurch es zum Auswachsen eines Wt $p53_{264-272}$-nicht-exprimierenden Tumors ($p53^-$) kommt.

Die Überprüfung der *Immunselektions-Hypothese* für die *in vivo* Situation erfolgte mit der Tetramer Studie.

5.2 Teil 2. Tetramer Studie

Da die Häufigkeit von Antigen- und somit auch $p53_{264-272}$-spezifischen T-Zellen im Peripheralblut in aller Regel gering ist, mußte zunächst eine sensitive und spezifische Durchflußzytometrie-Technik entwickelt werden. Durch die Anwendung von insgesamt vier Farben konnten einerseits unspezifische Ereignisse ausgeschlossen werden und andererseits die $CD8^+$/$Tetramer^+$ Ereignisse hochsensitiv bestimmt werden (Abb.5; Hoffmann et al. 2000[c]).

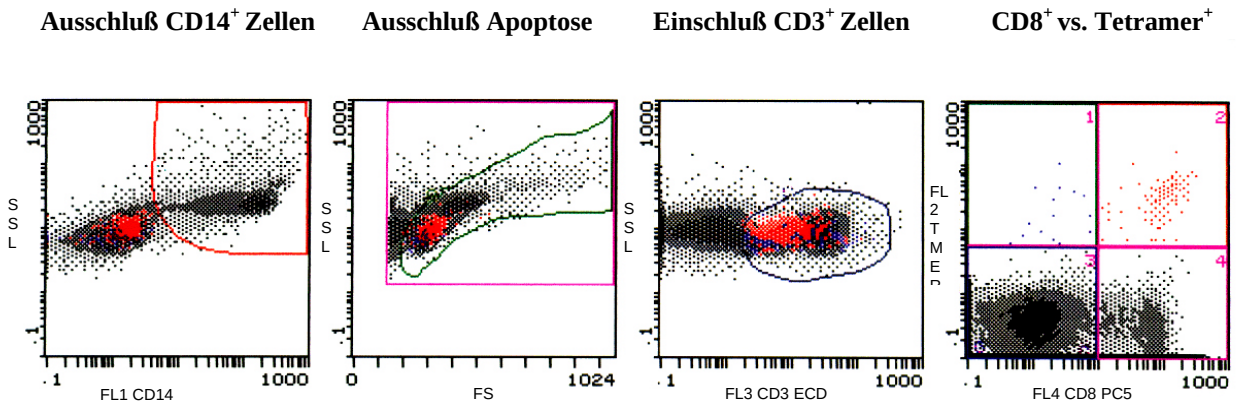

Abbildung 5. Vier-Farben-Durchflußzytometrie zur Erhöhung der Spezifität und Sensitivität. In der rechten Punktewolke kommen nach Ausschluß von $CD14^+$ sowie apoptotischen Zellen und dem Einschluß von $CD3^+$ T-Zellen im oberen rechten Quadranten die $Tetramer^+$ und $CD8^+$ T-Zellen zur Darstellung.

Mit dieser Vier-Farben-Durchflußzytometrie-Technik wurden PBMZ von 30 Patienten mit einem Kopf-Hals-Plattenepithelkarzinom und 31 gesunden Kontrollen auf die Häufigkeit sowie den Phänotyp der $p53_{264-272}$-spezifischen $CD8^+$ T-Zellen untersucht, und die Ergebnisse der Tumorgruppe mit dem jeweiligen p53-Status verglichen (Hoffmann et al. 2002[a]).

Patienten mit Kopf-Hals-Karzinomen hatten im Vergleich mit dem Kontrollkollektiv signifikant höhere Frequenzen von $p53_{264-272}$-spezifischen $CD8^+$ T-Zellen. Darüber hinaus zeigte die Korrelation mit dem p53-Tumorstatus ein eindrucksvolles Bild: Patienten mit

normaler p53-Proteinexpression des Tumors, also geringem Potential einer $p53_{264-272}$-Peptidpräsentation, oder mit einer p53-Mutation, die eine Epitop-Präsentation verhindert, wiesen sehr hohe Frequenzen von $p53_{264-272}$-spezifischen T-Zellen auf (Abb. 6).

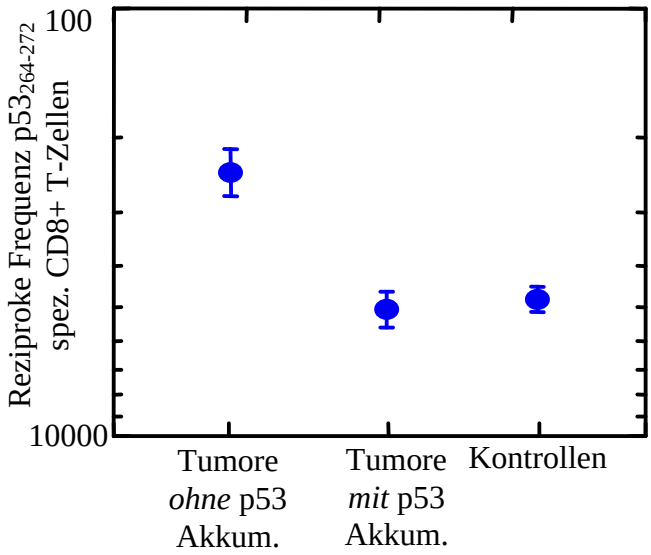

Abbildung 6. Reziproke Frequenz von Wt $p53_{264-272}$-spezifischen $CD8^+$ T-Zellen bei Patienten mit Plattenepithelkarzinomen des Kopf-Hals-Bereichs und Kontrollpersonen.

Ein beträchtlicher Anteil der $p53_{264-272}$-spezifischen T-Zellen bei Patienten mit Tumoren ohne p53-Akkumulation entsprach dem sog. Gedächtnis-Phänotyp ($CD45RO^+$), was auf einen stattgehabten Kontakt mit dem p53-Antigen hinweist. Im Gegensatz hierzu hatten Patienten mit p53-akkumulierenden Tumoren deutlich niedrigere Frequenzen von Wt $p53_{264-272}$- spezifischen T-Zellen, von denen außerdem die Mehrzahl dem naiven Phänotyp ($CD45RA^+$) zuzurechnen waren (Hoffmann et al. 2002[a]). Diese reziproke Verteilung lässt vermuten, dass *in vivo* durch die Präsenz von Wt $p53_{264-272}$-spezifischen T-Zellen solche Tumorzellen selektiert werden, die das relevante Epitop Wt $p53_{264-272}$ verloren haben und deshalb der Abwehr durch Wt $p53_{264-272}$-spezifische T-Zellen entkommen sind. Diese Konstellation bestätigt somit das entworfene Immunselektions-Modell (siehe Abb. 4), das ein erhebliches Problem bei der Entwicklung einer p53-basierten Immuntherapie darstellen dürfte.

Vor diesem Hintergrund bot sich eine Modifikation der verwendeten p53-Peptide an. In einer weiterführenden Serie von Experimenten sollte daher überprüft werden, wie sich die Immunogenität des ursprünglich verwendeten Wt $p53_{264-272}$-Peptids erhöhen läßt.

5.3 Teil 3. $p53_{264-272}$-Varianten

Unter Verwendung eines Computer-unterstützten, dreidimensionalen Modells des Wt $p53_{264-272}$-MHC-Klasse I Komplexes wurden durch den Austausch einzelner Aminosäuren 19 Varianten des ursprünglichen Wt $p53_{264-272}$ generiert. Zwei dieser $p53_{264-272}$-Varianten (270W und 269T) induzierten *in vitro* eine anti-p53 Immunantwort bei primär nicht reagierenden (toleranten) Patienten (Tab. 2). Damit erhöhte sich nicht nur die Gesamtfrequenz für die *in vitro* Induktion einer anti-p53 Immunantwort, sondern es konnte durch diesen Schritt auch das grundsätzliche Problem der Toleranz durchbrochen werden. So ergab sich die Induktion einer anti-Wt $p53_{264-272}$-Immunantwort für Patient #3 durch die *in vitro* Stimulation mit der 270W-Peptid-Variante (Umkreisung in Tab. 2). Dies war insofern beachtenswert, als der entsprechende Tumor eine p53-Akkumulation aufwies und sich die Lymphozyten dieses Patienten gegenüber der primären Stimulation mit Wt $p53_{264-272}$ refraktär verhielten. Zudem ließ sich die erfolgreich induzierte anti-p53 Immunantwort auch gegenüber der aus dem Tumor etablierten, autologen Zellinie (PCI-13) nachweisen. Basierend auf dieser *in vitro* Antwort würde sich der Patient gut für eine p53-basierte Immuntherapie mit der $p53_{264-272}$-Variante 270W eignen.

Patient	p53 Mutation	Wt $p53_{264-272}$-Präsentation	Antwort auf originalem $p53_{264-272}$	IVS mit variantem $p53_{264-272}$
1	E7-248 R-W	+	-	-
2	E5-157 V-F	+	-	-
3	E8-286 E-K	+	-	+
4	E6-213 stop	-	-	+
5	Wt	-	+	-
6	Wt	-	+	+
7	E8-273 R-H	-	+	+

Tab. 2. Beziehung zwischen p53-Tumorstatus und anti-p53 T-Zell Immunantwort unter Verwendung von Wt $p53_{264-272}$ und $p53_{264-272}$-Varianten bei sieben Patienten mit Plattenepithelkarzinomen des Kopf-Hals-Bereichs (Hoffmann et al. 2002[b]). (IVS, *in vitro* Stimulation; E7–248 R-W, Mutation in Exon 7 mit Austausch der Aminosäure R (Arginin) durch W (Tryptophan) bei Position 248).

Abschließend wurde das TZR-Repertoire der mit originalem und variantem $p53_{264-272}$-Peptid stimulierten T-Zellen untersucht. Die über eine sog. Limitierende Verdünnng gewonnenen oligoklonalen Zellinien wurden hinsichtlich der variablen β Kette des TZR überprüft (Tab. 3).

Zell-linie	IVS	Spezi-fität	Vb Familie	Vb-Sequenz	CDR3 Region	Joining Region
2	Wt	Wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3
4	Wt	Wt + 7W	Vβ1	LELGDSALYFCA	SSEGGL	ETQYFGPGTRL/BJ2-5
53	7W	7W	Vβ9	LGDSAVYFCA	SSAGTNT	YEQYFGPGTRLTVT/BJ2-7
68	7W	Wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3

Tab. 3. Aminosäure-Sequenz der monoklonalen TZR-Transkripte von vier originalen und/oder varianten $p53_{264-272}$-spezifischen, zytotoxischen T-Zellinien (Hoffmann et al. 2002[b]). (IVS, *in vitro* Stimulation)

Hierbei zeigte sich, dass die mit originalem $p53_{264-272}$-Peptid stimulierten T-Zellen hinsichtlich ihrer TZR Vβ Kette einen Vβ 13.6 oder Vβ 1 Subtyp aufwiesen (Zellinie 2 und 4). Andererseits wurde in den mit der 270W Peptid-Variante stimulierten T-Zellen ein Vβ 9 Subtyp vorgefunden, wenn deren Reaktivität lediglich gegen das 270W Peptid gerichtet war (Zellinie 53). Die gegen originales und variantes Peptid reaktive Zellinie 68 wies hingegen einen Vβ 13.6 Subtyp auf, ist also absolut identisch mit der von einem anderen Patienten etablierten Zellinie 2. Diese Beobachtung, dass spezifisch stimulierte und offensichtlich kreuzreagierende T-Zellen von unterschiedlichen Patienten identische Merkmale (TZR/CDR3/Joining-Region) tragen, verdeutlicht das immuntherapeutische Potential der 270W-Variante. Zudem können diese Informationen für die Entwicklung einer zweiten Generation von stärker immunogenen p53-Peptid-Varianten herangezogen werden.

6. ZUSAMMENFASSUNG

Mit den vorliegenden Arbeiten wurde untersucht, ob das Wildtyp $p53_{264-272}$ -Peptid sowie hiervon abgeleitete Peptid-Varianten in Verbindung mit Dendritischen Zellen möglicherweise als Tumorvakzine zur adjuvanten Immuntherapie von Kopf-Hals-Karzinomen eingesetzt werden können. Die Untersuchungen zeigten zwei immunologische Besonderheiten, die bei der Planung einer p53-basierten Immuntherapie berücksichtigt werden müssen: Lymphozyten von Patienten mit einem p53-überexprimierenden Tumor weisen eine Toleranz gegenüber der *in vitro* Stimulation mit dem Wildtyp $p53_{264-272}$ -Peptid auf, und sowohl *in vitro* als auch *in vivo* wurde eine „Immun-Konstellation“ vorgefunden, die als Immunselektions-Modell beschrieben wird. Durch den Einsatz von $p53_{264-272}$-Peptid-Varianten konnte die beobachtete Toleranz gegenüber dem Wildtyp $p53_{264-272}$-Antigen durchbrochen werden. Somit stellen Varianten des $p53_{264-272}$-Epitops einen Ansatz zur Toleranzüberwindung im Rahmen der p53-basierten Immuntherapie dar.

7. AUSBLICK

Eine von Brenman et al. im Jahre 1995 veröffentlichte Studie an Operationspräparaten von Kopf-Hals-Karzinomen beschrieb in etwa der Hälfte der untersuchten Fälle „p53-positive Tumorzellen“ im Bereich der Resektionsränder, obwohl in der alleinigen histopathologischen Beurteilung eine R0-Resektion beschrieben worden war. Diese Patienten hatten im Vergleich zu Patienten mit ”p53-negativen” Rändern eine deutlich erhöhte Rate von Lokalrezidiven. Die p53-positiven Zellen müssen demnach als histomorphologisch nicht detektierbare, kleinste Tumorzellnester angesehen werden, die einer weiteren Behandlung bedürfen – derzeit geschieht dies in aller Regel mit der postoperativ durchgeführten Strahlentherapie.

Die vorgestellten Untersuchungen bilden die entscheidende Grundlage für die Entwicklung einer p53-Tumorvakzine. Wildtyp $p53_{264-272}$-Peptid bzw. hiervon abgeleitete Peptid-Varianten in Verbindung mit einer wirkungsvollen Antigen Präsentation durch Dendritische Zellen eignen sich möglicherweise zur adjuvanten Immuntherapie von Patienten mit Kopf-Hals-Karzinomen, die nach kurativ intendierter Standardtherapie ein hohes Rezidivrisiko aufweisen.

8. LISTE DER VERWENDETEN ABKÜRZUNGEN

CD	Cluster of Differentiation
DZ	Dendritische Zelle
ECD	Phycoerythrin-Texas Red
FITC	Fluorescein Isothiocyanate
GM-CSF	Granulozyten-Makrophagen Kolonie-stimulierender Faktor
HLA	Human Leukocyte Antigen
HPV	Humanes Papilloma Virus
IFN	Interferon
IL	Interleukin
IVS	In vitro Stimulation
MHC	Major Histocompatibility Complex
PBMZ	Peripheral-Blut mononukleäre Zellen
PBS	Phosphate Buffered Saline
PC5	Phycoerythrin Cyanin-5.1
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll-a Protein
PGE	Prostaglandin E
RT-PCR	Reverse-Transkriptase-Polymerase-Kettenreaktion
TAP	Transporter associated with antigen processing
TNF	Tumor Nekrose Faktor
TZR	T-Zell-Rezeptor
Wt	Wildtyp

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10. DANKSAGUNG

Herrn Univ.-Prof. Dr. U. Ganzer danke ich verbindlichst für die Ermöglichung des Auslandsstipendiums und die großzügige Förderung meiner klinischen und wissenschaftlichen Laufbahn.

Mein besonderer Dank gilt Herrn Prof. Dr. H. Bier, der bei allen Arbeiten eine tragende Rolle spielte. Besonders zu erwähnen ist sein außergewöhnliches Engagement bei der Etablierung der amerikanischen Kooperation, der Planung und Verwirklichung des Auslandstipendiums wie auch die umfassende und großzügige Unterstützung der Arbeiten nach meiner Rückkehr an die HNO-Klinik der Universität Düsseldorf. Ich danke ihm für seine Zeit, seine Mühen und sein Vertrauen.

Den amerikanischen Kooperationspartnern und Mentoren am Cancer Institut der Universität Pittsburgh, namentlich Frau Prof. Dr. T.L. Whiteside, Herrn Prof. Dr. A.D. DeLeo, sowie Herrn Prof. Dr. A.D. Donnenberg und Frau Priv. Doz. Dr. V. Donnenberg bin ich zu großem Dank verpflichtet. Die wissenschaftlichen und auch freundschaftlichen Interaktionen ließen ein einzigartiges Arbeitsklima entstehen. An dieser Stelle sei auch den klinischen Kooperationspartnern Herrn Prof. Dr. E. Myers und Herrn Prof. Dr. J. Johnson gedankt.

Ferner danke ich Frau Dr. V. Balz und den Medizinisch Technischen Assistentinnen im HNO-Forschungslabor für die Weitergabe ihrer praktischen Erfahrungen und das angenehme Arbeitsklima.

Ich danke von Herzen meiner Frau Ulrike für die andauernde Unterstützung, die Motivation, sowie die partnerschaftliche und wissenschaftliche Kooperation bei der Durchführung der Studien.

Ich danke meinen Eltern.

Die vorliegende Arbeit wurde von der Deutschen Krebshilfe gefördert (Postgraduierten Stipendium der Dr. Mildred Scheel Stiftung für Krebsforschung).

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Drittmittel: Postgraduierten Stipendium der Dr. Mildred Scheel Stiftung für Krebsforschung „In vitro Versuche zur Entwicklung von p53-abgeleiteten Vakzinen für Plattenepithelkarzinome des Kopf-Hals-Bereichs“ (1.2.99-31.1.01)

Sachbeihilfe der Deutschen Krebshilfe „Humane Papillomviren in Plattenepithelkarzinomen des Oropharynx“ (70-2976-HoI, 2003 - 2006)

Im Begutachtungsverfahren: Neuantrag auf Einzelförderung durch die Forschungskommission der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf „Funktionelle Relevanz von Chemokinen im Metastasierungsprozeß von Kopf-Hals-Karzinomen“

Preise: Meidenbauer N, Hoffmann TK, Müller-Berghaus J, Whiteside TL. Cytokines augment the cross-presentation and cross–priming-capability of human dendritic cells internalizing apoptotic tumor cells. Presidential Award Session des 1st International Scientific Meeting of the Society for Biological Therapy, Berlin, 15. September, 2000.

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12. LITERATURVERZEICHNIS

Originalarbeiten

Hervorgehoben sind die im engen inhaltlichen und zeitlichen Zusammenhang erschienen Arbeiten, die als Grundlage für die vorliegende kumulative Habilitationsschrift dienten.

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Summe Impact Faktor (2001) – 85.4
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13. EINE AUSWAHL DER ORIGINALARBEITEN

[CANCER RESEARCH 60, 3542–3549, July 1, 2000]

Generation of Tumor-specific T Lymphocytes by Cross-Priming with Human Dendritic Cells Ingesting Apoptotic Tumor Cells[1]

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ABSTRACT

It has been suggested that dendritic cells (DCs) are capable of ingesting apoptotic tumor cells (ATCs) and presenting tumor-associated antigens to immune cells. We evaluated the potential of human DCs, which have ingested ATCs, to serve as a source of antigenic epitopes for presentation to T cells specific for PCI-13, a squamous cell carcinoma of the head and neck cell line. Immature DCs (DC_{imm}) generated in the presence of interleukin 4 and granulocyte machrophage colony-stimulating factor from peripheral blood monocytes of HLA-$A2^+$ healthy donors were incubated in the presence of ATCs. Uptake of ATCs by DCs was monitored by flow cytometry and confocal microscopy after 2–18 h of coincubation. When DCs were matured (DC_{mat}) in the presence of proinflammatory cytokines, their capacity to uptake ATCs was reduced. Responses of PCI-13-specific $CD8^+$ T cells to unmodified PCI-13 cells and to DC_{imm} or DC_{mat} +/− ATCs or +/− tumor lysates were tested in γ-IFN enzyme-linked immunospot and cytotoxicity assays. Unmodified tumor cells were found to be the best stimulators of antitumor activity of the established T-cell line, and ATCs alone were minimally stimulatory. However, DCs that ingested ATCs were able to present tumor antigens to CTLs, and DC_{imm} and DC_{mat} were almost equally stimulatory. When DCs plus various tumor-derived preparations were used as antigen-presenting cells with autologous HLA-$A2^+$ T cells obtained from normal donors, DCs that had ingested ATCs were more effective in generating $CD8^+$ CTLs than tumor cells alone or DCs pulsed with tumor lysates. The results indicate that human DCs fed with ATCs and then matured effectively generated T cell-mediated antitumor responses *in vitro*.

INTRODUCTION

CTLs[4] are a critical component of the immune response to human tumors, and induction of strong CTL responses is the goal of most current cancer vaccine strategies. CTL target tumors through the recognition of a self-MHC class I molecule and an antigenic peptide generally derived from endogenous tumor cell proteins. However, for CTL induction and expansion, the antigenic peptide has to be presented to precursor T cells in the context of costimulatory molecules usually provided by professional APCs. Delivery of exogenous antigens to the endogenous MHC class I-restricted processing pathway in professional APCs is a critical challenge in cancer vaccine designs. DCs are potent APCs, which can uptake exogenous proteins. Peptides generated from these proteins are cross-presented by DCs on class I MHC molecules to T cells and, on successful T-cell receptor-mediated recognition, can induce antigen-specific CTL responses (1–5). However, priming with DCs for generation of tumor-specific responses usually requires prior definition of tumor-derived antigens and characterization of the epitopes involved. With the exception of melanoma and renal cell carcinoma, however, few TAAs have been defined and cloned thus far (6). In the majority of human cancers, where the TAAs are unknown, DCs coincubated or pulsed with tumor cells or various tumor-derived preparations could be used as vaccines. This type of strategy, broadly referred to as cross-priming, could potentially result in polyvalent immunization of the host to multiple (unknown) TAAs.

Recently, it has been demonstrated that human DCs can acquire viral antigens from apoptotic cells and stimulate antigen-specific MHC class I-restricted $CD8^+$ T cells to mediate antiviral CTL responses (7, 8). In this and other *in vitro* models (9–11), apoptotic death was a critical trigger for the antigen processing pathway, and apoptotic cells were a preferred source of antigen, because antigens derived from necrotic cells were not presented on the MHC class I molecules. In addition, the use of ATCs fed to DCs led to effective priming of tumor-specific CTLs in several recent *in vivo* animal studies (11, 12).

In the present study, the ability of human DCs coincubated with ATCs or tumor cell lysates to stimulate tumor-specific T cells was evaluated in an *in vitro* TAA presentation model. We compared monocyte-derived DC_{imm} and DC_{mat} for their capacity to phagocytose apoptotic SCCHN and cross-present TAAs to tumor-specific CTLs. In a separate *in vitro* model of TAA cross-priming, we observed that DCs fed with apoptotic SCCHN were capable of cross-priming naïve T cells obtained from HLA-A2-matched healthy donors for tumor-specific responses.

MATERIALS AND METHODS

Cells and Cell Culture. The HLA-$A2^+$ PCI-13 cell line was established in our laboratory from freshly harvested squamous cell carcinoma of the retromolar trigone and characterized by Heo *et al.* (13). Tumor cells were cultured in plastic culture flasks (Costar, Cambridge, CA) under standard conditions (37°C, 5% CO_2 in a fully humidified atmosphere) using serum-free AIM-V medium (Life Technologies, Inc., Grand Island, NY). For subculturing cells were detached from plastic using 0.05% trypsin/0.02% EDTA solution (Life Technologies, Inc.). The cultures were routinely tested and found to be free of *Mycoplasma* contamination (GEN-PROBE, San Diego, CA).

Human DCs were generated according to a modified method by Sallusto and Lanzavecchia (14). Briefly, peripheral blood or a leukapheresis product was obtained from HLA-$A2^+$ normal donors, and PBMCs were isolated by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). The PBMCs were incubated for 1 h at 37°C in AIM-V medium, and nonadherent cells were removed by gentle washing with warm medium. The remaining (adherent) cells were incubated in AIM-V medium + 1000 units/ml granulocyte macrophage colony-stimulating factor (Immunex, Seattle, WA) and IL-4 (Schering Plough, Kennilworth, NJ). The cultures were supplemented with additional cytokines on day 4 of culture. DCs were harvested at day 6 using cold Hanks' solution (Life Technologies, Inc.).

The PCI-13-specific CTL bulk cell line was established from peripheral blood lymphocytes of a patient with SCCHN, as described previously (15).

Received 10/15/99; accepted 5/4/00.

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[1] Supported in part by NIH Grant PO1-DE-12321 (to T. L. W.). T. K. H. is funded by a postdoctoral training fellowship from the Dr. Mildred Scheel Stiftung fur Krebsforschung (Grant D/99/08916).

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[4] The abbreviations used are: CTL, cytolytic T lymphocytes; APC, antigen-presenting cell; Ab, antibody; ATC, apoptotic tumor cell; DC, dendritic cell; DC_{imm}, immature DC; DC_{mat}, mature DC; ELISPOT, enzyme-linked immunospot; IL, interleukin; IVS, *in vitro* stimulation; mAb, monoclonal Ab; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; PG, prostaglandin; SCCHN, squamous cell carcinoma of the head and neck; TAA, tumor-associated antigen; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; $DiOC_6$, 3,3′-dihexadecyloxacarbocyanine perchlorate.

The CTLs were thawed and maintained in the presence of IL-2 and IL-4 with repeated sensitization on tumor cell monolayers. The $CD8^+$ T cell line was derived from the original bulk T cell line by negative selection, using anti-CD4 Ab-coated magnetic beads. The $CD8^+$ cells recognized a shared antigen on SCCHN: they lysed autologous SCCHN targets as well as HLA-$A2^+$ allogeneic (but not HLA-$A2^-$) SCCHN targets, including PCI-13 (16). This lysis was blocked with anti-CD3, anti-CD8, anti-TCR α/β, and anticlass I MHC Abs (w6/32) as well as anti HLA-A2 Abs. The CTL line or clones derived from it did not lyse K562 or Daudi targets, normal tissue cells, or HLA-$A2^+$ phytohemagglutinin-stimulated T cells. For the experiments described here, the $CD8^+$ T cell line was cultured in AIM-V containing 10% FCS and 300 IU IL-2/IL-4. It was stimulated twice with γ-irradiated PCI-13 cells (10,000 rad) and incubated for 7 days before being used in ^{51}Cr-release assays or ELISPOT assays.

Cytokines and Antibodies. The following cytokines were used for cell cultures: IL-1β (National Cancer Institute, Biological Resources Branch, Frederick, MD), IL-2 (Chiron-Cetus, Emeryville, CA), IL-4 (Schering Plough), IL-6 (Sandoz, Basle, Switzerland), PGE_2 (Sigma Chemical Co., St. Louis, MO), IFN-γ (Genentech, San Francisco, CA), granulocyte macrophage colony-stimulating factor (Immunex), and TNF-α (Knoll Pharmaceuticals, Whippany, NJ).

The antibodies used for staining of cells or blocking of responses were either unlabeled or labeled with PE or FITC and included: anti-MHC class I mAbs (HB95; w6/32), as well as anti-HLA-A2 mAbs (BB7.2) obtained from Dr. Albert DeLeo (University of Pittsburg Cancer Institute); anti-MHC class II, anti-CD14, anti-CD25, and anti-CD80 (Becton Dickinson, San Jose, CA); anti-CD40 and anti-CD86 (Ancell, Bayport, MN), anti-CD83 mAbs (Immunotech, Marseille, France) and respective IgG isotype controls (either from Becton Dickinson or PharMingen, San Diego, CA).

DCs, lymphocytes, or tumor cells (2×10^5/200 μl) were incubated with mAbs on ice for 30 min and washed twice in PBS containing 0.1% (w/v) BSA and 0.1% (w/v) NaN_3. After staining, the cells were fixed with 1% (w/v) paraformaldehyde in PBS for 30 min at room temperature prior to flow cytometry. Flow cytometry analysis was performed as described previously (17), using a FACScan (Becton Dickinson) equipped with a single 488-nm argon ion laser. At least 10,000 events were acquired for each sample.

Apoptosis Induction and Detection. PCI-13 cells cultured in AIM-V were irradiated with 1500 $\mu W/cm^2$ UVB (UVB bulb BLE-GT 302; Spectronics Corp., Westbury, NY) for 2 min or 15 min. To minimize the UVB absorbing effect of phenol red in AIM-V, the medium level was reduced to a minimum during irradiation. Apoptosis was detected by $DiOC_6$ staining and in the TUNEL assay. Additionally, apoptotic bodies were stained by propidium iodide (10 μg/ml; Sigma Chemical Co.) for 15 min at room temperature after cell membrane permeabilization and examined by confocal microscopy, as described below.

For $DiOC_6$ staining, aliquots (50 nM) of the lipophilic cationic fluorochrome $DiOC_6$ (Molecular Probes, Eugene, OR) were added to 5×10^5 cells/ml of culture medium and incubated for 15 min at room temperature. In apoptotic cells, mitochondria show a decrease in *green* fluorescence intensity, which is quantitated by flow cytometry (18). For the TUNEL assay, tumor cells were fixed with 2% (w/v) paraformaldehyde in PBS and permeabilized with 0.1% (w/v) sodium citrate in PBS containing 0.1% (w/v) Triton X-100 for 7 min on ice. After washing, cells were incubated with FITC-conjugated dUTP in the presence of terminal deoxynucleotidyl transferase enzyme solution for 1 h at 37°C, using reagents purchased from Boehringer Mannheim (Indianapolis, IN). After incubation, the cells were washed, and 10,000 events were acquired and analyzed by flow cytometry. Negative controls included cells incubated without the enzyme in the labeling buffer, and positive controls included the same cells treated with DNase (Sigma Chemical Co.).

Tumor Lysates. Lysates were produced by exposing tumor cells to four rapid freeze-and-thaw cycles until the cell membrane integrity was lost. Cell debris was removed by centrifugation (30 min at $15{,}000 \times g$), and the protein content was measured by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of the lysate (1 mg/ml) were used to pulse DCs.

Tumor Uptake by DCs. To study their uptake by DCs, tumor cells were stained *green* with 2 μg/ml $DiOC_{16}$ (Molecular Probes) for 30 min at 37°C in PBS and washed three times in medium before induction of apoptosis. After a 12–24-h incubation in medium to allow for the tumor cells to undergo apoptosis, they were cocultured with DCs at various DC:tumor cell ratios. The cells were harvested 2–18 h later, and DCs were stained with PE-labeled anti-CD80 Ab. Two-color flow cytometry was performed to determine the percentage of cells that phagocytosed apoptotic SCCHN, based on the number of double-positive cells (*green*/*red*). The same experiments were also performed at 4°C to show that the uptake of tumor cells by DCs was inhibited at low temperatures.

To prepare cells for confocal microscopy, sterilized glass coverslips were placed on the bottom of a 6-well plate. $DiOC_{16}$-stained PCI-13 cells were added to these wells and exposed to UVB light as described above. DCs were added 12–24 h after induction of apoptosis. After overnight coculture, the glass coverslips were removed and washed with PBS. The DCs attached to glass were stained with anti-CD80 Ab in combination with a secondary Cy3-conjugated rabbit antimouse Ab (Jackson Immuno-Research Laboratories, West Grove, PA). After fixation with 1% (w/v) paraformaldehyde, coverslips were mounted on a slide and analyzed by confocal laser scanning microscopy at ×600 original magnification (Leica TCS NT confocal LSM; Leica Lasertechnik, Heidelberg, Germany). Images were edited using the Adobe Photoshop software program (Adobe Systems, Mountain View, CA).

Maturation of DCs. Maturation of DCs was induced by the addition of proinflammatory cytokines (10 ng/ml IL-1β, 1000 units/ml IL-6, 10 ng/ml TNF-α, and 1 μg/ml PGE_2), as described previously (19). Changes in expression of MHC class I and II molecules as well as CD14, CD25, CD40, CD80, and CD86 on DCs were monitored by flow cytometry, and the level of expression is shown as mean fluorescence intensity for DC_{imm} and DC_{mat}.

Processing and Cross-Presentation of Tumor-derived Epitopes. DC_{imm} and DC_{mat} that had phagocytosed ATCs or DCs pulsed with tumor lysates, as described above, were harvested, washed, and counted. To determine the ability of these DCs to process and cross-present tumor-derived epitopes to the PCI-13-specific $CD8^+$ CTL line and, thus, be recognized by the CTLs, the DCs were used as stimulators in 24-h ELISPOT assays for IFN-γ production or as targets in 4 h ^{51}Cr-release cytotoxicity assays.

IFN-γ ELISPOT Assay. The ELISPOT assay was performed as described elsewhere (20). Briefly, wells of 96-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA) were coated with 50 μl of primary Ab solution [10 μg/ml in 1× PBS (pH 7.4), clone MAB1-D1K; Mabtech, Nacka, Sweden] and incubated for 24 h at 4°C. Then, the plates were washed four times with PBS, and a 100-μl aliquot of AIM-V supplemented with 10% (w/v) human serum was added for 1–3 h to block nonspecific binding. Next, 1×10^4 to 2×10^4 responder T cells with an equal number of stimulator cells (PCI-13 or DC) were added in a final volume of 200 μl of AIM-V medium. The assay was performed in quadruplicate wells for each experimental condition. The plates were then incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 24 h. After the incubation period, cells were removed by washing the plates six times with 0.05% (w/v) Tween 20 in PBS (Fisher Scientific, Pittsburgh, PA). A 50-μl aliquot of biotinylated secondary anti-IFN-γ Ab (2 μg/ml, clone Mab7-B6–1; Mabtech) was added to each well. The plates were again incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 2 h. The washing steps were repeated, and after a 1-h incubation at room temperature with the avidin-peroxidase complex reagent (Vectastain Elite Standard ABC-Kit; Vector Laboratories, Burlingame, CA), the plates were washed again three times with PBS/0.05% Tween and then three times with PBS alone. Aliquots (100 μl) of the aminoethylcarbazole staining solution (Sigma Chemical Co.) were added to each well to develop the spots. The reaction was stopped after 4–6 min under running tap water. The spots were counted by computer-assisted image analysis (Zeiss ELISPOT 4.14.3.; Zeiss, Jena, Germany). If the mean number of spots against DCs plus tumor preparation (experimental values) was significantly different from the mean number of spots against nonpulsed DCs (background values), as determined by a two-tailed Wilcoxon rank sum test, the background values were subtracted from the experimental values.

For Ab-blocking experiments, PCI-13 cells were preincubated with w6/32 Ab, anti-HLA-A2 (BB7.2) Ab or anti-HLA-DR Ab (clone L 243; kindly provided by Dr. Albert DeLeo), or purified mouse IgG1 (clone S_1-68.1; PharMingen) for 30 min. As a control for the assay reproducibility, PBMCs obtained from the same normal donor and cryopreserved in a series of vials were used each time the assay was performed. These control cells were thawed, washed, and used at the concentration of 2×10^4/ml in AIM-V in the ELISPOT assay. The control PBMCs were stimulated with phorbol 12-myris-

tate 13-acetate (1 ng/ml) and ionomycin (1 μM), both from Sigma Chemical Co. The coefficient of variation for this assay was determined as 15% based on 30 independent determinations.

Cytotoxicity Assay. The 4-h ^{51}Cr-release assay was performed at four E:T ratios, as described previously (21). Briefly, targets (PCI-13, K562 or DC +/− different tumor preparations) were labeled with ^{51}Cr for 45 min at 37°C, washed, and added to wells of 96-well plates (1×10^4 cells/well). Effector T cells were then added to give various E:T ratios. The assays were performed in triplicate. The percentage of specific lysis was calculated according to the formula:

$$\% \text{ Specific lysis} = \frac{\text{Experimental cpm} - \text{control cpm}}{\text{Maximal cpm} - \text{control cpm}} \times 100$$

Enrichment of CD8$^+$ Cells. Cultured PBMCs or the bulk CTL line were enriched for CD8$^+$ cells by positive immunoselection, using magnetic beads (MiniMacs; Miltenyi Biotec, Auburn, CA) according to the manufacturer's recommendations. The purity of selected CD8$^+$ cell fractions was checked by flow cytometry.

Cross-Priming of T Cells. PBMCs were obtained as leukapheresis products from normal HLA-A2$^+$ donors, and monocytes were separated by adherence to plastic. The adherent cells were used for DC generation, whereas the recovered lymphocytes were stimulated with autologous DCs, which have ingested ATCs at the ratio of 10:1. The lymphocytes were cultured in AIM-V medium + 10% human serum supplemented with 25 ng/ml IL-7 for the first 72 h and then in AIM-V supplemented additionally with 20 IU/ml IL-2 for the remaining time in culture. The lymphocytes were restimulated after the first week and weekly thereafter for up to four total stimulations. Responses of T cells to PCI-13, PCI-13 + w6/32 Ab, PCI-13 + HLA-A2.1, PCI-13 + anti-HLA-DR, and PCI-13 + IgG or the controls HR (HLA-A2$^+$ gastric carcinoma), Fem-X (HLA-A2$^+$ melanoma), and HLA-A2$^+$ normal human fibroblasts were tested in 24-h ELISPOT and cytotoxicity assays.

Statistical Analysis. A two-tailed Wilcoxon rank sum test was performed to analyze ELISPOT data. Unpaired two-tailed Student's *t* test was used for statistical analysis of flow cytometry data.

Differences were considered significant when P was <0.05.

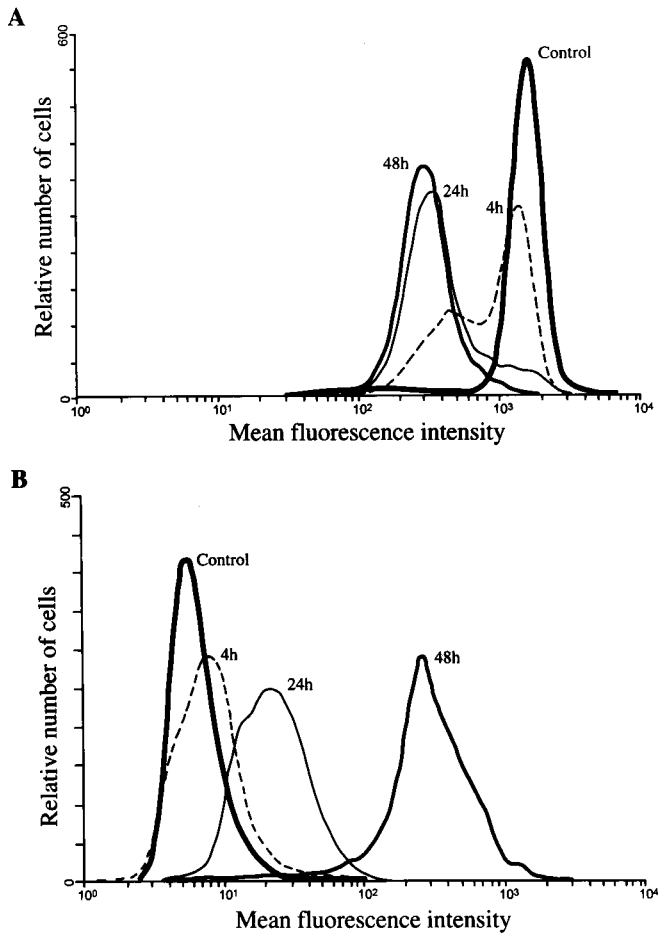

Fig. 1. UVB treatment results in apoptotic death of PCI-13 cells. Apoptosis was induced by a 15-min treatment with UVB (1500 μW/cm^2) and measured by flow cytometry after a 4-h, 24-h, or 48-h incubation of tumor cells in medium. *A*, $DiOC_6$-stained tumor cells show a decrease in the mitochondrial transmembrane potential during the apoptotic process, as evidenced by reduced fluorescence (FL1, X axis). In a TUNEL assay (*B*), FITC-labeled nucleotides are incorporated into DNA of apoptotic cells, leading to an enhancement in fluorescence (FL1, X axis).

RESULTS

Uptake of ATCs by Human DCs. For induction of apoptosis, PCI-13 cells were treated with UVB light for various periods of time. Apoptosis was already induced after 2 min of UVB exposure but was more pronounced using UVB for 15 min, as indicated by reduced $DiOC_6$ staining, reflecting a decrease in mitochondrial transmembrane potential (Fig. 1*A*) or by increased TUNEL reactivity, which measures DNA fragmentation (Fig. 1*B*). To allow for apoptosis to progress, UVB-treated tumor cells were incubated for 4–48 h before flow cytometry analysis. Optimal effects were observed following a 24–48-h incubation of UVB-treated PCI-13 cells (Fig. 1).

To obtain additional evidence for apoptosis at 24 h after exposure to UVB light for 15 min, PCI-13 cells were permeabilized, stained with propidium iodide, and examined in a confocal microscope. They showed typical morphological features of apoptosis: chromatin condensation and fragmentation of cell nuclei into apoptotic bodies (data not shown). UVB irradiation induced apoptosis in nearly all tumor cells.

We next coincubated apoptotic PCI-13 cells with DC_{imm} or DC_{mat} for various periods of time to determine the optimal conditions for internalization of ATCs. We observed that DC_{imm} ingested ATCs already after 2 h of coincubation (data not shown). Overnight coculture of viable PCI-13 cells with DCs resulted in a significant increase in the double-stained cell population (27%), as shown in Fig. 2. This proportion of double-stained DCs was further increased to 69% when DCs were cocultured with ATCs. After a 24-h coincubation, no "free" ATCs could be detected (Fig. 2*F*).

Several findings indicated an active uptake rather than a tight association of DCs with PCI-13 cells: (*a*) the remaining lymphocytes in the DC culture did not show a shift to green fluorescence (FL1, X axis); (*b*) the green fluorescence of DCs was always lower than that of tumor cells alone, presumably because the green color of tumor cells inside DCs was absorbed by the DC membrane; (*c*) the process of uptake was substantially inhibited at 4°C, a temperature that blocks phagocytosis (data not shown); and (*d*) by confocal microscopy, we were able to confirm that ATCs were internalized by DCs (Fig. 3).

In a separate series of experiments, DC_{mat} were generated by incubating DC_{imm} in the presence of proinflammatory cytokines for 36 h. This led to up-regulation of various molecules on DCs, including HLA-DR and CD83 as well as CD80 or CD86, as shown in Table 1.

Effects of DC maturation on the ability to uptake ATCs were next examined. When DCs were treated with the cytokine mixture at the beginning of coculture (0 h) with ATCs, the uptake of tumor material was slightly increased, judging by the percentage of double-positive DCs in these cocultures (78%). However, at 8–12 h after the start of DC maturation, the proportion of DCs ingesting ATCs was significantly and consistently reduced by about 50%. Because of this observation, the following procedure was selected to optimize the ability of DCs to present ATC-derived material to T cells: DC_{imm} were cocultured with ATCs overnight to guarantee sufficient uptake, and afterward they were matured for 36 h by the addition of the cytokine mixture prior to ELISPOT and cytotoxicity assays.

Cross-Presentation by DCs of TAAs to PCI-13-specific CD8$^+$ T Cells. To determine whether human DCs ingesting ATCs are able to process and present tumor-derived epitopes to T cells, we used an *in vitro* antigen-presentation model, consisting of a tumor-specific CTL line and monocyte-derived DCs. The semi-allogeneic, HLA-A2-restriced and PCI-13-specific CTL line was generated as described previously by us (15, 16), and cryopreserved T cells were thawed, maintained in culture, and restimulated with PCI-13 tumor cells at weekly intervals to expand the cells, as needed. The

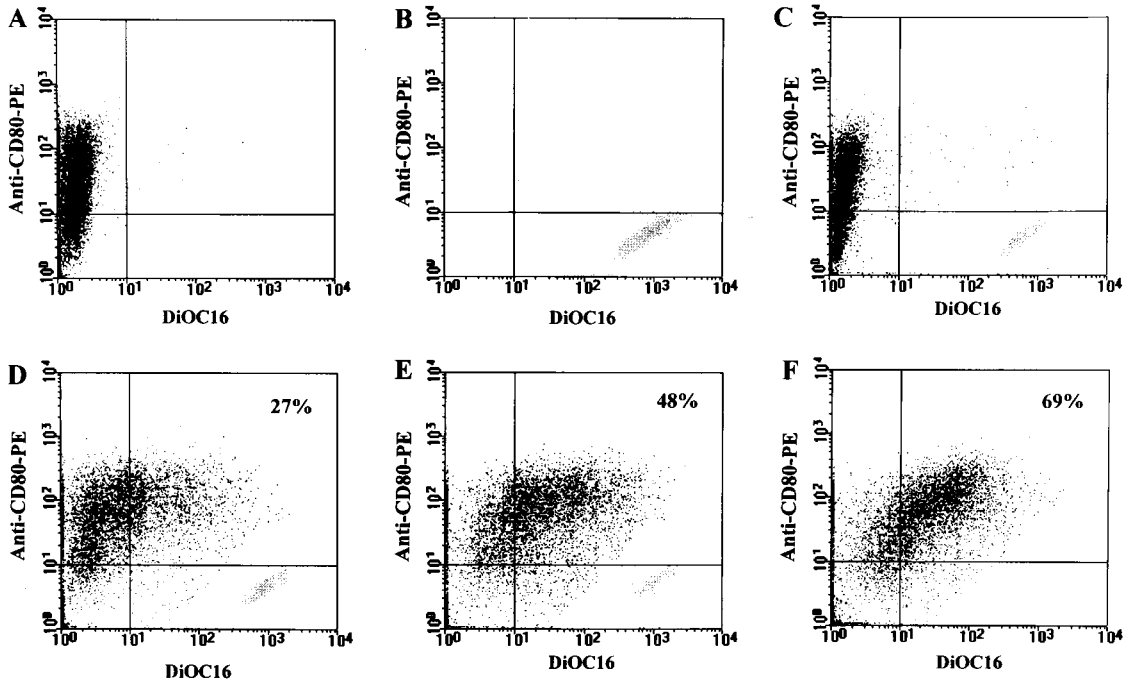

Fig. 2. Monocyte-derived DCs efficiently engulf ATCs. DCs, labeled with PE-conjugated anti-CD80 Ab (*A*), and PCI-13 tumor cells, stained with $DiOC_{16}$ (*B*), were analyzed by flow cytometry. A brief coincubation of DCs with ATCs at the 2:1 ratio resulted in two distinct populations (*C*). Overnight coculture of DCs with alive PCI-13 cells led to internalization of tumor cells in 27% of DCs (*D*). The number of double-positive DCs increased up to 69%, when ATCs [PCI-13 cells treated either for 2 min with UVB (*E*) or for 15 min with UVB (*F*)] were used. The higher proportion of double-positive DCs was accompanied by a decreased proportion of tumor cells, additionally suggesting an active uptake of tumor cells by DCs.

characteristics of this CTL line were extensively evaluated and described before (16, 22). Before their use as responders in cross-presentation experiments with DCs, the expanded CTLs were tested in 4-h ^{51}Cr-release assays against PCI-13 as well as K562 targets. As shown in Fig. 4, the CTLs were HLA-A2 restricted, and they efficiently killed HLA-$A2^+$ PCI-13 but not K562 targets. Not shown are results demonstrating that the CTLs did not lyse HLA-$A2^+$ T cell blasts, HLA-$A2^+$ tumor targets that were not SCCHN or HLA-$A2^-$ SCCHN targets.

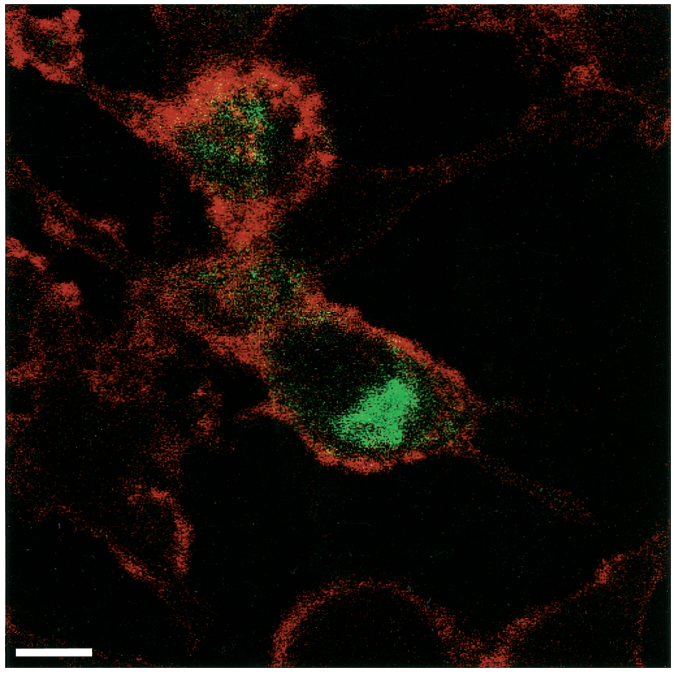

Fig. 3. Uptake of apoptotic PCI-13 cells by DCs. Tumor cells were stained with $DiOC_{16}$ (*green*) before induction of apoptosis and cocultured with DCs on glass coverslips. After an overnight incubation, attached cells were stained with an anti-CD 80 Ab in combination with a rabbit antimouse Cy3-conjugated secondary Ab (*red*). Apoptotic PCI-13 cells (*green*) were detected inside DCs (*red*) by confocal microscopy (midplane image). *Bar*, 10 μm.

Table 1 *Effects of proinflammatory cytokines on the maturation of monocyte-derived human DCs*[a]

	Without proinflammatory cytokines	With proinflammatory cytokines
IgG1 control	6.9 (0.7)	9.2 (2.3)
IgG2a control	7.3 (1.2)	8.9 (1.7)
IgG2b control	4.8 (0.8)	6.7 (1.4)
MHC I	224.8 (97.1)	295.0 (98.0)
MHC II	331.9 (93.0)	627.7 (99.0)
CD 14	16.5 (17.6)	11.8 (7.4)
CD 25	8 (1.6)	139.7 (92.6)
CD 40	330.9 (97.5)	354.2 (98.6)
CD 80	37.0 (58.4)	125.8 (94.5)
CD 83	11.1 (4.5)	85.3 (82.8)
CD 86	150.9 (53.7)	289.3 (98.2)

[a] Immature, monocyte-derived DCs were incubated in the presence or absence of proinflammatory cytokines (10 ng/ml IL-1β, 1000 units/ml IL-6, 10 ng/ml TNF-α, 1 μg/ml PGE2) for 36 h. The cells were stained with mAbs or isotype control Abs and examined by flow cytometry, as described in "Materials and Methods." The data are presented as mean fluorescence intensity with the percentages of positive cells shown in *parentheses.* A representative experiment of three performed is shown.

In cross-presentation experiments, an ELISPOT assay was used as the read-out. PCI-13-specific $CD8^+$ T cells were used as responders, and viable PCI-13 cells, ATCs alone, DC_{imm}+ ATCs, or DC_{mat}+ATCs were used as stimulators. As shown in Table 2, the highest number of IFN-γ spots was always observed with viable PCI-13 cells ($P < 0.05$), indicating that tumor cells were the best stimulators. This finding was confirmed in the cytotoxicity assays performed in parallel with ELISPOT assays (data not shown). This was not surprising because the CTL line was generated by coincubation with PCI-13 and had been repeatedly stimulated by irradiated PCI-13 cells. ATCs alone were poor stimulators of CTLs, possibly because these ATCs expressed significantly lower levels of MHC class I molecules than PCI-13 tumor cells (mean fluorescence intensity of 77 for viable PCI-13 cells *versus* 15 for ATCs and 10 for IgG control). DCs ingesting ATCs or pulsed with tumor lysates

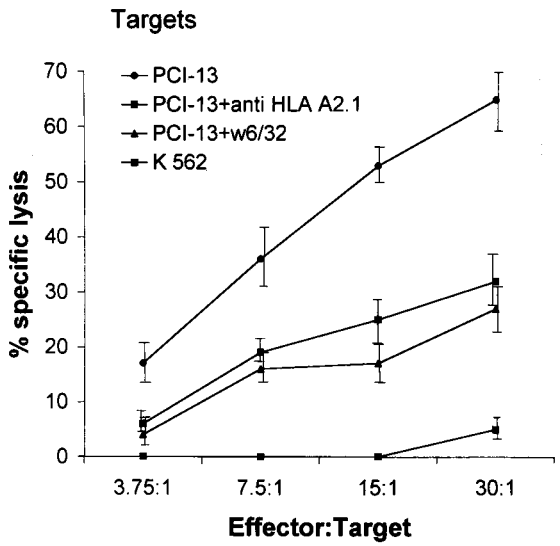

Fig. 4. Cytotoxicity of the CTL line against PCI-13 and K562 cells as control for nonspecific lysis. Anti-MHC-class I Ab and anti-HLA-A2 Ab were used in blocking experiments to confirm that the CTLs are HLA-A2 restricted. A representative experiment of four performed is shown.

were recognized better than ATCs alone, and DC_{mat} + ATCs tended to be somewhat more stimulatory than DC_{imm}, although the differences between experiments using different DC preparations were not statistically significant (Table 2).

Cross-Priming with DCs to Generate Tumor-specific CTLs. To demonstrate that cross-priming of naïve T cells with DCs, which have ingested ATCs, results in the generation of highly responsive antitumor CTLs, we obtained PBMCs from HLA-$A2^+$ normal donors ($n = 4$) and used them as a source of both DCs and T cells. Priming of T cells (two to four stimulations) was performed using γ-irradiated PCI-13 cells, ATCs alone, DCs + ATCs, DCs + nonapoptotic PCI-13, DCs pulsed with tumor lysates, or DCs alone as a control. Various priming conditions +/− DCs were compared for the best generation of tumor-reactive T cells, as detectable in ELISPOT assays. The data in Table 3 indicate that in three of four cultures the percentage of $CD3^+/CD8^+$ T cells was increased after priming with DCs + ATCs compared with the other priming conditions. Also, in two of four cultures primed with irradiated tumor cells alone, increased proportions of natural killer cells were observed. Overall, however, priming with DCs + ATCs favored an enrichment in T cells.

The CTL lines generated by cross-priming under different conditions were further enriched in $CD3^+/CD8^+$ T cells by positive selection with Ab-coated magnetic beads (purity >95% as detected by flow cytometry). These T cells were then used as responders in ELISPOT assays, whereas PCI-13 tumor cells served as stimulators. The representative data for donor 1 (Table 3) are shown in Fig. 5. The ELISPOT results indicate that T cells derived by priming with DCs + ATCs were significantly more responsive ($P < 0.05$) to the tumor (PCI-13) than were T cells derived by priming of PBMCs with γ-irradiated (viable) PCI-13 cells (Fig. 5*A*), ATCs alone (data not shown), or DCs alone (Fig. 5, *A* and *B*). More importantly, the response was blocked by anti-MHC class I and HLA-A2.1 mAbs but not isotype control immunoglobulin. These T cells did not respond to irrelevant HLA-$A2^+$ HR (gastric carcinoma) or Fem-X (melanoma) tumor cells, or HLA-$A2^+$ normal fibroblasts used as control (Fig. 5, *A* and *C*). In only one of four cross-priming experiments γ-irradiated PCI-13 cells were as stimulatory as DCs + ATCs.

Experiments performed to compare the priming capacity of DCs + ATCs with that of DCs pulsed with PCI-13 lysates show that DCs + ATCs, but not DCs pulsed with tumor lysate, were effective in cross-priming autologous T cells (Fig. 5*B*). Most important, blocking experiments with anti-HLA class I and anti-HLA-A2 mAbs confirmed that recognition of PCI-13 by these T cells was MHC class I and HLA-A2 restricted, respectively (Fig. 5*C*). The results obtained with T2 cells, expressing unoccupied HLA-A2 molecules, which were pulsed with fresh tumor lysate and used as stimulators in ELISPOT assays, confirmed the results obtained with PCI-13 or DC stimulators (data not shown). In a limited number of experiments, we had sufficient numbers of T cells available to perform cytotoxicity assays in addition to ELISPOT assays. Our data consistently showed that DCs + ATCs generated MHC class I-restricted CTLs, whereas the other priming regimens did not (data not shown). Overall, these results clearly indicated that the cross-priming protocol, using DCs + ATCs was optimal for the generation of tumor-specific CTLs *ex vivo*.

DISCUSSION

SCCHN are generally considered to be poorly immunogenic and/or immunosuppressive tumors (23). This perception is largely based on recent observations indicating that SCCHN show reduced expression of costimulatory molecules (24), have alterations in the MHC class I-associated epitope processing pathway (25), and are able to induce functional defects and apoptosis in immune cells (17). Therefore, there is a need for immune restoration or potentiation of the immune system in patients with SCCHN, perhaps via vaccination strategies involving DCs as APCs. This novel therapeutic modality could be considered today because it is now feasible to generate human DCs *ex vivo* and pulse them with antigenic peptides for delivery to patients with cancer (26, 27). However, only a few TAAs have been characterized in SCCHN, including CASP-8, SART-1, and p53 wild-type peptides (28–32). Vaccination trials with specific peptides ± DCs similar to those ongoing in patients with melanoma (26, 27) have to await the definition of immunogenic peptides in SCCHN. Meanwhile, it might be feasible to use DCs plus tumor cells or ATCs as vaccine components, provided preclinical studies confirm that such DCs can be successfully used for cross-priming of T cells.

Current evidence suggests that significant differences exist in the efficiency of TAA processing and presentation by human DCs, depending on the source or form of tumor-derived materials, the maturation stage of DCs, or responsiveness of T-cell populations available for stimulation with DCs (8–12, 33, 34). These differences are likely to be important for the outcome of immunizations, and, thus, there exists a need to optimize the design of tumor vaccines, using different tumor preparations (tumor cell lysates, ATCs, whole tumor cells, tumor cell fractions) and *ex vivo*-generated DCs. Clearly, a selection of the optimal method for antigen delivery to be available for future DC-based vaccine clinical trials is important for their success.

In the present study, we have evaluated human monocyte-derived DCs for their ability to: (*a*) take up ATCs; (*b*) present tumor-derived epitopes to already sensitized and committed tumor-specific T cells in the cross-presentation *ex vivo* model; and (*c*) prime T cells from normal donors to develop into antitumor effector cells in the cross-priming *ex vivo* model. To this end, two *in vitro* models had been developed. In the first model (cross-presentation) it was possible to evaluate tumor-specific T-cell responses to human DCs presenting

Table 2 *Responses of the PCI-13-specific CTLs to stimulation with the tumor, ATCs or ATCs as well as tumor lysates, presented by mature or immature human DCs*[a]

Stimulator	Exp 1	Exp 2	Exp 3	Exp 4	Mean ± SE
PCI-13	495	277	149	191	278 ± 77[b]
DC_{mat} + ATC	49	40	17	28	34 ± 7
DC_{imm} + ATC	39	0	0	49	22 ± 14
DC_{mat} + lysate	0	60	0	51	28 ± 17
DC_{imm} + lysate	0	22	18	0	10 ± 6
ATC	0	0	0	52	13 ± 13

[a] The data were obtained in four separate experiments, using 24-h ELISPOT assays. DCs used for CTL stimulation were derived from four different healthy HLA-$A2^+$ donors. In each experiment, the mean number of spots in quadruplicate control wells (nonpulsed DCs + CTLs) was subtracted from the mean number of spots in quadruplicate experimental wells (pulsed DCs/tumor cells + CTLs). The SD in the quadruplicate control or experimental wells was always lower than 10%.

[b] A significant difference ($P < 0.05$) between the number of spots obtained when tumor cells were used as stimulators and of the mean numbers of spots obtained with all other stimulators.

Table 3 *Phenotypic characteristics of T cells generated from PBMCs of healthy donors by priming with different stimulators*[a]

Priming stimulator	$CD3^+/CD4^+$	$CD3^+/CD8^+$	$CD3^-/CD16^+/CD56^+$
Donor 1			
Cytokines only	48	28	23
DC	50	47	0
DC + ATC	28	70	0
PCI-13 irradiated	23	14	45
Donor 2			
Cytokines only	52	32	10
DC	71	33	0
DC + ATC	48	52	0
DC + lysate	77	23	0
PCI-13 irradiated	48	38	13
Donor 3			
Cytokines only	65	26	5
DC	40	60	0
DC + ATC	38	62	0
DC + lysate	50	50	0
Donor 4			
Cytokines only	64	26	5
DC	75	18	1
DC + ATC	70	26	1
DC + lysate	82	14	1
PCI-13 irradiated	68	29	3

[a] T cells were derived from PBMCs of $HLA\text{-}A2^+$ healthy donors by using various priming conditions, as described in "Materials and Methods." The T cells were phenotyped prior to ELISPOT assays. The data are percentages of positive cells in the lymphocyte gate, as measured by flow cytometry.

TAAs derived from processed ATCs or tumor lysate. The CTLs used as responders in this model recognized a shared antigen on $HLA\text{-}A2^+$ SCCHN, as described previously (16), and again confirmed in the current experiments. Human DCs were $HLA\text{-}A2^+$ semi-allogenic monocyte-derived APCs, which were either immature or were matured by *ex vivo* culture in the presence of proinflammatory cytokines. ELISPOT assays for IFN-γ production or cytotoxicity assays were used to monitor responses of CTLs to tumor-derived epitopes presented by DCs, tumor cells alone, ATCs alone, or DCs + tumor lysates. This *in vitro* cross-presentation model was used to quantitate the magnitude of CTL responses, and it allowed for a comparison of variously pretreated DCs for their capability to present a tumor-derived epitope(s) to CTLs known to be able to recognize and efficiently kill tumor cells.

Initially, while developing the model, it was necessary to show that SCCHN cells, subjected to an apoptotic signal (UVB light), were taken up by human monocyte-derived DCs. We showed a high level of uptake of ATCs by these DCs, as determined by flow cytometry as well as confocal microscopy. In fact, nearly all ATCs were taken up by DCs during a 24-h period of coincubation. We also observed that maturation of DCs was not visibly affected by the uptake of ATCs at the ATC:DC ratio of 1:2. On the other hand, previous reports indicated that in mice DC maturation was induced at a much higher (5:1) ATC:DC ratio, whereas lower ratios, similar to those used in our experiments, failed to mature DCs (11). To achieve maturation of human DCs, we, therefore, resorted to the use of a mixture of proinflammatory cytokines (19). When the ability of DC_{mat} to uptake ATCs and present TAAs to T cells was compared with those of DC_{imm}, it seemed that the uptake of ATCs was found to be reduced after 8–12 h of coincubation, but the T-cell stimulatory activity of DC_{mat} was improved as compared with that of DC_{imm}. Conceivably, up-regulation of costimulatory molecules and MHC class I molecules or increased stability of peptide-MHC-class I complexes on DC_{mat} could be responsible for this effect. In addition, PGE_2 in combination with TNF-α could synergistically induce high levels of IL-12 production in human monocyte-derived DCs, stimulate T-cell proliferation (35), and increase IFN-γ production by responder $CD8^+$ T cells without inducing type 2 cytokines, as reported previously (19).

In our cross-presentation model, DC_{mat} ingesting ATCs were recognized by the PCI-13-specific T cells somewhat better than DC_{imm} and ATCs, but untreated PCI-13 cells were always eliciting the best T-cell responses. This observation is as expected because the CTL line used was generated by IVS with PCI-13 cells and was repeatedly stimulated with PCI-13 cells at weekly intervals. Sim-

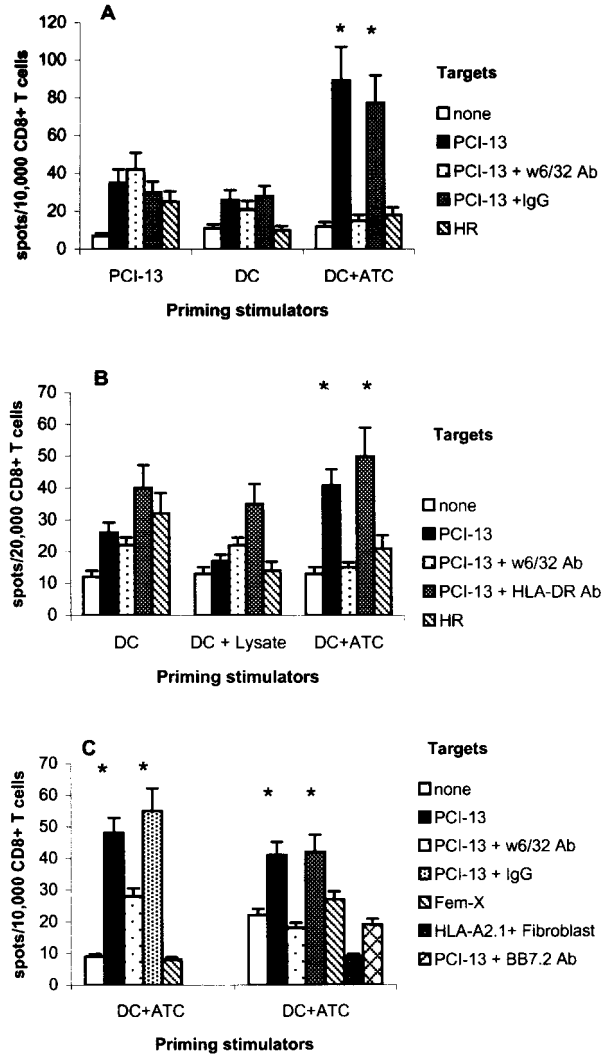

Fig. 5. Recognition of PCI-13 targets by T cells generated by cross-priming of PBMCs with DCs alone or DCs + various tumor preparations or tumor cells alone. IFN-γ ELISPOT assays were performed after two to four IVS cycles. T cells generated in these cocultures were enriched in $CD8^+$ T cells by positive selection with magnetic beads prior to ELISPOT assays. *A*, T cells (as responders) were tested against PCI-13 or HR cells ($HLA\text{-}A2^+$ gastric carcinoma used as a negative control) as stimulators at the ratio of 1:1. To confirm HLA-class-I restriction, PCI-13 cells were incubated with w6/32 or IgG Ab. Spots were counted by computer-assisted image analysis. A representative experiment (donor 1) of four performed with autologous DCs and T cells obtained from different $HLA\text{-}A2^+$ healthy donors is shown. *B*, PBMCs of a normal $HLA\text{-}A2^+$ donor (donor 3) were cross-primed with DCs, DCs + tumor lysate, or DCs + ATCs. The generated T cells were tested in ELISPOT assays against PCI-13 or HR cells at the ratio of 1:1. To confirm HLA-class-I restriction, PCI-13 cells were incubated with w6/32 or IgG Ab. Spots were counted by computer-assisted image analysis. A representative experiment of four performed with autologous DCs and T cells obtained from different $HLA\text{-}A2^+$ donors is shown. *C*, PBMCs of two normal $HLA\text{-}A2^+$ donors were cross-primed with DCs + ATCs. To confirm specificity of the generated T cells were tested in ELISPOT assays against PCI-13 +/− anti-MHC class I mAb (w6/32), PIC-13 +/− anti-HLA-A2 mAb (BB7.2), or against irrelevant targets such as $HLA\text{-}A2^+$ melanoma (Fem-X) or $HLA\text{-}A2^+$ normal human fibroblasts. *Asterisks* indicate that the number of spots obtained when T cells were cross-primed with DCs + ATCs was significantly higher ($P < 0.05$) than the numbers of spots obtained with all other priming protocols and the use of PCI-13 or PCI-13 + IgG as stimulator resulted in a significantly higher number of spots ($P < 0.05$) compared with controls (PCI-13 + w6/32 Ab; PCI-13 + BB7.2 Ab; HR cells; Fem-X; fibroblasts).

ilar results were reported by Bellone *et al.* (9) in a murine system. It was interesting to note that ATCs alone were not stimulatory, possibly due to a dramatic decrease in the expression of MHC class I molecules in ATCs relative to PCI-13 cells, as observed by flow cytometry. Several recent reports indicated that ATCs have reduced immunogenicity compared with live cells (12, 36, 37). Many different mechanisms have been proposed to account for this low immunogenicity (12, 36, 37), but our findings of low levels of MHC class I expression in ATCs is a novel observation that seems to fit well with the requirement for DCs, which express both MHC and costimulatory molecules, to process and present these ATCs to generate an effective immune response. It is reasonable to assume that ATCs become immunogenic, when they are cross-presented to T cells by professional APCs equipped with an efficient antigen-processing and presenting machinery and expressing costimulatory molecules. Processing of phagocytosed ATCs by DCs yields epitopes that can access the MHC class I pathway via TAP (transporter of antigen-processing)-dependent mechanisms and are ultimately presented to and recognized by antigen-specific CTLs. This type of effective cross-presentation of murine TAAs or viral antigens by macrophages and DCs has been described previously in *ex vivo* experiments (7–10).

In the second *in vitro* model (cross-priming), we used DCs, which had internalized ATCs or other tumor-derived epitopes, to generate effector T cells able to recognize and kill PCI-13 targets. In the recent *in vivo* experiments in rodents, Henry *et al.* (11) and Ronchetti *et al.* (12) described generation of tumor-specific CTLs, which were able to mediate tumor rejection and induce long-term memory, when using DCs + ATCs but not DCs pulsed with tumor extracts. These studies in rodents indicated a superior ability of DCs, which had internalized ATCs, to stimulate antitumor responses. Using human DCs and autologous T cells obtained from the circulation of normal HLA-$A2^+$ donors, we demonstrated in the cross-priming model that stimulation with DCs + ATCs yielded T-cell lines strongly responsive to the tumor. To reach this conclusion, we performed several cycles of IVS, under conditions designed to compare γ-irradiated viable tumor cells, non-ATCs, ATCs alone, DCs + ATCs, DCs ± non-ATCs, DCs + lysate, or DCs alone for the ability to prime naïve T cells. This type of cross-priming was successful in generating tumor-specific CTLs in all four attempts using HLA-$A2^+$ PBMCs of normal donors. Lymphocytes cross-primed with DCs + ATCs contained the highest frequency of IFN-γ-producing T cells specific for the tumor. In only one of four experiments (donor 4) γ-irradiated (viable) PCI-13 cells were as stimulatory as DCs + ATCs. ATCs alone did not induce the outgrowth of tumor-reactive T cells in our experiments. Interestingly, DCs pulsed with tumor lysates showed lower, if any, cross-priming capacity compared with DCs + ATCs. This finding was somewhat surprising in view of the widespread practice of using tumor lysates pulsed on DCs as a potentially effective antigen-delivery procedure for CTL generation. On the other hand, it has been shown that the administration of exogenous class I-restricted antigens in the form that requires phagocytosis is essential for their effective presentation to T cells (7, 8). Moreover, Inaba *et al.* (33) have shown that phagocytosed cellular fragments are 3000 times more efficient in forming MHC-peptide complexes than the preprocessed peptide. Because Herr *et al.*[5] observed the induction of a strong CD4 response, but only a weak CD8 response, when loading DCs with lysates in an EBV/viral model, it is possible that tumor-derived lysates primarily induce CD4 responses. This possibility is currently under investigation in our laboratory.

Overall, this study demonstrates that human monoctye-derived DCs that internalize and process ATCs can cross-prime T cells and generate more effective antitumor-specific T cells *in vitro* than viable tumor cells or tumor cell lysates pulsed on these DCs. Although these studies were performed in a semi-allogeneic setting, which could enhance T-cell activation and proliferation of both HLA-A2-restricted tumor-specific T cells as well as nonspecific T cells, the antitumor responses we measured in ELISPOT and cytotoxicity assays were mediated by tumor-specific, HLA-A2-restricted T cells. The results available from vaccination experiments in tumor-bearing rodents indicate that a similar approach may be successful *in vivo* (11, 12). Therefore, vaccination of cancer patients with ATCs + autologous DCs should be considered in the future as a reasonable therapeutic strategy, especially applicable when immunogenic tumor epitopes are not available.

ACKNOWLEDGMENTS

We thank Dr. Albert DeLeo and Robbie B. Mailliard for helpful discussions as well as Audra Notalio and Sean Alber for assistance with cell imaging performed in the Biological Imaging Facility at the University of Pittsburgh, directed by Dr. Simon Watkins.

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Generation of T Cells Specific for the Wild-Type Sequence $p53_{264-272}$ Peptide in Cancer Patients: Implications for Immunoselection of Epitope Loss Variants[1]

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Alterations in the *p53* gene occur frequently and can lead to accumulation of p53 protein in squamous cell carcinomas of the head and neck (SCCHN). Since accumulation of p53 is associated with enhanced presentation of wild-type sequence (wt) p53 peptides to immune cells, the development of pan vaccines against SCCHN has focused on wt p53 epitopes. We used the HLA-A2.1-restricted wt $p53_{264-272}$ epitope to generate CTL from circulating precursor T cells of HLA-A2.1$^+$ healthy donors and patients with SCCHN. Autologous peptide-pulsed dendritic cells were used for in vitro sensitization. CTL specific for the wt $p53_{264-272}$ peptide were generated from PBMC obtained from two of seven normal donors and three of seven patients with SCCHN. These CTL were HLA class I restricted and responded to T2 cells pulsed with $p53_{264-272}$ peptide as well as HLA-A2-matched SCCHN cell lines naturally presenting the epitope. Paradoxically, none of the tumors in the three patients who generated CTL could adequately present the epitope; two had a wt *p53* genotype and no p53 protein accumulation, while the third tumor expressed a point mutation (R to H) in codon 273 that prevents presentation of the $p53_{264-272}$ epitope. In contrast, patients who did not generate CTL had tumors that accumulated altered p53 and potentially could present the $p53_{264-272}$ epitope. These findings suggest that in vivo, CTL specific for the wt $p53_{264-272}$ peptide might play a role in the elimination of tumor cells expressing this epitope and in immunoselection of epitope-loss tumor cells. Immunoselection of tumors that become resistant to anti-p53 immune responses has important implications for future p53-based vaccination strategies. ***The Journal of Immunology,*** **2000, 165: 5938–5944.**

Current therapies for patients with squamous cell carcinoma of the head and neck (SCCHN)[3] consist of surgery or combinations of surgery with radiotherapy and/or chemotherapy (1). Unfortunately, survival of patients with SCCHN treated with these therapies has not improved in the last 30 years (2). New therapies are needed to improve patient survival, and vaccine development is considered a promising therapeutic strategy. Since missense mutations of *p53* occur in a wide range of human tumors (3, 4), this tumor suppressor gene product has been an attractive candidate for vaccines potentially capable of inducing anti-tumor Ags immune responses in a broad population of cancer patients (5, 6). Initially, individual p53 missense mutations, which are tumor specific in nature, were considered as promising vaccines. These vaccines, however, would have limited clinical usefulness, because they require that the p53 mutation occurs within or creates an epitope that could be presented by the HLA class I molecules expressed by the individual patient. In many tumor cells, however, missense mutations of p53 result in accumulation (overexpression) of the altered p53 molecules (3–5). Since most mutations of p53 involve the alteration of a single amino acid, it follows that the majority of p53 epitopes processed and presented to immune cells by tumors would be wild type (wt) in sequence. Attention has shifted, therefore, to the targeting of wt sequence p53 epitopes as potential immunogens.

The ability to induce CTL recognizing wt p53 epitopes has established a basis for future development of a broadly applicable p53-based immunotherapy (7–14). The two HLA-A2.1-restricted, human wt p53 epitopes most often used in these studies are $p53_{149-157}$ and $p53_{264-272}$. We and others have previously reported on the generation of CTL recognizing the wt $p53_{264-272}$ epitope from PBMC obtained from healthy donors (7, 13–18). However, it is unconfirmed whether similar effector cells can be generated from PBMC of patients with cancer. Although it is reasonable to expect that patients with SCCHN, especially those who accumulate p53, have a higher frequency of p53-responsive precursor T cells than normal donors, it is equally probable that these T cells are neither present nor functional. Patients with SCCHN are known to be immunosuppressed (19–21), their T cells have signaling defects (21), and a higher proportion of apoptotic T cells has been detected in the peripheral blood of these patients compared with that in healthy individuals (22). Therefore, it is important to determine whether stimulation of PBMC with the wt $p53_{264-272}$ leads to the generation of p53-specific CTL in patients with SCCHN.

The status of p53 has been shown to be critical for the ability of the tumor to process and present the wt $p53_{264-272}$ epitope. To date, most tumor cell lines shown to be sensitive to CTL specific

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Received for publication May 15, 2000. Accepted for publication August 16, 2000.

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[1] This work was supported in part by the National Institutes of Health Grant PO1-DE12321 (to T.L.W.) and Grant D/99/08916 from the Dr. Mildred Scheel Stiftung für Krebsforschung (to T.K.H.).

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[3] Abbreviations used in this paper: SCCHN, squamous cell carcinoma of the head and neck; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; IVS, in vitro sensitization; wt, wild-type sequence.

0022-1767/00/$02.00

for wt $p53_{264-272}$ accumulate mutant p53 molecules, whereas tumor cells expressing wt p53 (exons 5–8) with no p53 accumulation are not sensitive to these CTL (17). In this report, we show that CTL specific for the wt $p53_{264-272}$ epitope could be generated from PBMC of patients with SCCHN using peptide-pulsed autologous dendritic cells (DC) for in vitro sensitization (IVS). The frequency and activity of these T cells were evaluated relative to the presence of p53 gene mutations and protein expression in the patients' tumors and to the presence of anti-p53 Abs in their sera.

Materials and Methods

Cells and cell culture

The HLA-$A2^+$ SCCHN cell lines SCC-4 and SCC-9 were obtained from the American Type Culture Collection (Manassas, VA). The SCC-4 cell line expresses and accumulates p53 with a missense mutation/point mutation in position 151, but does not present the wt $p53_{264-272}$ epitope (16). The SCC-9 cell line expresses, but does not accumulate, p53 molecules, in which codons 274–285 have been deleted, and presents the wt $p53_{264-272}$ epitope (16). The SCCHN cell line PCI-13 was established in our laboratory (23). PCI-13 was previously described to express a p53 missense mutation in codon 286 (Glu to Lys) and to present the wt $p53_{264-272}$ epitope (18). The p53-null osteosarcoma cell line, SaOS-2, was purchased from American Type Culture Collection, and the $p53^+$ cell line SaOS-2cl3 was derived by transduction of p53-null SaOS-2 cells with p53 cDNA expressing a p53 missense mutation in codon 143 (9, 18). Tumor cells were cultured in plastic culture flasks (Costar, Cambridge, CA) under conditions described previously (18), using DMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin (all from Life Technologies, Grand Island, NY). For subculturing, cells were detached from plastic using 0.05% trypsin/0.02% EDTA solution (Life Technologies). The hybrid (T×B) T2 cell line (24), which is deficient in TAP protein, was obtained from American Type Culture Collection and maintained in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics. The cultures were routinely tested and found to be free of mycoplasma contamination (Gen-Probe, San Diego, CA).

$p53_{264-272}$ peptide

The HLA-A2.1-binding peptide, LLGRNSFEV (7, 8), corresponding to $p53_{264-272}$, was synthesized by standard F-moc methodology, purified, and stored as a lyophilized preparation. The peptide was purified by reverse phase HPLC, and its amino acid sequence was confirmed by mass spectroscopy. The peptide was dissolved in DMSO (Fisher Scientific, Pittsburgh, PA) at 1 mg/ml and diluted with PBS just before use.

Generation of anti-p53 CTL using peptide-pulsed autologous DC

Peripheral blood or a leukapheresis product was obtained from HLA-$A2^+$ SCCHN patients or normal donors, respectively, and PBMC were isolated by sedimentation over Ficoll-Hypaque gradients (Amersham Pharmacia Biotech, Piscataway, NJ). The study was approved by the institutional review board at the University of Pittsburgh, and written informed consent was obtained from each individual. Human DC were generated according to a modified method of Sallustro and Lanzavecchia (25). Briefly, PBMC were incubated for 1 h at 37°C in AIM-V medium, and nonadherent cells were removed by gentle washing with warm medium. The remaining (adherent) cells were incubated in AIM-V medium containing 1000 U/ml GM-CSF (Immunex, Seattle, WA) and IL-4 (Schering Plow, Kennilworth, NJ). The cultures were supplemented with additional IL-4 and GM-CSF on day 4 of culture. DC were harvested on day 6 using cold Hanks' solution (Life Technologies) and were used as APCs. DC were resuspended at the concentration of 2×10^6 cells/ml in AIM-V medium containing 10 μg/ml peptide and incubated at 37°C for 4 h. Subsequently, the peptide-pulsed DC were cocultured with autologous PBMC in 24-well tissue culture plates (Costar, Corning, NY) in a final volume of 2 ml/well of AIM-V medium supplemented with 10% human AB serum (Pel-Freeze, Brown Deer, WI) and 25 ng/ml of IL-7 (Genzyme, Cambridge, MA) for the first 72 h and additionally with 20 IU/ml IL-2 (Chiron/Cetus, Emeryville, CA) for the remaining time in culture. The lymphocytes were restimulated weekly with the peptide-pulsed autologous DC. Irradiated (3000 rad) autologous PBMC were used as APCs after the second round of restimulations. The reactivity of generated T cells was tested against various targets in 24-h enzyme-linked immunospot (ELISPOT) assays as well as cytotoxicity assays. The specificity was determined in Ab blocking experiments and was confirmed by tetramer staining (see below).

ELISPOT assay for IFN-γ

The ELISPOT assay was performed in 96-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA) exactly as previously described by us (26). The capture and detection Abs were purchased from Mabtech (Nacka, Sweden). The spots were counted by computer-assisted image analysis (ELISPOT 4.14.3; Zeiss, Jena, Germany). For Ab blocking experiments, target cells were preincubated with anti-HLA class I-specific mAb, W6/32 (HB95; American Type Culture Collection), anti-HLA-A2-specific mAb, BB7.2 (HB82; American Type Culture Collection), or the respective IgG isotype controls (IgG2a and IgG2b, respectively, both from PharMingen, San Diego, CA) for 30 min. The assay reproducibility was controlled using PBMC obtained from a normal donor, cryopreserved in a series of vials, and was tested each time the assay was performed after stimulation with PMA (1 ng/ml) and ionomycin (1 μmM; both from Sigma, St. Louis, MO). The interassay reproducibility of the assay was acceptable, with a coefficient of variation of 15% ($n = 30$).

Cytotoxicity assay

The 4-h ^{51}Cr release assay was performed at four E:T cell ratios as previously described (27). Briefly, sensitized targets were labeled with ^{51}Cr for 45 min at 37°C, washed, and added to wells of 96-well plates (1×10^4 cells/well). Effector T cells were then added to give various E:T cell ratios. When Ab blocking experiments were performed, target cells were incubated with anti-HLA class I Ab or anti-HLA-A2 Ab at a final concentration of 9 μg/ml for 30 min before adding effector cells. The percent specific lysis was calculated according to the formula: percent specific lysis = (experimental cpm − control cpm)/(maximal cpm − control cpm) × 100.

Tetrameric peptide/HLA-A2.1 complexes (tetramers)

The streptavidin-PE-labeled tetramers used in this study were obtained from the tetramer core facility at the National Institute of Allergy and Infectious Disease (Atlanta, GA). Three-color flow cytometry assays (FACScan; Becton Dickinson, San Jose, CA) were performed with anti-CD3-peridinin chlorophyll protein, anti-CD8-FITC, and tetramer-PE. The specificity of the $p53_{264-272}$/HLA-A2.1 tetramer was confirmed by staining of a CTL line specific for p53 (18) and by the lack of staining of irrelevant CTLs or HLA-A2-negative PBMC of healthy donors. Additionally, the streptavidin-PE-conjugated HIV peptide (ILKEPVHGV)/HLA-A2.1 tetramer was used as a negative control. Cells were considered positive for tetramer staining when populations clustered and were at least 1 log above the mean fluorescence of the negative population. For each sample, 75,000 events were collected progressively after live gating on lymphocytes by forward and side scatter.

p53 mutation analysis, immunohistochemistry, and detection of p53 Abs

All cases of SCCHN included in this study were available as paraffin blocks archived at the University of Pittsburgh Medical Center. The histology of each case was reviewed by a pathologist (S.D.F.), and representative tissue sections containing areas of invasive SCCHN were selected for microdissection. Normal-appearing salivary gland tissue or skeletal muscle was microdissected separately to serve as an internal nontumor control. Using 4-μm-thick recut unstained histologic sections, normal and malignant tissue samples were removed under stereomicroscopic observation. Sufficient material was collected from a single histologic section to afford replicate analysis. Samples were treated with proteinase K at a final concentration of 100 μg/ml for 2 h and then boiled for 5 min to remove protease activity. Sets of amplification primers flanking exons 5 through 8 of the *p53* gene were used in four separate PCR (28). Amplified DNA from microdissected tissues also included splice sites. PCR products were electrophoresed in 4% agarose, and the ethidium bromide-stained bands were excised and then isolated with glassmilk. DNA sequencing used antisense PCR primers for each exon with [^{33}P]dATP as the reporter molecule, and sequence analysis was read from overnight-exposed autoradiograms of 6% polyacrylamide gels.

For p53 immunohistochemistry, Formalin-fixed, paraffin-embedded tumor tissues were sectioned (3–5 μm), air dried overnight at 37°C, deparaffinized, dehydrated, and stained with a mAb against p53, D0-7 (Dako, Carpinteria, CA), which recognizes an epitope in the N terminus between aa 35 and 45 and reacts with wt and most mutant forms of p53 protein. The avidin-biotin-peroxidase method was used to visualize the p53, according to the instructions supplied by the manufacturer (Dako). The immunostained slides were evaluated by light microscopy for p53 accumulation.

The tumor was considered p53 positive when >25% of the tumor cells showed staining intensity of 2+ and higher on a scale of 0–4+. IgG isotype mAb used at the same concentration as the primary mAb served as a negative control.

Ab to p53 in the patients' and control sera was detected by an ELISA purchased from PharmaCell Immunotech Coulter (Miami, FL) using microtiter plates coated with recombinant human wt p53 protein. Peroxidase-conjugated goat anti-human IgG was used for detection of human anti-p53 Ab by a colorimetric reaction. Staining intensity was compared with a standard curve, and anti-p53 levels ≥1.1 U/ml were considered positive. Assays were performed twice in triplicate and included sera obtained from seropositive as well as seronegative individuals as internal positive/negative controls.

HLA-A subtyping

PBMC or tumor cell lines were phenotyped for expression of HLA-A2 molecules by flow cytometry using the anti-HLA-A2 mAb, BB7.2, and IgG isotype as a control. Verification of the A0201 subtype was performed using PCR with sequence-specific primers as previously described (29). Briefly, DNA was obtained from PBMC using SDS and proteinase K, and after removal of protein contaminants with a saturated salt solution, DNA was precipitated using 2 vol of ethanol. Following washing and drying, the DNA pellet was reconstituted and quantitated by reading OD at 260 nm. The extracted DNA was subjected to PCR with sequence-specific primers (Dynal, Oslo, Norway) with primers that distinguish the specific allelic polymorphisms. Each primer set was designed to give an amplified fragment of a specific size, which was detected by gel electrophoresis and ethidium bromide staining. Patterns of positive and negative amplifications yielded the relevant genotype.

Statistical analysis

A two-tailed Wilcoxon rank sum test was performed to analyze ELISPOT data. Differences were considered significant at $p < 0.05$.

Results

Generation of HLA-A2.1-restricted T cells reactive against wt p53

DC populations generated from the patients' adherent mononuclear cells in the presence of GM-CSF and IL-4 were routinely phenotyped by flow cytometry and found to be $CD14^-$, $CD40^+$, $CD80^+$, $CD83^-$, $CD86^+$, DR^+, HLA class I^+ (data not shown). The phenotypes and yields of DC were comparable to those of DC generated from PBMC of normal donors using the same procedure. The DC were pulsed with the wt $p53_{264-272}$ peptide and tested for the ability to stimulate autologous T cells in IVS. The outgrowing T cells were evaluated for anti-p53 epitope activity against various targets in ELISPOT and 4-h ^{51}Cr release assays and were found to be specific and HLA-A2 restricted. The ELISPOT data obtained for one representative patient (patient 2) are shown in Fig. 1*A*. The generated T cells were HLA class I restricted and reacted against T2 cells pulsed with $p53_{264-272}$ peptide and, to a lesser extent, against an HLA-A2.1-matched sarcoma cell line, SaOS-2Cl3, as well as against a SCCHN cell line, SCC-9, both of which naturally present the epitope (Fig. 1*B*). It is generally observed that the reactivity of CTL is greater against peptide-pulsed targets than tumor cells, presumably because peptide-pulsed target cells are presenting the epitope at physiologically abnormal high levels compared with levels of the epitope naturally presented by tumors.

To exclude the possibility that memory anti-$p53_{264-272}$ T cells did not survive multiple rounds of stimulation in nonresponders to IVS, T cells were tested before and after the first round of IVS in some cases. However, there was no evidence for stimulation in ELISPOT. Since a response usually increased with the number of IVS, the ability of bulk effector cell populations to lyse peptide-pulsed T2 cells or HLA-$A2^+$ human tumor cells was evaluated after the last round (four times) of IVS. T2 cells pulsed with an irrelevant peptide, gp100, were not recognized by these effector cells. Lysis of T2 cells pulsed with $p53_{264-272}$ peptide by the effector T cells was blocked by anti-HLA-A2 mAb, as shown for T cells of patient 2 in Fig. 2*A*. Among tumor cell lines tested in 4-h ^{51}Cr release assays were the sarcoma lines SaOS-2 and SaOS-2Cl3 and the SCCHN cell lines SCC-9 and PCI-13, which naturally present the wt $p53_{264-272}$ epitope. All of these cell lines were lysed by the effector cells (Fig. 2*B*), and this lysis was blocked by anti-HLA-A2 mAb. In contrast, the SCCHN cell line SCC-4 and the sarcoma cell line SaOS-2, which do not express the epitope, were only minimally lysed, and their killing was not blocked by anti-HLA-A2 mAb. These data demonstrate that CTL generated ex vivo in response to the wt $p53_{264-272}$ peptide were able to eliminate tumor cells expressing this epitope, but not the targets unable to present it.

Table I summarizes the results obtained with PBMC of patients with SCCHN and of normal donors after IVS with the $p53_{264-272}$ peptide. T cells of three of seven patients with SCCHN were found

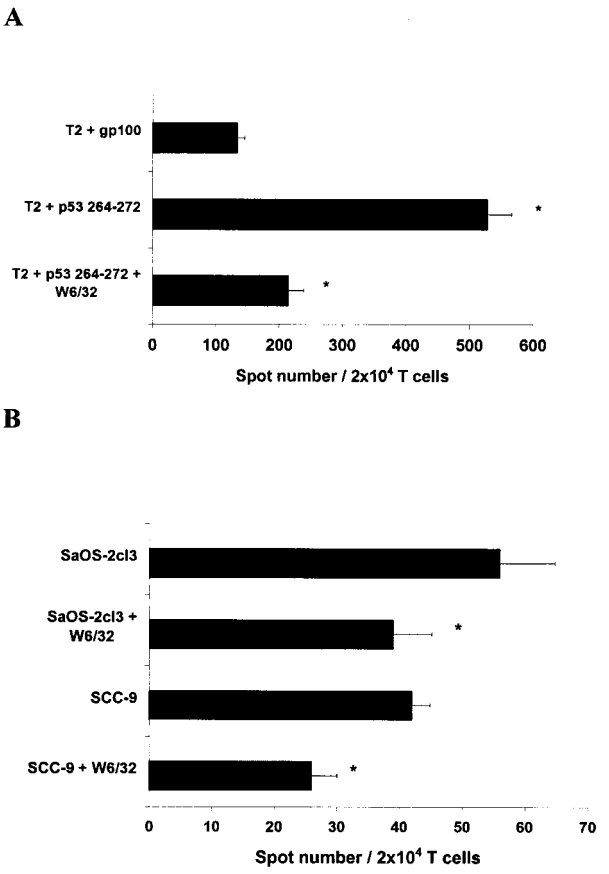

FIGURE 1. *A*, Results of a representative ELISPOT assay for IFN-γ production. T cells were generated by priming of PBMC with the wt $p53_{264-272}$ peptide pulsed onto autologous DC. The ELISPOT assay was performed after three IVS cycles. The CTL were tested against T2 cells pulsed with an irrelevant peptide (gp100) or the wt $p53_{264-272}$ peptide. Spots were counted by computer-assisted image analysis. A representative experiment performed with PBMC obtained from patient 2 is shown. *B*, Results of a representative ELISPOT assay (patient 2) for IFN-γ production in which tumor cell lines SaOS-Cl3 and SCC-9 were used as targets at a ratio of 1:1. Spots were counted as described above. *, Significant difference ($p < 0.05$) between the number of spots obtained from T cells incubated with T2 cells pulsed with the wt $p53_{264-272}$ peptide vs that obtained using T2 cells pulsed with the irrelevant gp100 peptide. In all experiments, blocking with anti-HLA class I Ab W6/32, but not with isotype control Ig (data not shown), resulted in a significant decrease ($p < 0.05$) in the number of spots.

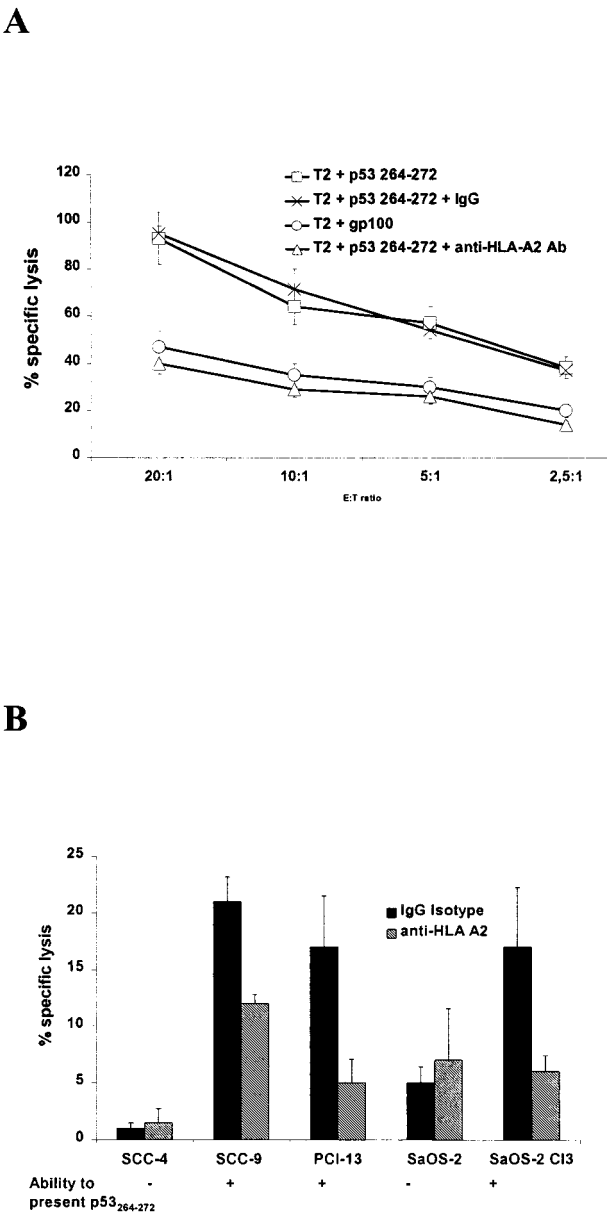

FIGURE 2. *A*, Lysis of T2 cells pulsed with the wt $p53_{264-272}$ peptide by responder T cells derived from the PBMC of patient 2. T2 cells were labeled with ^{51}Cr and preincubated with 10 μg/ml of the relevant or irrelevant (gp100) peptide for 1 h. They were then added to responder cells at various ratios. The results shown are representative of two independent experiments. Cytotoxicity was blocked in the presence of anti-HLA-A2 Ab, but not in the presence of isotype control Ig. *B*, Cytolytic activity of anti-$p53_{264-272}$ bulk CTL (patient 2) against a panel of human tumor cells. Targets were labeled with ^{51}Cr, and CTL were added at the indicated E:T cell ratios in the presence of anti-HLA-A2 Ab or isotype control Ig.

to be reactive against peptide-loaded T2 cells as well as against tumor cells naturally presenting the wt $p53_{264-272}$ epitope. This reactivity was HLA class I restricted, as it was blocked by anti-HLA class I or anti-HLA-A2 mAb. Similar responses were obtained in two of seven healthy donors (data not shown). Two additional normal donors who did not show any response after the fourth stimulation with autologous DC pulsed with $p53_{264-272}$ peptide were found to express the HLA-A2.7 allele and were excluded from the study.

Tetramer staining of T cells specific for the wt $p53_{264-272}$ peptide

Tetrameric $p53_{264-272}$/HLA-A2.1 complexes were used to confirm the anti-$p53_{264-272}$ specificity of CTL present in bulk IVS cultures. Fig. 3 shows representative results of three-color flow cytometry performed with T cells generated from PBMC of patient 2 after two to four successive restimulations. Only 0.03% tetramer-positive $CD8^+$ T cells of gated $CD3^+$ lymphocytes were present in fresh PBMC obtained from this patient. After four IVS cycles, up to 16.4% of all $CD3^+$ lymphocytes were $CD8^+$/$tetramer^+$. Similarly robust responses were observed with cells obtained from healthy donor 2 and, to a lesser extent, patients 1 and 3 and healthy donor 1 (Table I). The frequencies of $p53_{264-272}$-specific precursor CTL in unstimulated lymphocytes obtained from patients or healthy donors were above background, as established in HLA-A2.1-negative individuals or by using a tetramer with an irrelevant peptide (HIV-1 reverse transcriptase peptide, pol 476–484). The frequencies ranged between 0.005 and 0.04% of the $CD3^+$ T cells in all samples analyzed. They were higher for SCCHN patients 1 (0.04%), 2 (0.03%), and 3 (0.02%) than for the nonresponding patients or the nonresponding normal controls, both of which had a mean of 0.01%. The tetramer-based method appeared to be more sensitive for detection of responder T cells than the ELISPOT assay. As shown in Figs. 1 and 3, a higher frequency of tetramer-positive cells (6.6% of $CD3^+$ T cells) was obtained after the third stimulation compared with that of IFN-γ-secreting T cells measured by ELISPOT ($\sim$300/20,000 T cells = 1.5%), as also reported by others (30).

Mutation analysis, p53 overexpression, and the presence of p53 autoantibodies

Although most HLA-$A2^+$ tumor target cells sensitive to lysis by CTL recognizing the wt $p53_{264-272}$ epitope do accumulate mutant p53, this phenotype is not an absolute prerequisite for their recognition by the CTL. In particular, a mutation at codon 273 is known to prevent the processing and presentation of the $p53_{264-272}$ epitope due to interference with the proteasome pathway (31). Immunohistochemistry for p53 as well as sequencing of the p53 gene in the patients' tumors were performed, therefore, to investigate a possible association between the presence of a CTL response specific for the $p53_{264-272}$ epitope and the p53 status of the tumor. Tumor samples were available for all patients studied, and the results of the analysis are shown in Table I.

Of the three patients who showed CTL responses (patients 1, 2, and 3), the tumors of patients 1 and 2 had the wt epitope in exons 5–8 of the p53 gene and no p53 accumulation, whereas that of patient 3 accumulated p53 expressing a missense mutation at codon 273. All three tumors were, therefore, unlikely to present the wt $p53_{264-272}$ epitope. In contrast, mutations in p53 exons 5–8 were detected in all tumors obtained from the four patients who did not show CTL responses to wt $p53_{264-272}$ epitope (patients 4–7). The tumors of patients 4, 5, and 6 accumulated p53 and could present the epitope, whereas the tumor of patient 7 had a mutation in codon 213 (exon 6), resulting in a stop codon, and no detectable p53 protein. Therefore, it would appear that the presence of a CTL response to the epitope under study is more prevalent in patients bearing a tumor unable to present the epitope.

It is important to note that CTL responses were also generated in two of seven HLA-$A2.1^+$ normal donors, but not in two donors who were found to express the HLA-A2.7 subtype. This observation is consistent with the finding that most HLA-2.1-restricted peptides do not bind to HLA-A2.7 molecules (32). None of the patients with SCCHN or normal controls included in this study

Table I. *Summary of responses to wt $p53_{264-272}$ in patients with SCCHN as well as normal donors and the p53 status of patients with SCCHN*[a]

Patient	Anti-p53 Response After 3 × IVS	*p53* Status		
		Tumor *p53* genotype	Tumor p53 protein	Serum *p53*-Ab
1	0.4%; reactivity against $p53^{++}$ tumors lysis of $p53^{++}$ tumors 0.78% $tetramer^+$ T cells	wt in exons 5–8	–	–
2	1.5%; reactivity against tumor cells lysis of $p53^{++}$ tumors 6.6% $tetramer^+$ T cells	wt in exons 5–8	–	–
3	0.26%; reactivity against $p53^{++}$ tumors ND[b] 0.61% $tetramer^+$ T cells	Mutation in exon 8 R 273 H	Accumulation[c] No presentation	–
4	–	Mutation in exon 7 R 248 W	Accumulation	–
5	–	Mutation in exon 5 V 157 F	Accumulation	–
6	–	Mutation in exon 8 E 286 K	Accumulation	–
7		Mutation in exon 6 213 stop	–	–
	Normal donors (n = 9) 7 were HLA-A2.1$^+$ 2 were HLA-A2.1$^-$		Anti-p53 response after IVS +2/7 −2/2	

[a] PBMC were obtained from SCCHN patients or normal blood donors and stimulated with the peptide-pulsed DC or autologous peptide-pulsed PBMC for two to four cycles. Effector cell reactivity was tested in ELISPOT or cytotoxicity assays, and T cell specificity for the $p53_{264-272}$ epitope was confirmed using tetramer technology. The results after the third IVS are shown in the second column as: 1) frequency of $p53_{264-272}$-specific T cells and their reactivity against p53 overexpressing ($p53^{++}$) tumor targets (ELISPOT); 2) reactivity against peptide-pulsed T2 cells and $p53^{++}$ tumor targets (cytotoxicity assay); and 3) frequency of $p53_{264-272}$-specific $CD8^+$ T cells out of total $CD3^+$ T cells (tetramer technology). *p53* gene mutations were determined by sequencing, p53 protein expression by immunohistochemistry and anti-p53 autoantibodies in the patient's sera by ELISA. The order in which the data are presented does not reflect the real time points at which patient samples were obtained and processed.

[b] ND, Not done.

[c] R 273 H mutation has been shown to prevent presentation of $p53_{264-272}$ epitope (31).

was p53 seropositive (Table I), and thus no insights were obtained into possible interactions between humoral and cellular anti-p53 immune responses in these patients.

Discussion

The primary objective of this study was to determine whether CTL specific for the wt $p53_{264-272}$ epitope could be induced from PBMC obtained from patients with SCCHN. The presence of CTL precursors responsive to the wt p53 epitope had been previously demonstrated in PBMC obtained from normal donors, but not patients with SCCHN (12–18). Our data show that it was possible to generate anti-$p53_{264-272}$ responses from PBMC of SCCHN patients with a frequency comparable to that obtained with PBMC of healthy donors. Like the CTL generated from normal donors, these CTL recognized and killed T2 cells pulsed with the wt $p53_{264-272}$ epitope as well as several tumor cell targets, including SCCHN, which naturally present the $p53_{264-272}$ epitope.

However, in this as well as our previous study (18), we noted that CTL responses to wt p53 epitopes could not be induced from PBMC obtained from all the normal donors or patients with SCCHN tested. The failure to induce CTL recognizing wt p53 epitopes in certain individuals might be due to a variety of reasons, including technical limitations of the methods used to induce and detect the CTL as well as their HLA-A2 subtype. However, biological events, such as clonal deletion or anergy of T cells specific for self-epitopes, appear to be primarily responsible for the paucity of these effector cells (33–35). The estimated precursor frequencies of CTL specific for $p53_{264-272}$ peptide ranged from 1:33,000

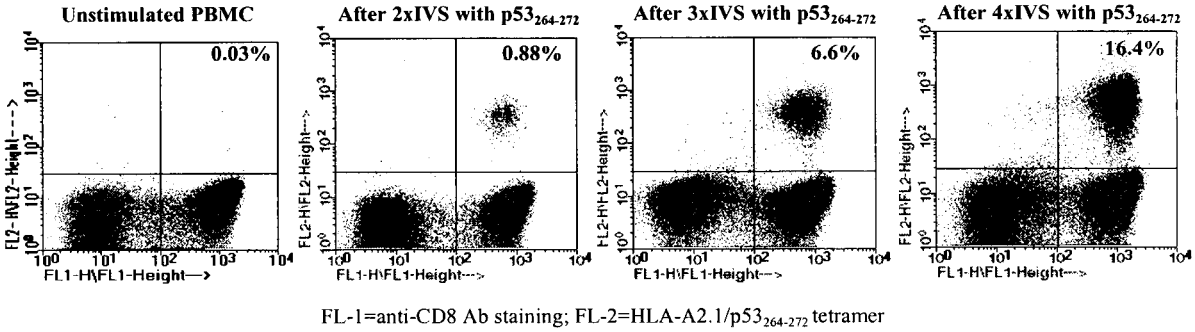

FIGURE 3. Identification of wt $p53_{264-272}$-specific $CD8^+$ T cells by tetramer staining from PBMCs of SCCHN patient 2 before and after IVS (two to four times). Cells were gated by forward and side scatter for lymphocytes and by $CD3^+$ for T cells. Gated populations are plotted as CD8 staining (horizontal axis) vs tetramer staining (vertical axis). In the *right upper quadrant*, the number of $tetramer^+/CD8^+$ T cells of all gated $CD3^+$ lymphocytes is shown as a percentage.

to <1:300,000 in PBMC of three normal donors tested by limiting dilution (15). In the current study, we applied for the first time tetramer technology to detect human T cells specific for the wt $p53_{264-272}$. The use of tetramers is particularly attractive, because it allows a direct quantification of Ag-specific T cells from blood without the need for their in vitro expansion (30, 35). Using three-color flow cytometry, we detected relatively high frequencies (up to 1:2500) of T cells specific for the epitope in patients with SCCHN who responded to IVS. Using the tetramer technology, we are currently involved in determining the proportions of anti-p53 precursor T cells in the circulation of a larger number of HLA-$A2.1^+$ SCCHN patients compared with healthy donors by four-color flow cytometry (T. K. Hoffmann, A. D. Donnenberg, T. L. Whiteside, and A. B. DeLeo, manuscript in preparation). The preliminary data confirm our previous observations, indicating that the precursor frequencies are higher in patients with SCCHN who responded to IVS with the wt $p53_{264-272}$ peptide than in those who did not respond or in normal donors. Studies by Sherman's group (34, 35) demonstrated that tolerance to self-p53 epitopes in mice is associated with deletion of high-avidity T cells and retention of low to intermediate affinity T cells. In humans, comparable anti-self-p53 CTL have been isolated following IVS (18). Based on our detection of nonexpandable tetramer-positive anti-$p53_{264-272}$ T cells in PBMC of non-IVS responders, these cells can be considered to have been tolerized. Presently, however, one can only speculate as to the mechanism(s) contributing to their anergy. It is possible that, due to their inherited repertoire, the TCRs capable of recognizing this self-p53 epitope (tetramer positive) in nonresponding individuals have a lower affinity for the epitope and/or a lower density than the TCRs expressed in responding individuals, which could prevent their appropriate stimulation and expansion (33). An area under investigation, therefore, is a comparative analysis of the tetramer binding affinity of precursor CTL in PBMC obtained from nonresponding and responding individuals.

In the current study, we observed that CTL specific for the wt $p53_{264-272}$ epitope were generated only from PBMC of SCCHN patients with tumors unable to present this epitope (Table I). The tumors either expressed a wt p53 genotype (exons 5–8) with no p53 accumulation or accumulated mutant p53 molecules with a point mutation (R to H) at codon 273. This mutation, which occurs in ~12% of human cancers and is considered at a hot spot, has been shown to block processing of the $p53_{264-272}$ epitope (31). It is tempting to speculate, therefore, that tumor cells harboring the codon 273 mutation might be able to evade recognition by CTL in HLA-$A2^+$ individuals and thus have a competitive edge for growth. The presence of epitope-specific CTL precursors in PBMC of such individuals might well facilitate the outgrowth of the epitope-loss tumors. This type of immunoselection could lead to the development of tumors that successfully escape the host immune system (36, 37). The results of our analysis of the protective and therapeutic effects of p53-based vaccines in mice exposed to the chemical carcinogen, methylcholanthrene, are supportive of this concept. We observed a higher incidence of epitope-loss tumors in animals immunized against a single wt p53 epitope than in control groups of mice (V. Cicinnati, G. Dworacki, and A. B. DeLeo, unpublished observations).

Although based on an analysis of a limited number of patients, the observation that CTL recognizing the wt $p53_{264-272}$ epitope could not be generated from PBMC of patients with tumors likely to present this epitope suggests that another form of immune evasion might have taken place in vivo. By preventing CTL generation or by elimination of p53-specific precursor T cells, these tumors could have escaped from immune intervention. The capability of SCCHN and other human tumors to disarm the host immune system by inducing dysfunction or apoptosis of effector cells has been well recognized (21, 22, 38–40) and is under extensive investigation in our laboratories. However, for patient 7 no inverse relationship between IVS responsiveness and the p53 status of the tumor was observed. This patient's PBMC did not respond to IVS, yet this individual's tumor can be considered to be p53-null (one p53 allele was deleted, and the other had a stop codon at 213). Until other similar cases are found, it is difficult to speculate on the possible basis for this observation. Unlike the other patients studied, however, this patient recently underwent extensive tumor resection and adjuvant postoperative therapy. As a result, this individual may be immunocompromised to a greater extent than any of the other patients studied.

We anticipate that the results of the analysis of PBMC obtained from a larger population of patients with SCCHN using tetramers will provide further insights into the intriguing possibility that the frequency of $p53_{264-272}$ CTL precursors in the peripheral blood of patients with SCCHN, particularly those whose tumors have a low potential of presenting the epitope, is significantly different from that in healthy donors. If a confirmation of this emerging pattern is obtained, it would suggest that the wt $p53_{264-272}$ epitope is immunogenic in some HLA-$A2^+$ individuals, and that CTL specific for this epitope may well have influenced the outgrowth of epitope-loss tumors that are able to avoid these effector cells. The possibility that immunoselection of such tumor cells might occur during p53-based immunotherapy merits consideration in designing future clinical trials. It implies that the use of vaccines capable of targeting multiple p53 epitopes expressed on different class I HLA molecules might be necessary to prevent tumor escape from the immune system.

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Cytometry 41:321–328 (2000)

Rapid Communication

Competition of Peptide-MHC Class I Tetrameric Complexes With Anti-CD3 Provides Evidence for Specificity of Peptide Binding to the TCR Complex

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Received 30 June 2000; Revision Received 15 August 2000; Accepted 16 August 2000

Background: Major histocompatibility complex (MHC)-peptide tetrameric complexes (tetramers) are valuable tools for detecting and characterizing peptide-specific T cells. Because the frequency of these cells is generally very low, it may be difficult to discriminate between nonspecific and specific tetramer binding.

Methods: A four-color flow cytometric assay that simultaneously measures tetramer, CD3, CD8, and CD14 was used to investigate the sensitivity and specificity of MHC class I tetramer staining. This was accomplished by using the influenza virus matrix protein peptide, GILGFVFTL (FLU), as a model recall antigen and the human immunodeficiency virus (HIV) reverse transcriptase peptide, ILKEPVHGV (HIV), as a model novel antigen. Peripheral blood mononuclear cells (PBMC) from 31 HLA-A2.1$^+$ and 10 HLA-A2.1$^-$ healthy individuals were stained with the tetramers.

Results: The lower limit of detection was established at approximately 1/8,000. In HLA-A2$^+$ PMBC, frequencies of tetramer-positive CD8$^+$ T cells were log normally distributed and were high for FLU (1/910) but low for HIV (1/6,067). A novel competition assay, in which tetramer binding was shown to diminish subsequent staining with anti-CD3 antibody, was used to confirm the specificity of tetramer binding to the T-cell receptor (TCR) complex. The competition assay was validated by evaluating several anti-CD3 antibodies and showing that in PBMC from HLA-A2$^-$ subjects, spurious tetramer-positive events (1/20,000) failed to compete with CD3 binding. For the "recall" FLU tetramer, the degree of competition was proportional to the frequency, suggesting a selection of high avidity cells. Although CD3 competition was also highly correlated with the intensity of tetramer staining, competition allowed the identification of false positive cases with relatively high tetramer staining intensity.

Conclusion: The data indicate that competition of CD3 binding allows confirmation of the specificity of tetramer binding to the TCR, extending the usefulness of tetramers in the frequency analysis of peptide-specific T lymphocytes. Cytometry 41:321–328, 2000.

Key terms: MHC-class I tetramer; CD3; influenza virus; human immunodeficiency virus

Soluble peptide-major histocompatibility complexes (MHC) have a fast dissociation rate from the T-cell receptor (TCR). Multimeric peptide-MHC complexes bind to more than one TCR on a specific cell and, therefore, have slower dissociation rates (1,2). Multimeric peptide-MHC complexes (generically referred to as tetramers because of their 4:1 molar ratio of biotinylated protein complexes to streptavidin) are proving invaluable as fluorescent reagents for enumeration, characterization, and isolation of peptide-specific T cells. They have afforded many advantages over previous techniques, particularly the ability to directly quantify and phenotype antigen-specific T cells with minimal in vitro manipulation (3–11). However, few

Grant sponsors: National Institutes of Health; Grant numbers: 1RO1-AI41408, RO1-AI 14870, 1PO1-A143664 (ADD), PO-1 DE-12321 (TLW, ABD), CA 72914 (ABD); Grant sponsor: Dr. Mildred Scheel Stiftung für Krebsforschung; Grant number: D/99/08916 (TKH).

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studies have investigated physical characteristics that underlie tetramer binding. Wheelan et al. (12) described temperature as a variable affecting the specificity and intensity of tetramer staining. Bodinier et al. (13) determined that nonspecific tetramer binding can occur through interaction with the CD8 coreceptor. In addition, Yee et al. (14) correlated tetramer staining intensity with T-cell avidity and demonstrated that tetramers can be used to selectively identify high avidity cytotoxic T lymphocytes (CTL). The majority of investigators have used relatively simple two- or three-color staining strategies to identify $CD8^+$ tetramer-positive events. Lee et al. (15) used multiparameter flow cytometry to hone the specificity of tetramer staining by eliminating $CD4^+$, $CD19^+$, and $CD14^+$ cells from their analysis. However, this approach has not been widely adopted, owing perhaps to the complexity of their 10-color flow assay. Multiparameter analysis may not be necessary when the goal is to characterize the tetramer binding properties of T-cell lines or clones or to evaluate the in vivo response to strong antigens such as viral peptides. However, when the objective is to evaluate the in vivo frequency of T cells to novel antigens, self-antigens, or tumor antigens (14–22), for which tetramer binding frequencies are low and staining patterns may be ambiguous, it is important to establish a method that minimizes background staining and allows tetramer binding specificity to be evaluated as an independent parameter.

In this study, we addressed these issues using MHC class I tetrameric complexes containing a human immunodeficiency virus (HIV) peptide and an influenza virus (FLU) peptide as models for novel and recall antigens, respectively. The aim was to optimize analysis for future studies with expected low frequencies of peptide-specific T cells. A four-color flow cytometry-based technique is described, allowing the reduction of background staining and the confirmation of the specificity of tetramer binding to the TCR.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells

Peripheral blood or a leukapheresis product was obtained from 31 HLA-$A2.1^+$ and 10 HLA-$A2.1^-$ normal healthy donors. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficoll-Hypaque gradients (Amersham Pharmacia Biotech, Piscataway, NJ). Leukapheresis products were prepared by the Institute of Transfusion Medicine (Pittsburgh, PA). The study was approved by the institutional review board (IRB) at the University of Pittsburgh and written informed consent was obtained from each individual. All products were tested and found negative for HIV-I antigens and antibodies to HIV.

Cryopreservation

PBMC were frozen at a concentration of 50×10^6 cells/mL in freezing medium consisting of human AB serum (Pel-Freeze, Brown Deer, WI) plus 10% dimethylsulfoxide (DMSO; Fisher Scientific, Pittsburgh, PA). Cells were held overnight at -80°C and transferred to liquid N_2. Cells were rapidly thawed at 37°C and an excess of cold Aim-V medium (Life Technologies, Grand Island, NY) supplemented with 20% human AB serum was added to the cells. The cells were washed twice, adjusted to 5×10^6/mL, and maintained for 2–4 h at room temperature. Clumps were carefully removed and viability of the remaining cells was measured by the trypan blue dye exclusion test. Average viability was 70–80%.

HLA-A Subtyping

PBMC were phenotyped for expression of HLA-A2 molecules by flow cytometry, using the anti-HLA-A2 monoclonal antibody (mAb), BB7.2 (ATCC, Manassas, VA), and an IgG isotype as a control. The verification of the A2.1 subtype was performed in the laboratory of Dr. Adriana Zeevi using polymerase chain reaction with sequence-specific primers (PCR-SSP) as previously described (23). Briefly, DNA was obtained from PBMC using sodium dodecyl sulfate (SDS) and proteinase K. After removal of protein contaminants with a saturated salt solution, DNA was precipitated using 2 volumes of ethanol. Following washing and drying, the DNA pellet was reconstituted and quantified by reading the optical density (OD) at 260 nm. The extracted DNA was subjected to PCR-SSP (Dynal, Oslo, Norway) with primers that distinguish the specific allelic polymorphisms. Each primer set was designed to give an amplified fragment of a specific size, which was detected by gel electrophoresis and ethidium bromide staining. Patterns of positive and negative amplification yield the relevant genotype. A total of 31 HLA-$A2.1^+$ and 10 HLA-$A2^-$ healthy individuals were identified and included in the study.

Tetrameric Peptide-MHC Class I Complex (Tetramer) Assay

Tetramers were obtained through the National Institute of Allergy and Infectious Diseases (NIAID) Tetramer Facility and the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program; stock solutions contained 0.5 μg monomers per milliliter. Tetramer preparation was described in detail by Altman et al. (1). Peptides provided to the NIAID were either GILGFVFTL, an influenza matrix immunodominant peptide (residues 58–66), or the HIV-1 reverse transcriptase peptide (pol 476–484), ILKEPVHGV.

To minimize background staining, each tetramer was titered and used at the lowest concentration that still gave a clearly discernible positive population in a donor vaccinated for influenza (FLU 58-66 tetramer) and in an HIV-infected individual (pol 476–484; 10). The final dilution of both preparations during staining, relative to the stock reagent supplied by the NIH, was 1/300. Within a twofold range of tetramer concentrations bracketing the concentrations used here, the frequency of tetramer-positive events and competition of CD3 binding were stable and tetramer fluorescence intensity was within 80% of that obtained at saturating concentrations. At saturating concentrations, CD3 competition decreased, fluorescence intensity of tetramer-positive cells increased, and tetramer

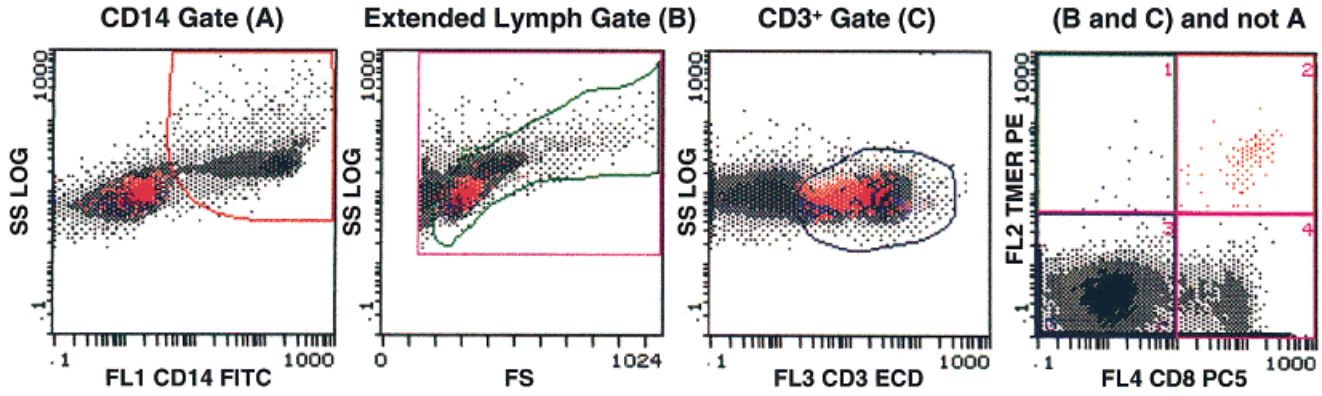

FIG. 1. Compound gating strategy used to identify tetramer-positive $CD8^+$ T cells. PBMC from an HLA-$A2.1^+$ healthy donor were stained with FLU tetramer followed by anti-CD14, anti-CD3, and anti-CD8 mAbs using four-color flow cytometry. Tetramer-positive cells were evaluated within a Boolean gate excluding $CD14^+$ events and including cells within an extended lymphoid light scatter gate and a generous $CD3^+$ gate. Tetramer-positive $CD8^+$ $CD3^+$ cells are "color evented" in red, revealing their light scatter and staining properties. In this example, $CD3^+$ $CD8^+$ tetramer-positive events were present at a frequency of 1/313 $CD3^+$ $CD8^+$ T cells (0.320%).

frequency increased, the latter due chiefly to an increased number of tetramer-dim events.

Antibodies

The default panels for these studies were CD14-FITC (RMO52; Immunotech, Miami, FL), tetramer-PE, CD3-ECD (HIT3a; Beckman Coulter, Miami, FL), and CD8-PC5 [SFCI21Thy2D3(T8), Beckman Coulter]. Additionally, the following antibodies were used: anti-human α/β FITC (WT31; Becton Dickinson, Mountain View, CA); anti-human CD2 FITC (Leu-5b; Becton Dickinson); anti-human CD3 FITC (HIT3a; PharMingen, San Diego, CA); anti-human CD3 FITC (UCHT1; Immunotech); anti-human CD3 FITC (SP34; PharMingen); anti-human CD3 FITC (SK7; Becton Dickinson); anti-human CD5-FITC (BL1a; Immunotech); anti-human CD14-ECD (RMO52; Coulter); anti-human CD45RA-PC5/TC (MEM56; Caltag, Burlingame, CA); and anti–CD45RO-FITC (UCHL1, Caltag).

Flow Cytometry Analysis

Immediately prior to staining, cells were washed twice with the staining medium consisting of phosphate-buffered saline (PBS) + 0.1% (w/v) bovine serum albumin (BSA) + 0.1% (w/v) sodium-azide and resuspended at a concentration of 5×10^6/mL in a volume of 150 μl. Tetramer (5 μl of 1:10 dilution of stock solution) was added at room temperature for 30 min, followed by a 30-min incubation with antibodies (7.5 μL of each) at 4°C. After two additional washes, the cells were resuspended in approximately 1 mL of 0.5% methanol-free formaldehyde in PBS. At least 1×10^6 events were collected using a four-color Coulter Epics XL cytometer set on low or medium flow rate at a maximum of 1,000 events per second. Flow cytometry data were analyzed in real time using Beckman-Coulter System II software. In initial experiments, the region defining tetramer-positive events was determined by evaluating PBMC stained with the antibody panel, but without tetramer. This region was held constant throughout the analysis. Data were saved as FCS 2.0 Listmode files for subsequent reanalysis in System II or WinList (Verity Software House, Topsham, ME).

Statistical Analysis

Tetramer-positive cells were quantified by flow cytometry and expressed as frequencies (e.g., 1/1,000) or reciprocal frequencies (e.g., 1,000). We examined raw reciprocal frequency data and log-transformed reciprocal frequency data using normal probability plots (24). For both tetramers, the log-transformed data were better modeled by the normal distribution. Accordingly, descriptive statistics (means, SD, confidence intervals [CI]) and statistical analyses (Student's *t*-test, two-tailed) were performed on log-transformed reciprocal frequencies. For tetramer frequencies as well as competition of antibody binding, lower limits of detection were established by estimating the 99th percentile (geometric mean plus 2.6 SD) of responses measured in PBMC from HLA-A2- subjects. Linear regression was performed by the methods of least squares. In regression plots, the line of best fit and the 95% CI about the line of best fit are shown. The coefficient of correlation (r^2), the slope of the regression line, and the *P* value associated with the slope are reported as regression summary statistics. Statistical analysis and statistical graphics were performed using Systat Version 9 (SPSS, Chicago, IL).

RESULTS

Elimination of Nonspecific Tetramer Binding Events

To increase the specificity of tetramer binding, we developed a gating strategy to eliminate monocytes and apoptotic and necrotic cells, all of which can potentially bind tetramer or antibody nonspecifically. PBMC from a healthy HLA-$A2.1^+$ donor were incubated with the FLU tetramer (PE), followed by mAbs against CD14 (FITC), CD3 (ECD), and CD8 (PC5). Figure 1 shows that sources of nonspecific binding (e.g., $CD14^+$ monocytes in Fig. 1A,

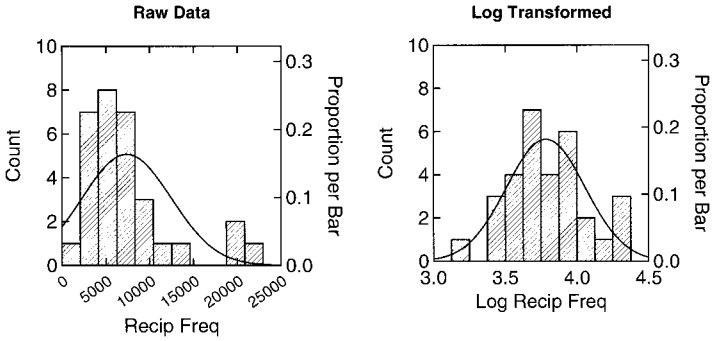

FIG. 2. HIV and FLU tetramer frequency data are log normally distributed. Reciprocal frequency data (**left**) and log-transformed reciprocal frequency data (**right**) from 31 independent analyses using the HIV tetramer are displayed as frequency histograms overlayed with the normal distribution predicted by the sample mean and SD (solid line). For data drawn from a normally distributed population, the shape and location of the bar histogram approximate that of the normal distribution curve (right). Without log transformation (left), the data are poorly represented by the normal distribution. This invalidates the use of parametric tests such as Student's *t*-test and renders the mean and SD poor descriptors of the data set.

dead cells in Fig. 1B, or other $CD3^-$ cells in Fig. 1C) were eliminated by compound gating. Cells with low tetramer staining intensity were also eliminated. A clearly discernible cluster of tetramer-positive/$CD8^+$ T cells emerged with a mean fluorescence intensity (MFI), which was at least one log above the MFI of the negative population. Compound gating also permitted us to use a liberal lymphocyte scatter gate (Fig. 1B) that would not exclude large activated T cells. Without these compound gates, a "smear" of tetramer-positive $CD8^+$ cells is often detected, including a significant number of cells with low tetramer staining intensity. For these reasons, the described gates were used for all subsequent experiments performed with the panel of antibodies including anti-CD14, anti-CD3, and anti-CD8.

Log Transformation of the Data and the Use of the Geometric Mean

It is essential to understand the distribution of data before descriptive statistics can be generated and statistical tests can be applied. Figure 2 shows a histogram of the raw and log-transformed HIV frequency data. Superimposed over the data are the normal distributions inferred from the sample mean and SD. The raw data are left skewed and does not conform to a normal distribution, invalidating the use of parametric statistics. Log transformation resulted in a more symmetric data distribution, with the mode and median values closer to the mean. For the two peptides used in this study and for others (data not shown), we have found that taking the log of the reciprocal frequency is optimal for bringing the data into conformation with a normal distribution.

Definition of the Lower Limit of Detection of Tetramer-Positive T Cells

In order to establish the lower detection limit for tetramer binding in HLA-$A2.1^+$ individuals, we stained PBMC obtained from 10 HLA-$A2^-$ individuals. Tetramer binding to peptides representing a recall antigen (FLU) and a novel antigen (HIV) was examined. Despite the application of the gating strategy described above, low levels of FLU and HIV tetramer-positive $CD3^+$ $CD8^+$ T cells were detected in PBMC of HLA-$A2^-$ donors (geometric means = 1/22,131 and 1/23,551, respectively). Because these events were nonspecific by definition, we established a cutoff for the lower detection limit of this assay at the upper 99th percentile of tetramer-positive $CD8^+$ T cells in HLA-$A2^-$ individuals. Using this cutoff, the lower limit of detection was 1/7,805 (pooled data for FLU and HIV tetramers). This cutoff was applied to all data obtained from testing the 10 HLA-$A2^-$ and 31 HLA-$A2.1^+$ individuals (Fig. 3). None of the HLA-$A2^-$ individuals had frequencies of FLU or HIV tetramer-positive $CD8^+$ T cells exceeding the cutoff. However, all HLA-$A2.1^+$ individuals had detectable frequencies of FLU tetramer-positive $CD8^+$ T cells (mean = 1/910) and 23 of 31 had detectable frequencies of HIV tetramer-positive $CD8^+$ T cells (mean = 1/6,067).

CD45 Isoform Expression

In order to discriminate between putative naive or memory subsets in tetramer-positive T cells, we determined CD45 isoform expression in the PBMC of five subjects. Cells were stained with either FLU or HIV tetramer followed by CD45RO-FITC, CD8-ECD, and CD45RA-PC5. Monocytes and high side scatter NK cells were eliminated from the analysis by using a tight lymphocyte light scatter gate. For the recall antigen, FLU, the majority of $CD8^+$ tetramer-positive cells were $CD45RO^{bright}/CD45RA^-$ (76.7 ± 2.7%). For the novel antigen, HIV, $CD8^+$ tetramer-positive cells were predominantly $CD45RO^-/CD45RA^{bright}$ (71.9 ± 9.4%).

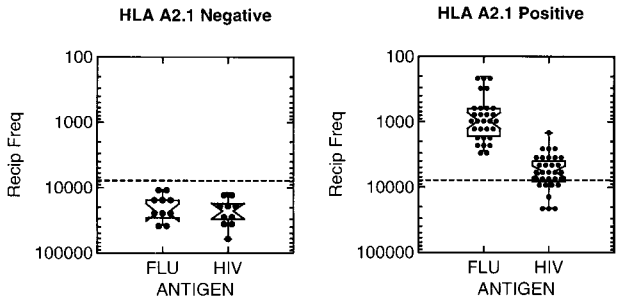

FIG. 3. Reciprocal frequencies of tetramer-positive $CD8^+$ $CD3^+$ T cells. Filled dots show individual data points. Superimposed notched box plots display nonparametric descriptive statistics. The waist indicates the group median and the hinges (upper and lower boundaries of the box) indicate interquartile distances. The notches show simultaneous 95% CI intervals about the median. The whiskers (bars) give the ranges. Reciprocal frequencies of tetramer-positive $CD8^+$ T cells in HLA-$A2.1^-$ (n = 10, **left**) and HLA-$A2.1^+$ (n = 30/31 [FLU/HIV], **right**) healthy donors. The dashed line represents the cutoff for the lower detection limit as determined in HLA-$A2.1^-$ donors (geometric mean + 2.6 SD).

Competition Between Tetramer and Anti-CD3 Antibody Binding

During analysis of the data set, we consistently noted that in PBMC from HLA-A2.1$^+$ subjects, tetramer-positive events stained dimmer for CD3 than did tetramer-negative events (Fig. 1C). This was not the case for the spurious tetramer-positive events seen in the PBMC from HLA-A2$^-$ subjects (see below). This observation suggested that tetramer binding to the TCR interfered with binding of anti-CD3 antibody to CD3. To determine whether this phenomenon could be exploited as a marker for specificity of tetramer binding to the TCR complex (as opposed to nonspecific binding to some other surface structure), we quantified the extent to which tetramer binding competed with CD3 binding on CD8$^+$ T cells according to the formula:

$$\text{Percent competition} = \frac{[(\text{CD3Mnl}_{\text{CD3}^+\text{CD8}^+\text{tmer}^-}) - (\text{CD3Mnl}_{\text{CD3}^+\text{CD8}^+\text{tmer}^+})]}{(\text{CD3Mnl}_{\text{CD3}^+\text{CD8}^+\text{tmer}^-})} \times 100$$

where CD3Mnl is the log MFI of CD3 staining of the population designated by the subscript.

To confirm and further characterize competition between tetramer and anti-CD3 antibody binding, we performed a series of experiments in which the anti-CD3 antibody (clone HIT3a) was exchanged for a variety of other antibodies directed to the epsilon chain of the TCR, TCR α/β, CD2, or CD5. Results from testing PBMC obtained from three HLA-A2.1$^+$ donors indicated that binding of anti-CD3 clones HIT3a, UCHT 1, and SP34 was comparably inhibited by the FLU tetramer (Table 1). No consistent effect was observed with the anti-CD3 mAb SK7, anti-CD2, or anti-CD5. Interestingly, binding of antibodies to TCR α/β was consistently enhanced. The data indicate that tetramer binding to the TCR results in steric changes that influence binding of antibodies directed at some, but not all, epitopes of CD3 epsilon.

Optimal competition of anti-CD3 binding was dependent on the staining sequence. When HLA-A2.1$^+$ PBMC were incubated with the FLU tetramer first, followed by the incubation with the anti-CD3 antibody, competition was significantly greater ($P = 0.008$, paired t-test) than that obtained with the inverse approach (first antibodies, then tetramer; Table 2). Accordingly, CD3 staining intensity was significantly lower ($P = 0.007$) and tetramer staining was significantly brighter ($P = 0.0004$). In this small series, the estimated frequency of FLU tetramer-positive CD8$^+$ T cells was also somewhat lower when cells were stained first with anti-CD3. However, the difference was not statistically significant ($P = 0.06$). These findings further support the conclusion that the diminution of CD3 binding is due to competition by the tetramer.

Because there was no detectable competition between the tetramer and anti-CD3 antibody binding for CD8$^+$ T cells in 9 of 10 HLA-A2.1$^-$ individuals (Fig. 4), we were able to define a cutoff based on the 99th percentile of CD3 competition in these HLA-A2$^-$ subjects (3.2%). CD3 competition in excess of this cutoff was considered significant. For the FLU tetramer, 28 of 30 subjects displayed significant CD3 competition. For the novel HIV peptide, there was significant competition of CD3 binding in only 11 of 31 subjects. Competition was strongest on CD8$^+$ FLU

Table 1
Competition of Anti–T-Cell Antibodies With FLU Tetramer in PBMC From HLA-A2.1$^+$ Donors[†]

	Percent competition in CD8$^+$ T cells ($n = 3$ HLA-A2$^+$ normal donors)
Anti-CD3 mAb clone	
HIT3a (ECD, Coulter)	50.9 ± 10.6*
HIT3a (FITC, PharMingen)[a]	52.8 ± 9.4**
UCHT 1 (FITC, Immunotech)[a]	52.6 ± 9.1**
SP34 (FITC, PharMingen)[a]	49.9 ± 11.4*
SK 7 (FITC, Becton Dickinson)[a]	17.7 ± 8.8
Anti-CD5 mAb (FITC, clone BL1a, Immunotech)[a]	9.7 ± 13.8
Anti-CD2 mAb (FITC, clone Leu-5b, Becton Dickinson)[a]	−57.0 ± 29.5
Anti-α/β (FITC, clone WT31, Becton Dickinson)[a]	−25.4 ± 2.0***

[†]Cells were stained with FLU tetramer-PE followed by mAbs. Results are means ± SD of results from three individuals. Negative competition, reported for CD2 and TCR α/β, indicates that anti–T-cell mAb staining was brighter in tetramer-positive events. Student's t-test (two-tailed) was used to determine whether competition was significantly different than 0%: *$P < 0.025$, **$P < 0.01$, ***$P < 0.005$.

[a]In addition to the designated anti–T-cell mAb, which was FITC conjugated, cells were stained with anti–CD14-ECD and anti–CD8-PC5.

Table 2
Optimal Competition of Anti-CD3 Binding by Tetramer Requires That Tetramer Binding Precede Staining With Anti-CD3 (HIT3a-ECD)[†]

	Order of reagent addition	
	1. Tetramer 2. Antibodies	1. Antibodies 2. Tetramer
Competition of anti-CD3 binding (%)	49.0	20.8*
CD3 staining fluorescence intensity of tetramer$^+$ CD8$^+$ T cells (geometric mean channel)	16.8	33.6*
Tetramer staining fluorescence intensity (geometric mean channel)	36.4	23.9**
Frequency of tetramer$^+$ CD8$^+$ T cells	1/239	1/274

[†]Results are means of four independent experiments. Student's paired t-test (two-tailed) was used to determine whether competition was significantly different when the order of staining was changed: *$P < 0.01$, **$P < 0.001$.

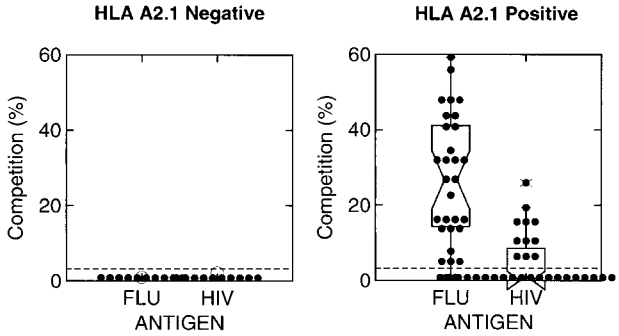

FIG. 4. Competition of anti-CD3 binding by tetramer on $CD8^+$ $CD3^+$ T cells. Notched box plots show individual data points and nonparametric statistics as described in Figure 3. Outliers (more than 1.5 times the hingespread from the median) are shown by asterisks and far outliers (more than three times the hingespread from the median) are shown by double circles. **Left:** Competition in $CD8^+$ T cells from HLA-$A2.1^-$ (n = 10) subjects. **Right:** Results obtained in HLA-$A2.1^+$ (n = 30–31) individuals. The dashed line represents the cutoff for the lower limit of competition as determined in HLA-$A2.1^-$ normal controls.

tetramer-positive T cells (mean = 26.5%) and was lower for $CD8^+$ HIV tetramer-positive T cells (mean = 4.7%).

When reciprocal frequencies of tetramer-positive $CD8^+$ T cells were plotted against tetramer competition of anti-CD3 binding, a strong correlation was noted for FLU tetramer-positive $CD8^+$ T cells (r^2 = 0.425, slope = -34.7, P = 0.0004, Fig. 5, left panel). This relationship was not seen for the HIV tetramer (Fig. 5, right panel). This observation is consistent with the interpretation that T-cell expansion driven by repeated in vivo exposure to antigen results in selection of T cells that bind the tetramer more avidly and thus compete anti-CD3 binding more effectively.

In keeping with this interpretation, CD3 competition was plotted as a function of the median fluorescence intensity of tetramer-positive events (Fig. 6, left panel). The strong correlation indicates that individuals with brighter tetramer staining also have higher CD3 competition. T cells that bind greater numbers of tetramer per cell, and are brighter, are interpreted to express a high avidity TCR (14). CD3 competition can be seen to increase both as a function of tetramer brightness and frequency (Fig. 6, right panel). More importantly, CD3 competition can be used to screen out cases with bright tetramer staining at low frequency (Fig. 6, red circles). In this data set, 13 of 61 cases (most of them, the HIV tetramer) fell below the CD3 competition cutoff, despite having frequencies in excess of 1/7,805.

DISCUSSION

Tetramer staining is widely used today for the detection of antigen-specific T cells. By analyzing the binding of tetramers representing prototype recall and novel peptides and using T cells from HLA-2.1^+ and HLA-$A2.1^-$ subjects, this report details some of the potential pitfalls and provides guidance for interpretation of results. In addition to describing a new method to determine the specificity of tetramer binding, we have addressed several technical issues that bear on the interpretation of peptide-MHC tetramer results. This is especially important when the population of interest is present at a low frequency and is subject to several sources of artifact that are characteristic of rare event problems (25). Tetramer-positive events were defined in six-parameter space. Because of this, we were able to identify our event of interest as $CD3^+$/$CD8^+$/tetramer positive and eliminate non-T cells, monocytes, debris, and dead cells, which is particularly important when cryopreserved cells are used. CD14 was useful as a "dump" parameter because any event that was positive in FL1, whether a $CD14^+$ monocyte or broadly autofluorescent debris, was eliminated as a potential tetramer-positive event (15). The only alternative to multiparameter flow cytometry is to perform a preseparation step in which sources of interference are eliminated by negative selection (9,17,19,20), with the attendant risk of nonrandom cell loss or alteration. Another technical issue bearing on data analysis addressed here is the use of geometric, rather than arithmetic means to summarize the data and determine cutoffs that define the lower limits of detection. This is analogous to antibody titers, for which the use of geometric mean reciprocal frequency is standard practice (26). Several reports in the current literature have used arithmetic means of percent tetramer-positive cells (6,9,27). As a result, mean values are biased by a left shoulder of high frequencies and are therefore overestimated. Similarly, estimates of the sensitivity (lower limit of detection) of the assay would be underestimated. Finally, having applied this methodology, a cohort of HLA-$A2.1^-$ individuals was used to define the level of nonspecific tetramer binding, and hence the lower limit of detection of this assay. For FLU and HIV tetramers (as well as others, not shown), the frequency of false $CD3^+$/$CD8^+$/tetramer-positive events was remarkably consistent at approximately 1/20,000 (0.005% positive). Therefore, the limit of detection (the 99th percentile of false-positive events) was estimated at approximately 1/8,000 (0.0125% positive).

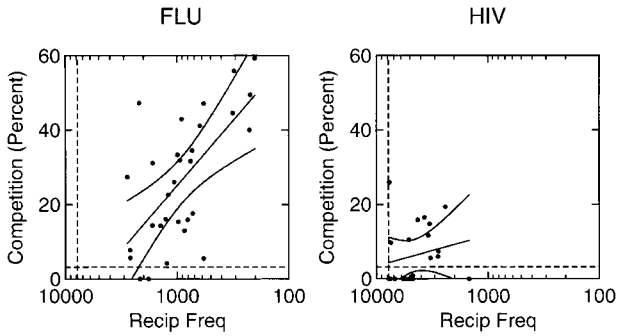

FIG. 5. Correlation between the frequency of tetramer-positive T cells and the magnitude of competition for CD3 binding. PBMC from 30 and 31 HLA-$A2.1^+$ subjects (FLU and HIV, respectively) were tested; data were censored from the analysis if the frequency of tetramer-positive T cells was lower than the cutoff established in HLA-$A2.1^-$ subjects. Dashed lines represent the cutoffs for the lower detection limits of tetramer frequency and CD3 competition. A strong correlation between frequency of tetramer-positive T cells and magnitude of competition was obtained for the FLU tetramer (r^2 = 0.425, slope = -34.7, P = 0.0004, **left**) but not for the HIV tetramer (**right**).

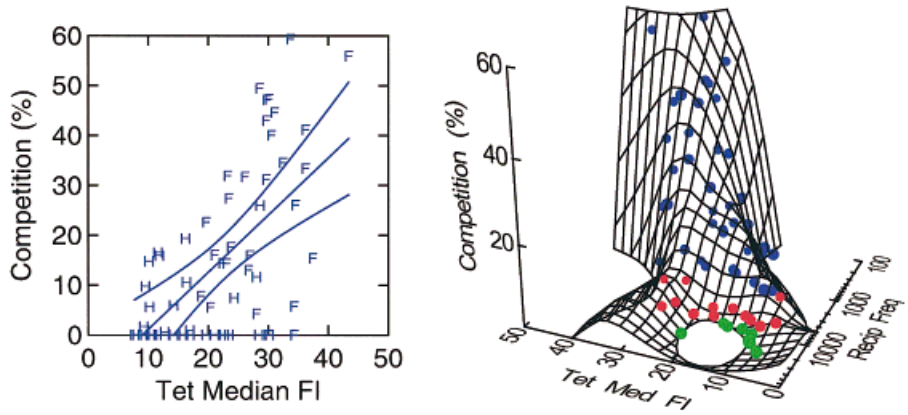

FIG. 6. Correlation between the magnitude of competition for CD3 binding, the median staining intensity of tetramer-positive T cells, and the frequency of tetramer-positive events. PBMC from 30 and 31 HLA-A2.1$^+$ subjects (FLU and HIV, respectively) were tested. The two-dimensional plot shows a strong correlation between the median staining intensity of tetramer-positive T cells and the magnitude of competition ($r^2 = 0.363$, slope = 1.144, $P = 0.0004$, **left**). FLU and HIV data are indicated by the symbols F and H, respectively. In the three-dimensional plot (**right**), values that lie below the CD3 competition cutoff (3.2%) and the frequency cutoff (1/7,800) are shown in green. Those that lie below the CD3 competition cutoff but not the tetramer frequency cutoff are shown in red. There were no data points in which CD3 competition was above the cutoff and tetramer frequency was below the cutoff. A distance-weighted least-squares curve-fit surface superimposed on the data (grid) indicates that CD3 competition is correlated with both tetramer frequency and brightness.

We consistently observed that tetramer-positive cells had diminished staining with anti-CD3 mAb, compared to tetramer-negative T cells. It is not likely that the observed diminution in brightness of anti-CD3 was due to internalization, down-regulation, or capping of the CD3/TCR complex. The reason is that staining was performed in the presence of sodium azide and staining with anti-TCR α/β was actually enhanced by tetramer binding. This unexplained, but highly significant, enhancement also rules out the possibility that tetramer-positive cells have inherently lower TCR expression. Further, competition of anti-CD3 binding was dependent on the sequence of staining events: incubation with tetramer prior to anti-CD3 gave the greatest inhibition. In contrast, the frequency of tetramer-positive cells was not significantly affected by the sequence of staining. Finally, for the recall FLU peptide, the magnitude of competition was proportional to the frequency of tetramer-positive cells. This suggests that successive rounds of in vivo exposure to antigen result in selection of T cells that bind peptides more avidly (14) and therefore compete with anti-CD3 antibody binding to a greater extent. This was not the case for the novel HIV antigen. CD45 isoform expression on tetramer-positive cells is consistent with this interpretation as the majority of FLU tetramer-positive cells were CD45RA$^-$/CD45RO$^+$, whereas the majority of HIV tetramer-positive cells were CD45RA$^+$/CD45RO$^-$. Analysis of tetramer brightness alone did not appear to be sufficient to ensure specificity, as a number of cases were identified where tetramer bound brightly at low frequency, but which were CD3-competition negative.

Although the epitope specificity of the commercial antibodies used in this study are not known, competition appeared to be epitope dependent. Binding of one of five anti-CD3 epsilon mAbs was not competed by tetramer. The competition also appeared to be unique to antibodies directed against the CD3 epsilon chain; mAbs directed against CD5 or CD2 were not competed by tetramer binding and, as mentioned, anti-α/β binding was actually enhanced. These data support the idea that CD3 competition requires TCR engagement by tetramer. We suggest that CD3 competition can be used as a marker for tetramer specificity, particularly in cases where the frequency or avidity of tetramer-positive cells is low. Consistent with this interpretation, PBMC from HLA-A2.1$^-$ subjects displayed low frequencies of tetramer-positive events (e.g., 1/21,000 for FLU) but failed to compete CD3 binding. This suggests a low level of nonspecific tetramer binding to other sites on the cell surface. We were able to exploit this finding by setting cutoffs for CD3 competition at the upper 99th percentile of competition observed in the HLA-A2.1$^-$ cohort (3.2%). By this definition, 93% and 36% of HLA-A2.1$^+$ subjects had detectable frequencies of cells specific for the recall FLU peptide and the novel HIV peptide, respectively. Further, competition of CD3 binding by tetramer appears to be of broader applicability. Our own investigations of tumor-specific immunity in squamous cell carcinoma of the head and neck validated CD3 competition using T-cell lines specific for a tumor-associated peptide (high competition) and an irrelevant peptide (no competition). The assay was then used to evaluate the peptide specificity of T cells freshly isolated from the peripheral blood of patients (unpublished data). Our data indicate that competition of CD3 binding represents a marker for the specificity of tetramer binding to the TCR. Such a marker is needed, as it has been shown that nonspecific tetramer binding may occur by TCR-independent interactions between CD8 and the MHC class I portion of the tetrameric complex (13). In this regard, CD3 competition may prove particularly useful for defining the response to tumor or self- antigens where the frequency of peptide-specific T cells is low and tetramer-positive populations are not always discrete.

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Journal of Immunotherapy
24(2):162–171 © 2001 Lippincott Williams & Wilkins, Inc., Philadelphia

Proinflammatory Cytokines and CD40 Ligand Enhance Cross-Presentation and Cross-Priming Capability of Human Dendritic Cells Internalizing Apoptotic Cancer Cells

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Summary: Human monocyte-derived dendritic cells (DC) can ingest apoptotic tumor cells (ATC) and present tumor-associated antigens (TAA) to T cells, leading to the generation of tumor-specific cytotoxic effector cells (*Cancer Res* 2000;60:3542–9). To further augment antitumor effector cell responses, attempts were made to modify antigen presentation and cross-priming of T cells by DC fed with ATC. Proinflammatory cytokines (PC), CD40 ligand (CD40L) and/or interferon-γ (IFN-γ) were found to markedly enhance the immunogenicity of TAA presented by DC. While PC upregulated expression of major histocompatibility complex class I/II and costimulatory molecules on the surface of DC, CD40L ± IFN-γ increased interleukin (IL)-12 and to a lesser extent, IL-15 production by DC. Additionally, lactacystin, a specific proteasome inhibitor, significantly abrogated the effects of IFN-γ and, in part, also those of CD40L or PC. The ability of DC + ATC to cross-prime TAA-inexperienced ("naive") T cells was significantly enhanced by PC and CD40L or CD40L + IFN-γ, but not by IFN-γ alone. These results indicate that future vaccines for patients with cancer incorporating DC fed with ATC could be made more effective by the addition of proinflammatory cytokines or CD40L ± IFN-γ to improve the DC function. **Key Words:** Cancer—Dendritic cells—Cytotoxic T cells—Cross-presentation/priming—CD40L.

Immunotherapy strategies using dendritic cells (DC), professional antigen-presenting cells capable of initiating potent primary and sustained secondary antitumor immune responses, have focused on the use of tumor-derived peptides for the induction of antitumor cytotoxic T lymphocytes (CTLs) (1,2). However, this approach requires previous identification and characterization of immunogenic tumor-derived epitopes. An alternative strategy, in which whole tumor cells or various tumor preparations instead of single peptides are taken up and presented by DC to T cells, does not require epitope identification and could potentially result in polyvalent immunization of the host to multiple (unknown) tumor associated antigens (TAA), many of which may be idiotypic to the patient's own tumor (3,4). Ex vivo experiments recently performed in our laboratory have demonstrated that antitumor CTL responses can be generated using human DC that acquired antigens from apoptotic tumor cells (ATC) (5). In this model system, ATC but not tumor cell lysates yield antigenic peptides, which were cross-presented to T cells by class I major histocompatibility complex (MHC) molecules expressed by autologous DC, resulting in the induction of antitumor CTL responses in vitro (5).

In general, TAA are weakly immunogenic, and the successful generation of antitumor responses requires strategies designed to amplify one or more mechanisms

Received August 8, 2000; accepted November 15, 2000.
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involved in antigen presentation. In the current study, we augmented antitumor CTL responses induced by DC fed with ATC by using various cytokines or agonists, known to modify the cross presentation and cross-priming ability of DCs. Human monocyte-derived immature DC fed with ATC were either directly used for antigen presentation or were additionally treated with: 1) proinflammatory cytokines (PC), 2) CD40 ligand (CD40L), and/or 3) interferon-γ (IFN-γ). These DC stimuli were used based on their well-described capability to enhance the immunogenicity of DC-presented antigens. Thus, the cocktail of PC, including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and prostaglandin E_2, has been shown to induce maturation in monocyte-derived DC, as evidenced by their phenotype and functional characteristics (6). Matured DC are known to be more effective than immature DC in eliciting T-cell stimulation (7,8). The interaction between soluble trimeric CD40L and the CD40 receptor is known to play an important role in DC-induced T-cell activation and CTL priming (9–13). In DC, high levels of CD40 crosslinking by its ligand or the combination of CD40L with IFN-γ have been shown to induce production and release of the bioactive IL-12 p70 heterodimer, an important molecule in the effector phase of an immune response (14–17). In addition, soluble CD40L has been reported to induce IL-15 production by DC, which augmented stimulation of antigen-specific cytolytic T cells (18). Treatment of DC with IFN-γ has been observed to alter the processing kinetics, quantity, and quality of MHC class I–bound peptide ligands by affecting the expression of the IFN-γ-inducible proteasomal apparatus in DC (19,20). To study the effects of these various agents on the ability of human DC fed with ATC to present TAA, we reevaluated a previously described *cross-presentation* model, in which a tumor- specific $CD8^+$ CTL line was monitored for class I MHC-restricted response to TAA (5). In addition, DC fed with ATC were also tested in a *cross-priming* model, where antitumor CTL responses were generated, using TAA inexperienced ("naive") T cells as responder cells.

MATERIALS AND METHODS

Cells and Cell Culture

The HLA-$A2^+$ PCI-13 cell line was established from freshly harvested squamous cell carcinoma of the retromolar trigone and characterized by Heo et al. (21). Tumor cells were cultured in plastic culture flasks (Costar, Cambridge, CA, U.S.A.) under standard conditions (37°C, 5% CO_2 in a fully humidified atmosphere), using serum-free AIM-V medium (Life Technologies, Grand Island, NY, U.S.A.). For subculturing, cells were detached from plastic, using a 0.05% trypsin and 0.02% EDTA solution (Life Technologies). The cultures were routinely tested and found to be free of mycoplasma contamination (GEN-PROBE, San Diego, CA, U.S.A.).

Human DC were generated according to a modified method by Sallusto and Lanzavecchia (22). Briefly, peripheral blood or a leukapheresis product was obtained from HLA-$A2^+$ normal donors, and peripheral blood mononuclear cells (PBMCs) were isolated by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). The PBMCs were incubated for 1 h at 37°C in AIM-V medium, and nonadherent cells were removed by gentle washing with warm medium. The remaining (adherent) cells were incubated in AIM-V medium + 1,000 U/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, Immunex, Seattle, WA, U.S.A.) and IL-4 (Schering Plough, Kennilworth, NJ, U.S.A.). On day 4, the cultures were supplemented with additional cytokines. DC were harvested on day 6, using cold Hanks' solution (Life Technologies).

The PCI-13-specific CTL bulk cell line was established from peripheral blood lymphocytes of a patient with squamous cell carcinoma of the head and neck (SCCHN), as described previously (23–25), and it was maintained in the presence of IL-2 and IL-4 for 10 to 12 weeks, with repeated sensitization on the tumor cell monolayers. The $CD8^+$ T cell line was derived from the original bulk T-cell line by negative selection, using anti-CD4 antibody-coated magnetic beads. The $CD8^+$ cells recognized a shared antigen on SCCHN: they lysed autologous SCCHN targets as well as HLA-$A2^+$ allogeneic (but not HLA-$A2^-$) SCCHN targets, including PCI-13 (23). This lysis can be blocked with anti-CD3, anti-CD8, anti-TCR α/β, and anti-class I monomorphic MHC antibodies (W6/32, American Type Culture Collection [ATCC], Manassas, VA, U.S.A.), as well as anti-HLA-A2 antibodies (BB7.2, ATCC), but not by immunoglobulin G (IgG) isotype controls ($IgG2_a$ and $IgG2_b$, respectively, both Pharmingen, San Diego, CA, U.S.A.). For the experiments described here, the $CD8^+$ T cell line was cultured in AIM-V containing 10 % fetal calf serum and 300 IU IL-2/IL-4. It was stimulated twice with gamma-irradiated PCI-13 cells (10,000 rad) and incubated for 7 days before testing in ^{51}Cr-release or enzyme linked immunospot (ELISPOT) assays.

Cytokines and Antibodies

The following cytokines were used for cell cultures: IL-1β (National Cancer Institute, Biological Resources

Branch, Frederick, MD, U.S.A.), IL-2 (Chiron-Cetus, Emeryville, CA, U.S.A.), IL-4 (Schering Plough), IL-6 (Sandoz, Basle, Switzerland), PGE_2 (Sigma, St. Louis, MO), IFN-γ (Genentech, San Francisco, CA, U.S.A.), GM-CSF (Immunex, Seattle, WA, U.S.A.), and TNF-α (Knoll Pharmaceuticals, Whippany, NJ). Soluble recombinant human CD40L trimer was provided by Immunex.

The antibodies used for staining of cells or blocking of responses were either labeled with phycoerythrin or fluorescein isothiocyanate or unlabeled, respectively, and included anti-MHC class I monoclonal antibodies (mAbs) (obtained from Dr. A. DeLeo, UPCI and prepared from HB95 [W6/32] hybridoma, which was purchased from the ATCC), anti-MHC class II, anti-CD14, anti-CD25, and anti-CD80 (all from Becton Dickinson, San Jose, CA, U.S.A.); anti-CD40 and anti-CD86 (Ancell, Bayport, MN, U.S.A.); anti-CD83 (Immunotech, Marseille, France); and respective IgG isotype controls (all from Becton Dickinson).

Staining and Flow Cytometry

DC, lymphocytes, or tumor cells (2×10^5/200 μL) were incubated with mAbs on ice for 30 min and washed twice in phosphate-buffered saline (PBS) containing 0.1% (wt/vol) bovine serum albumin and 0.1% (wt/vol) NaN_3. After staining, the cells were fixed with 1% (wt/vol) paraformaldehyde in PBS for 30 min at room temperature before flow cytometry. Flow cytometry analysis was performed using a FACScan (Becton Dickinson) equipped with a single 488-nm argon ion laser. At least 10,000 events were acquired for each sample. Controls consisting of PBS and isotypes were included in all experiments.

Cross-Presentation of Tumor-Derived Epitopes by DC After Uptake of ATC + Cytokines

Apoptosis in PCI-13 cells was induced by ultraviolet B (UVB) light, confirmed by TUNEL and DiOC6 staining (flow cytometry), and the uptake of ATC by DC was evaluated by flow cytometry and confocal microscopy as recently described (5). Because cytokine-treated DC tended to show a decreased uptake of DC, we generally co-incubated ATC with DC overnight and subsequently incubated the DC with cytokines: 1) proinflammatory cytokines (IL-1β, IL-6, TNFα, PGE_2a); 2) CD40L; and/or 3) IFN-γ. Also, supernatants of necrotic PCI-13 cells were produced by freeze and thaw cycles, according to a previously published protocol (26), and were used instead of cytokines to modify the antigen-presenting function in DC. DC that had phagocytosed ATC and were then treated with various preparations of cytokines were harvested, washed, and counted. To determine the ability of these DC to process and cross-present tumor-derived epitopes to the PCI-13-specific $CD8^+$ CTL line, they were used as stimulators in 24-h ELISPOT assays at an equal effector:target cell ratio.

IFN-γ ELISPOT Assay

The ELISPOT assay, confirmed in our laboratory to be a reliable test for the detection of T-cell function, was used as previously reported by us (5,27). Briefly, wells of 96-well plates with nitrocellulose membrane inserts were precoated with a primary antibody (clone 1D1K, Mabtech, Nacka, Sweden) solution and incubated for 24 h at 4°C. Effector and target cells were added after a washing and a blocking step in quadruplicate wells for each experimental condition. After a 24-h incubation period, cells were removed, and a biotinylated secondary anti-IFN-γ antibody (7-B6-1, Mabtech) was added for 2 h. After another washing step, the plates were incubated with the avidin–peroxidase complex reagent and stained with aminoethylcarbazole solution. The reaction was terminated after 4 to 6 min, and spots were counted by computer-assisted image analysis (Zeiss ELISPOT 4.14.3., Jena, Germany). If the mean number of spots against DC + tumor preparation (experimental values) was significantly different from the mean number of spots against nonpulsed DC (background values) as determined by two-tailed Wilcoxon rank-sum test, the background values were subtracted from the experimental values. Otherwise, the difference was regarded as zero.

For antibody blocking experiments, PCI-13 cells were preincubated with W6/32 antibody or purified mouse IgG1 as control (clone S_1-68.1, Pharmingen) for 30 min. To control for the assay reproducibility, PBMC obtained from the same normal donor and cryopreserved in a series of vials were used at the concentration of 2×10^4/mL in AIM-V each time the assay was performed. The control PBMC were stimulated with phorbol 12-myristate 13-acetate (1 ng/mL) and ionomycin (1 μM; both from Sigma).

Blocking of the Antigen-Processing Pathway

DC were incubated with the specific proteasome inhibitor lactacystin (Alexis, San Diego, CA) at 0.1 to 10 μM lactacystin for 24 h to determine nontoxic concentrations of the inhibitor. The concentration equal to or smaller than 2.5 μM of lactacystin was found to be nontoxic to DC, as determined by a trypan blue exclusion

test. For the subsequent experiments, DC were incubated with the proteasome inhibitor 2 h before cytokine addition.

The percent inhibition was calculated as:

$$\frac{[(DC + ATC \pm cyto.) - (DC_{alone} \pm cyto.)] - [(DC + ATC \pm cyto. + lacta.) - (DC_{alone} \pm cyto. + lacta.)]}{[(DC + ATC \pm cyto.) - (DC_{alone} \pm cyto.)]} \times 100$$

Enzyme-Linked Immunosorbent Assay for IL-12 or IL-15

Supernatants of DC (5 10^5) treated with various cytokines were collected, and either diluted or concentrated with Microcon 10-spin filters (Amicon, Beverly, MA, U.S.A.) before enzyme-linked immunosorbent assay (ELISA). To establish a time course of cytokine production after washout of cytokines, the medium was replaced after 24-h cytokine treatment and was replaced by AIM-V alone. Determinations of IL-12 or IL-15 proteins were performed after 6, 24, and 48 h after washout in duplicate, using a quantitative sandwich enzyme immunoassay technique (R&D Systems, Minneapolis, MN, U.S.A.). The lower limit of detection for IL-12 was 5 pg/mL and for IL-15 it was 1 pg/mL.

Blocking of IL-12 and IL-15

Blocking of IL-12 and IL-15 was performed by addition of neutralizing monoclonal antibody, C8.6 (10 μg/mL, Pharmingen) and M112 (10 μg/mL, Genzyme, Cambridge, MA, U.S.A.), respectively, according to the procedures previously described (17). Blocking was performed during ELISPOT as well as during the culture period of DC with cytokines. As a control, an IgG-antibody (clone A112.2, Pharmingen) was used at the same concentration.

Cross-Priming of T Cells by DC After Uptake of ATC + Cytokines

PBMC were obtained as leukapheresis products from normal HLA-$A2^+$ donors, and monocytes were separated by adherence to plastic. The adherent cells were used for DC generation, while the recovered lymphocytes were cryopreserved. At the appropriate time, the lymphocytes were thawed, washed, and cocultured with autologous DC at a ratio of 10:1. The cultures were performed in AIM-V medium + 10% human AB serum supplemented with 25 ng/mL of IL-7 for the first 72 h and then in AIM-V supplemented additionally with 20 IU/mL of IL-2 for the remaining time in culture. The lymphocytes were restimulated with DC ± ATC ± cytokines after the first week and weekly thereafter for up to three to four total stimulations. The cultured lymphocytes were phenotyped by flow cytometry, and their specificity was tested in 24-h ELISPOT assays. Responses of cultured T cells to PCI-13 or to the following controls—HR (HLA-$A2^+$ gastric carcinoma), Fem-X (HLA-$A2^+$ melanoma), and HLA-$A2^+$ normal human fibroblasts—were tested. Blocking with neutralizing class I MHC antibodies (W6/32) and anti-HLA-A2.1 antibodies (BB7.2) or isotype control IgG was performed to confirm T-cell specificity.

Enrichment of CD8+ T Cells

Cultured PBMC or the bulk CTL lines derived from cocultures with DC ± ATC ± cytokines were enriched for $CD8^+$ cells by positive immunoselection, using magnetic beads (MiniMacs, Miltenyi Biotec, Auburn, CA, U.S.A.) according to the manufacturer's recommendations. The purity of selected $CD8^+$ cell fractions was determined by flow cytometry and always exceeded 95%.

Statistical Analysis

A two-tailed Wilcoxon rank sum test was performed to analyze ELISPOT data. Unpaired two-tailed Student *t* test was used for statistical analysis of flow cytometry data. Differences were considered significant when p was less than 0.05.

RESULTS

Antigen-Presentation by DC After Uptake of ATC to PCI-13-Specific $CD8^+$ T Cells

When ATC were fed to DC in the presence or absence of cytokines, we observed that the cytokine treatment resulted in decreased antigen uptake capacity by DC, as shown in Figure 1. This decrease was already detectable after 2 h of treatment with PC, and it was further diminished after 12 h of incubation (a reduction of 42% compared to the initial uptake). A similar observation was made after DC were matured with CD40L. Therefore, to maximize uptake of ATC, untreated DC were cocultured with ATC overnight to guarantee uptake, and only afterward, they were further incubated with various cytokines to promote their maturation.

To determine whether the treatment of human DC ingesting ATC with various cytokines was able to augment processing and presentation of tumor-derived epitopes to T cells, we used an in vitro antigen-presentation model,

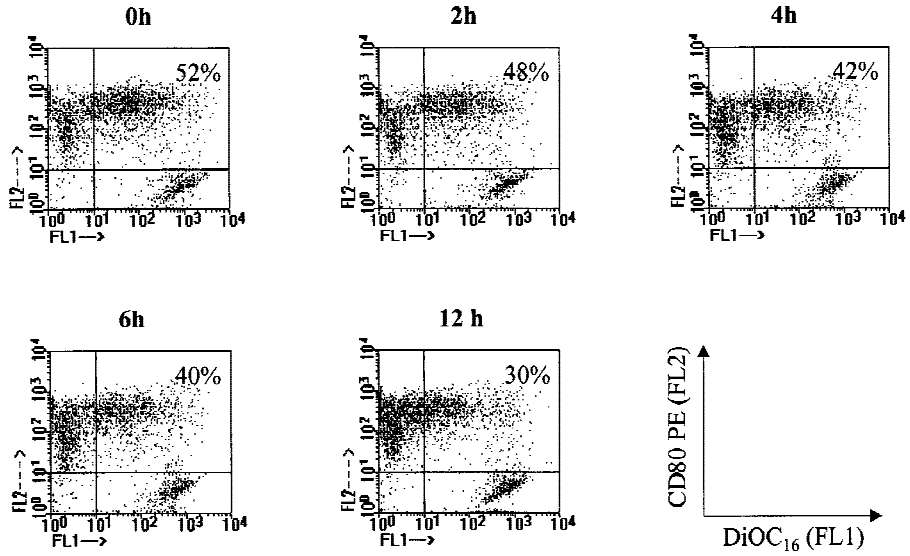

FIG. 1. Reduced tumor-uptake capacity of monocyte-derived dendritic cells (DC) after induction of maturation by proinflammatory cytokines. DC were treated with proinflammatory cytokines during the indicated intervals, washed, and then co-incubated with $DiOC_{16}$ (FL-1)-stained apoptotic PCI-13 tumor cells. After an 8-h co-incubation, DC were "counter"-stained with phycoerythrin-conjugated anti-CD80 antibody (FL-2), and two-color flow cytometry was performed. A representative experiment of three performed is shown.

in which a tumor-specific $CD8^+$ CTL line served as a source of responder cells. This HLA-A2-restricted and PCI-13-specific CTL line was generated as previously described by us (23–25). Recognition of DC + ATC by the CTL with or without cytokine pretreatment was tested in the IFN-γ ELISPOT assay.

ATC alone were found to be poor stimulators of CTL (Fig. 2), possibly because these ATC showed a significant decrease in MHC class I molecules compared to viable PCI-13. Figure 3 shows the mean fluorescence intensity (MFI) of MHC class I molecules on PCI-13 cells stained with W6/32 antibody and measured before, 1 h, 8 h, and 24 h after UVB treatment. UVB has been previously shown by us to induce apoptosis in PCI-13 cells (5). Initially, just after UVB treatment, expression of MHC class I molecules is not altered, but a significant decrease in their expression was noted after 8 h. By 24 h, the MFI was reduced by a factor of 5.

We observed that ATC+DC were significantly better in stimulating specific T cells than ATC alone (Fig. 2). Furthermore, the transient addition of cytokines to ATC + DC was followed by an increase in T-cell responsiveness for all cytokines and their combinations studied (Fig. 2). Although the assay background increased with the addition of cytokines (i.e., DC + cytokines compared

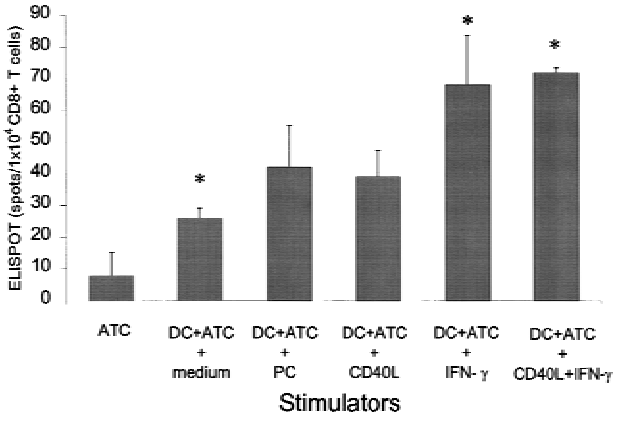

FIG. 2. Recognition of apoptotic PCI-13 cells or dendritic cells (DC) fed with apoptotic tumor cells (ATC) with or without their treatment with proinflammatory cytokines (PC), CD40L, and/or interferon-γ (IFN-γ) as measured with IFN-γ ELISPOT assays ($n \geq 5$). Error bars indicate the standard error of the mean. Asterisks indicate that the number of spots was significantly higher ($p < 0.05$) for DC + ATC versus ATC alone or for DC + ATC + cytokines versus DC +ATC alone.

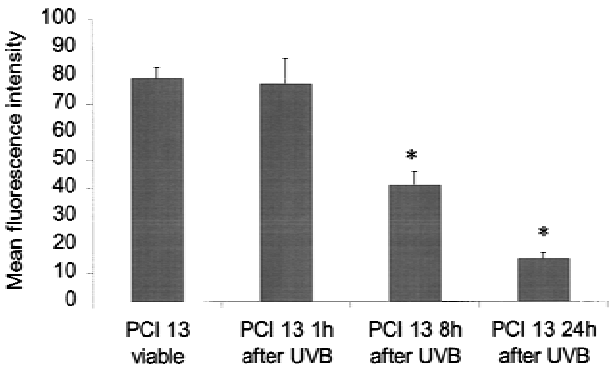

FIG. 3. Major histocompatibility complex (MHC) class I expression on PCI-13 cells before, 1 h, 8 h, and 24 h after ultraviolet B (UVB) irradiation. MHC class I molecules were detected by flow cytometry after staining with W6/32 primary antibody and fluorescein isothiocyanate–conjugated anti-mouse secondary antibody. The data are expressed as mean fluorescence intensity (MFI) determined by flow cytometry. MFI of immunoglobulin G control antibody was 1.3. The error bars indicate the standard deviation ($n = 4$). Asterisks indicate that the MFI was significantly lower ($p < 0.05$) for tumor cells 8 h and 24 h after UVB irradiation compared to untreated PCI-13 cells.

with DC alone; data not shown), the difference—(DC + ATC + cytokines) − (DC alone + cytokines)—was found to be significantly higher for CD40L ± IFN-γ as compared to (DC + ATC) − (DC alone). However, there was no increase in recognition when supernatants of necrotic PCI-13 tumor cells were used instead of cytokines (data not shown). It is important to note that the morphology and the yield of DCs was markedly changed on cytokine treatment: the treatment with IFN-γ alone or in combination with CD40L led to an extreme elongation of the dendrites in DC and to a yield that was decreased by half compared to that with untreated DC. When DC were incubated with PC and, to a lesser extent with CD40L, the cells were observed to round up, and the yield of DC was doubled, as compared to that with untreated DC. This increase in the yield was mainly caused by the decreased plastic adherence of DC.

Mechanisms Responsible for the Cytokine-Induced Increase in Antigen Recognition

To investigate the underlying mechanisms responsible for the observed augmentation in TAA recognition induced by cytokines, several possibilities were considered. First, DC were phenotypically characterized by flow cytometry using a panel of antibodies (Table 1). Clearly, DC incubated with PC and, to a lesser extent, with CD40L, showed prominent changes in phenotype, including the upregulation of MHC-class I/-II molecules, costimulatory molecules CD80 and CD86, as well as the maturation marker CD83. Each of these alterations could contribute to the observed increase in the stimulatory potential of DC + ATC + PC/CD40L. However, DC treated with IFN-γ alone displayed no significant changes, and no alterations in the DC phenotype were observed for DC treated with supernatants of necrotic cells (data not shown).

TABLE 1. *Effects of proinflammatory cytokines, CD40L, and/or interferon-γ (IFN-γ) on the phenotype of monocyte-derived human dendritic cells (DC)*[a]

	Untreated	Proinflammatory cytokines	CD40L	IFN-γ	CD40L + IFN-γ
Isotype control	5	4	6	4	5
MHC I	334	**743**	412	470	420
MHC II	1,305	**3,852**	**2,972**	1,820	**3,413**
CD14	20	8	5	12	5
CD25	4	**321**	11	4	15
CD40	768	**3,125**	**3,212**	1,073	**3,637**
CD80	61	**487**	**233**	68	**252**
CD83	8	**210**	**52**	10	**57**
CD86	113	**1,098**	**446**	132	**580**

[a] Representative data obtained in one of three experiments performed. Monocyte-derived, day 6 DC were incubated in the absence or presence of 3 μg/mL CD40L and/or 1,000 IU IFN-γ or proinflammatory cytokines (10 ng/mL interleukin [IL]-1β, 1,000 U/mL IL-6, 10 ng/mL tumor necrosis factor α, 1 μg/mL prostaglandin E_2) for 24 h before flow cytometry. The cells were stained with monoclonal antibodies or isotype control antibodies and examined by flow cytometry as described in Materials and Methods. The data are presented as mean fluorescence intensity and ≥1.5-fold increases are shown in boldface.

To test the possibility that increased production of IL-12 and/or IL-15 produced by activated DC were responsible for enhanced recognition of DC + ATC, we measured the levels of both cytokines in supernatants of DC that had been treated for 24 h with various cytokines. No IL-12 was detectable when DC were matured with PC or treated with IFN-γ. However, DC treatment with CD40L alone led to detectable IL-12 levels in the supernatant, which were dramatically increased by the combination of CD40L and IFN-γ (Table 2). Only immature DC were found to produce IL-12 in the presence of CD40L, and no detectable IL-12 levels were observed when DC were matured with PC before the addition of CD40L or CD40L and IFN-γ (data not shown). Because of low levels of IL-15 in the supernatants of DC, we performed a concentration step before ELISA analysis and found a small amount of IL-15 in all samples, with a subsequent increase in supernatants of DC cultured in medium alone, PC, IFN-γ, CD40L, and CD40L + IFN-γ. Interestingly, a two- to threefold increase in IL-15 levels was observed in 24-h and 4-h supernatants of DC, which were treated with PC, CD40L, and CD40L + IFN-γ (Table 2).

To confirm a possible role of IL-12 and IL-15 in the enhancement of antigen presentation by DC + ATC, we attempted to neutralize both cytokines, using specific antibodies during the coculture of DC + ATC with cytokines as well as in the ELISPOT assay. The enhancement in T-cell recognition of DC + ATC induced by CD40 ± IFN-γ was not blockable by either of these anti-cytokine antibodies. However, we observed a nonspecific decrease in the number of spots in ELISPOT for DC + ATC treated with CD40 ± IFN-γ, when using IL-12 neutralizing antibody, but not IL-15 blocking antibody or IgG controls (data not shown). Therefore, neither IL-12 nor IL-15 seems to contribute significantly to the CD40 ± IFN-γ mediated enhancement in the recognition of DC + ATC by our long-term cultured T cells.

We next studied the effect of lactacystin on the immunogenicity of DC, which had ingested ATC. In preliminary experiments, a concentration of 1–2.5 μg/mL was found to be nontoxic to DC, with more than 95% of DC remaining viable for 24 h of treatment. Therefore, this biologically active concentration of lactacystin was chosen for subsequent experiments. In the presence of

TABLE 2. *Interleukin (IL)-12 and IL-15 production by human* immature *monocyte-derived dendritic cells (DC) in the presence of cytokines before and 6 h, 24 h and 48 h after the removal of PC or CD40L ± interferon (IFN)-γ*[a]

	Medium	Proinflamm. cytokines	CD40L	IFN-γ	CD40L + IFN-γ
IL-12	<5 ± n.a.	13 ± 7	44 ± 24	<5 ± n.a.	478 ± 183
(pg/ml)	(<5; <5; <5)	(<5; <5; 8 ± 7)	(22 ± 28; <5; <5)	(<5; <5; <5)	(341 ± 127; 272 ± 200; 237 ± 205)
IL-15	3 ± 1	5 ± 2	12 ± 4	7 ± 3	18 ± 7
(pg/ml)	(3 ± 1; 3 ± 1; 5 ± 2)	(2 ± 1, 12 ± 8; 19 ± 7)	(10 ± 7; 21 ± 9; 29 ± 8)	(3 ± 2; 3 ± 1; 5 ± 3)	(9 ± 3; 36 ± 12; 43 ± 12)

[a] Monocyte-derived, day 6 DC (5×10^5 cells/ml) were incubated in the presence or absence of proinflammatory cytokines (10 ng/mL IL-1β, 1,000 U/mL IL-6, 10 ng/mL tumor necrosis factor α, 1 μg/mL prostaglandin E_2), 3 μg/mL CD40L and/or 1,000 IU IFN-γ for 24 h before assays for respective cytokines in the supernatants. The data are means ± SD of at least three experiments. The *first line* shows IL-12/IL-15 levels directly after the 24-h incubation period and *in parentheses below* are the results of cytokine production 6 h, 24 h, and 48 h after the removal of proinflammatory cytokines (PC) or CD40L ± IFN-γ. The supernatants were concentrated fivefold for IL-15 determinations. Neither IL-12 nor IL-15 were detectable if DC were matured with PC before further incubation in the presence of various cytokines. n.a., not applicable.

lactacystin, antigen presentation by DC + ATC to the PCI-13 specific $CD8^+$ CTL line was significantly inhibited (33% ± 20%), as determined in ELISPOT assays (Table 3). However, cytokine-induced augmentation in antigen recognition was further inhibited in the presence of PC (67% ± 27%) and nearly abrogated, when IFN-γ was used alone (80% ± 18%; Table 3). These data may indicate that in the presence of cytokines, TAA processing of ATC-derived antigens by DC was upregulated.

Cross-Priming With DC to Generate Tumor-Specific CTL

To demonstrate that cross-priming of naïve T cells with DC + ATC results in the generation of highly reactive antitumor CTL, we obtained PBMC from HLA-$A2^+$ normal donors and used them as a source of both DC and T cells. We have previously shown that priming of T cells was successful in four of four attempts using DC + ATC but never with ATC alone, DC + nonapoptotic PCI-13, DC pulsed with tumor lysates, or DC alone (5). We next attempted to enhance the generation of tumor-reactive T cells from their PBMC precursors by the addition of various cytokines to DC + ATC, which were used as stimulators. After three cycles of restimulations of PBMC with DC + ATC ± cytokines, the total yield of T cells was different for various priming conditions. Considering T-cell response to DC + ATC as the norm (i.e., 100% response), we obtained twice as many lymphocytes with PC, CD40L, and CD40L + IFN-γ-stimulated DC, but only three-fourths using IFN-γ alone treated DC from two normal donors tested.

The T-cell lines generated by cross-priming under different conditions were mixtures of $CD4^+$ and $CD8^+$ T cells. After enrichment in $CD3^+/CD8^+$ T cells by positive selection with antibody-coated magnetic beads, the purity of cultures exceeded 95%, as detected by flow cytometry. These T cells were then used as responders in ELISPOT assays, whereas PCI-13 tumor cells served as stimulators. The representative data for donor 1 are shown in Figure 4. The ELISPOT results indicated that T-cell cultures derived by priming with either DC + ATC + PC or CD40L or CD40L + IFNγ obtained significantly ($p < 0.05$) higher frequencies of $CD8^+$ T cells reactive with the tumor (PCI-13) than T-cell cultures derived by priming with DC + ATC. Similar results were generated from a second HLA-$A2^+$ donor.

The $CD8^+$ T-cell response was blocked by anti-MHC class I and HLA-A2 mAbs but not isotype control Ig. These T cells did not respond to irrelevant HLA-$A2^+$ HR (gastric carcinoma) or HLA-$A2^+$ Fem-X (melanoma) tumor cells, or HLA-$A2^+$ normal fibroblasts used as control target cells (data not shown). However, using DC + ATC + IFN-γ as stimulator cells, the total number of "antigen-specific" T cells was reduced compared to that generated in the presence of DC + ATC, and their response was not inhibited by inclusion of neutralizing antibodies reactive against class I MHC or HLA-A2 molecules. In additional experiments, T-cell priming was performed with DC + ATC stimulator cells after transient addition of lipopolysaccharide or the supernatant of necrotic cells. The lipopolysaccharide-treated DC were able to generate a robust immune response, but this re-

TABLE 3. *Inhibition (percent) of antigen processing in human monocyte-derived dendritic cells by specific proteasome inhibitor, lactacystin*[a]

	Medium	Proinfl. cytokines	CD40L	IFN-γ	CD40L + IFN-γ
% Inhibition	33 ± 20	67 ± 27	53 ± 24	80 ± 18	55 ± 24

[a] The data are means ± SD of at least three experiments. Monocyte-derived, day 6 DC (5×10^5 cells/mL) were briefly preincubated with 2.5 μM lactacystin and subsequently with 3 μg/mL CD40L and/or 1,000 IU interferon (IFN)-γ or proinflammatory cytokines (10 ng/mL IL-1β, 1,000 U/mL interleukin-6, 10 ng/mL tumor necrosis factor α, 1 μg/mL prostaglandin E_2) for 24 h before ELISPOT assays. The percent inhibition was calculated as described in Materials and Methods.

sponse appeared non-MHC restricted and was not blocked by MHC-class I reactive antibodies. Treatment of DC with supernatants of necrotic tumor cells did not affect T-cell responses detected after repeated rounds of in vitro sensitization (Fig. 4).

DISCUSSION

Current therapies for patients with solid neoplasms often consist of three standard treatment options, surgery and/or radiotherapy and/or chemotherapy. Because survival rates have not significantly changed during the past years/decades for various solid tumors, including SCCHN, new therapies are needed, and vaccine development is currently considered a promising therapeutic strategy (2,28,29). However, in contrast to viral antigens, TAA are often weakly immunogenic. The recent therapeutic use of DC, professional antigen-presenting cells, as "biologic adjuvants" to enhance the immunogenicity of TAA has focused attention on the selection of best immunogens and on the ex vivo generation of human DC with optimal therapeutic potential. In most human malignancies, tumor-derived immunogenic peptides have

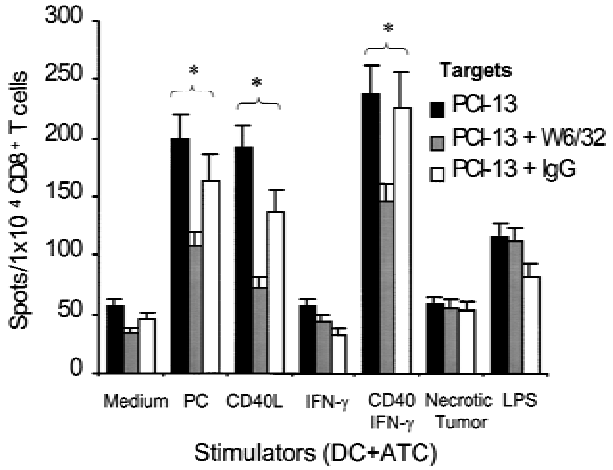

FIG. 4. Recognition of PCI-13 cells by $CD8^+$ T cells generated under different priming conditions (dendritic cells [DC] + apoptotic PCI-13 cells [ATC] ± cytokines). A representative ELISPOT experiment of two performed with autologous DC and T cells obtained from an HLA-$A2^+$ healthy donor after three in vitro sensitization cycles is shown. The cytotoxic T lymphocytes (CTL) were enriched in $CD8^+$ T cells by positive selection with magnetic beads before ELISPOT assays. The CTL (as responders) were tested against PCI-13 as stimulators at the ratio of 1:1. To confirm HLA class I restriction, PCI-13 cells were preincubated with W6/32 or immunoglobulin G (IgG) antibody. Spots were counted by computer-assisted image analysis. Asterisks indicate that the number of spots obtained when CTL were primed with DC + ATC + cytokines was significantly higher ($p < 0.05$) than that obtained with DC + ATC alone, and the use of PCI-13 +IgG as stimulators led to a significantly higher number of spots ($p < 0.05$) compared to controls (PCI-13 + W6/32). PC, proinflammatory cytokines; IFN-γ, interferon-γ; LPS, lipopolysaccharide.

yet to be defined. Therefore, DC pulsed with tumor cells or ATC as vaccine components represent a reasonable alternative. Current evidence suggests that significant differences exist in the efficiency of TAA processing and presentation by human DC, depending on the source or form of tumor-derived materials, the maturation stage of DC, or responsiveness of T-cell populations available for stimulation with DC (5,7,30–36). These differences are likely to be important for the outcome of future immunizations in patients with cancer. Thus, there exists a need to optimize the design of DC-based vaccines using different forms of tumor-derived antigens. Clearly, a selection of the optimal method for antigen delivery to DC, allowing for effective antigen processing and presentation to T cells, will prove critical to the prospective clinical benefit of these approaches.

In the present study, we have evaluated human monocyte-derived DC that have ingested ATC after treatment with various cytokines for their ability to *present* tumor-derived epitopes to previously sensitized, "memory" tumor-specific T cells, as well as to *prime* T cells from normal donors to develop into antitumor effector cells. In our as well as in other model systems, apoptotic cells served as a source of antigenic epitopes (5,30–35). Recently, the role of apoptosis in providing immunogenic peptides was questioned (26,37,38). In fact, Steinman suggested that instead of facilitating TAA presentation, DC ingesting apoptotic cells could be tolerogenic (38). It has also been stated that a second step was necessary to activate DC function after antigen uptake to obtain optimal cross-presentation (26). Furthermore, necrotic cells or their supernatants were shown to be able to provide a suitable level of DC activation after uptake of apoptotic target cells leading to the induction of DC maturation and to optimal cross-presentation of antigens (26). In contrast to these reports, we were unable to show any beneficial effect in terms of DC maturation, enhanced DC presentation, or DC priming capability, when using supernatants of necrotic cells prepared as described by Sauter et al. (26). Instead, our observations are in agreement with a recent study reported by Lanzavecchia's group that failed to demonstrate the maturation of DC by stimuli provided by necrotic cells, unless the cells were mycoplasma contaminated (39).

Our ex vivo results do not contradict the "danger or activation concept" proposed by Matzinger and colleagues (37,40), because secondary necrosis can be the result of the apoptotic process. However, the use of necrotic cells to mature or activate DC was found to be unsatisfactory. Instead, the addition of PC or CD40L ± IFN-γ was most efficient in inducing activation of DC. From a practical viewpoint, this is a relatively simple

approach to implement in DC-based antitumor therapy. We were able to show that treatment of DC + ATC with these cytokines induced a mature DC phenotype, enhanced presentation of TAA and, more importantly, increased the cross-priming capability of DC. In contrast, the addition of IFN-γ alone only led to minor changes in the expression of MHC class I and II molecules on DC and did not seem to be responsible for the observed enhanced antigen presentation. More likely, IFN-γ induces proteasomal subunits, leading to an alteration of the processing kinetics, quantity, and quality of MHC class I–bound peptide ligands (19). This is supported by results of our own experiments, in which the specific proteasome inhibitor, lactacystin, was shown to elicit the most prominent inhibition of antigen presentation in the DC + ATC, treated with IFN-γ alone. As expected, lactacystin-mediated inhibition was also observed in DC + ATC in the absence of cytokines, demonstrating the expected, "rate-limiting" importance of the processing apparatus in DC for successful antigen presentation of $CD8^+$ T-cell-defined epitopes.

To explore possible mechanisms responsible for the augmented TAA presentation by CD40L+IFN-γ, we investigated the induction of IL-12 and IL-15 production by DC in the presence of this combination of cytokines. Previously, the combination of CD40L with IFN-γ was shown to induce high levels of bioactive IL-12 p70 heterodimer in DC (14–17), whereas only low, but significant levels of IL-15 were found (18). The levels of both interleukins were remarkably stable up to 48 h after the removal of CD40L ± IFN-γ and, for IL-15, a time-dependent increase in production was actually observed. To provide evidence that these cytokines, believed to be important for the induction of antigen-specific CTL response, were responsible for the enhanced recognition of DC + ATC treated with CD40L ± IFNγ, we attempted to block the augmentation in T-cell response with specific anti-cytokine antibodies. Blocking was performed not only in the readout system ELISPOT, but also during the 24-h period of coculture of DC with the cytokines to prevent any cytokine-mediated effects occurring during the co-incubation. However, the cytokine-induced enhancement in recognition of DC + ATC by T cells was not significantly affected by this treatment, indicating that alternative dominant mechanisms are likely involved. For example, the direct effects of soluble trimeric CD40L, interacting with CD40 expressed on DC, and known to play an important role in DC-induced T-cell activation and CTL priming (9–12), could be responsible for the observed enhancement in TAA presentation.

Overall, this study demonstrates that DC that have internalized and processed ATC generate more effective antitumor-specific T cells in vitro, when these antigen-fed DC are subsequently activated by cytokines. These results indicate that a similar approach may prove successful in vivo, when ATC + autologous DC treated with activating cytokines are adoptively transferred to patients with cancer as a vaccine.

Acknowledgment: This work was supported in part by the National Institutes of Health grant PO1-DE 12321 to T.L.W. and a postdoctoral training fellowship from the Dr. Mildred Scheel Stiftung für Krebsforschung to T.K.H. (grant D/99/08916) and to J.M.-B. (D/98/14794).

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The Ability of Variant Peptides to Reverse the Nonresponsiveness of T Lymphocytes to the Wild-Type Sequence $p53_{264-272}$ Epitope[1]

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Recently, we observed that CTL specific for the wild-type (wt) sequence $p53_{264-272}$ peptide could only be expanded ex vivo from PBMC of a subset of the HLA-A2.1$^+$ normal donors or cancer patients tested. Surprisingly, the tumors of the responsive patients expressed normal levels of wt p53 and could be considered unlikely to present this epitope. In contrast, tumors of nonresponsive patients accumulated mutant p53 and were more likely to present this epitope. We sought to increase the responsive rate to the wt $p53_{264-272}$ peptide of PBMC obtained from normal donors and patients by identifying more immunogenic variants of this peptide. Two such variants were generated by amino acid exchanges at positions 6 (6T) and 7 (7W) of the peptide. These variants were capable of inducing T cells from PBMC of nonresponsive donors that recognized the parental peptide either pulsed onto target cells or naturally presented by tumors. TCR Vβ analysis of two T cell lines isolated from bulk populations of effectors reactive against the wt $p53_{264-272}$ peptide, using either the parental or the 7W variant peptide, indicated that these T cells were expressing identical TCR Vβ13.6/complementarity-determining region 3/J region sequences. This finding confirms the heteroclitic nature of at least one of the variant peptides identified in this study. The use of variant peptides of the wt $p53_{264-272}$ epitope represents a promising approach to overcoming the nonresponsiveness of certain cancer patients to this self epitope, thereby enhancing its potential use in tumor vaccines for appropriately selected cancer patients. ***The Journal of Immunology*****, 2002, 168: 1338–1347.**

Genetic alterations in p53 occur in a wide range of human tumors, including oral squamous cell carcinomas (OSCC)[3] (1). The most common type of genetic alteration in p53 involves a missense mutation that is usually accompanied by accumulation of the altered molecules in the cytosol of tumor cells. Initially, the effort to develop p53-based vaccines focused on these missense mutations, which are tumor specific in nature. However, missense mutations have limited clinical usefulness, because of the requirement that they occur within or create epitopes that could be presented by MHC molecules expressed by the individual patient. On the other hand, the majority of p53 epitopes derived from these altered p53 molecules would be wild type in sequence, representing a new class of tumor-associated self Ags that are candidates for use in the development of broadly applicable cancer vaccines (1–5).

To date, five MHC class I-restricted, naturally presented human wild-type (wt) sequence p53 epitopes have been identified. They have been shown to be able to induce epitope-specific CTL from PBMC obtained from healthy individuals (1, 6–11). The $p53_{125-134}$ epitope is HLA-A24 restricted (11), while the other four, $p53_{65-73}$, $p53_{149-157}$, $p53_{217-225}$, and $p53_{264-272}$, are HLA-A2.1 restricted. Among these, the wt $p53_{264-272}$ peptide has been the most intensively investigated (1, 2, 6–8, 12).

The potential of wt p53 epitopes as targets for immunotherapy, however, remains uncertain due to the several critical concerns related to immunological recognition of this truly self tumor Ag. Using HLA-2.1-transgenic wt ($p53^{+/+}$) and $p53^{null}$ ($p53^{-/-}$) mice, Sherman and colleagues (13–15) have demonstrated that the CTL repertoire available for wt p53 self epitopes in $p53^{+/+}$ mice is limited to intermediate affinity T cells, because the higher affinity CTL are either deleted or tolerized. Apparently, this situation occurs in humans as well, as only CTL with intermediate affinity for the wt $p53_{264-272}$ epitope have been generated to date from PBMC obtained from normal donors as well as cancer patients (7, 12). This observation raises the question of whether such CTL are potent enough to be effective in tumor eradication.

Another concern relates to our experience that PBMC obtained from only some HLA-A2.1$^+$ healthy donors and patients with OSCC were responsive to in vitro stimulation (IVS) with the wt $p53_{264-272}$ peptide pulsed onto autologous dendritic cells (DC) (7, 12). Furthermore, CTL reactive against this epitope could only be generated from T cell precursors in PBMC of patients whose tumors were not likely to present this epitope. The analysis of these

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Received for publication August 7, 2001. Accepted for publication November 27, 2001.

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[1] This work was supported in part by National Institutes of Health Grant PO1-DE12321 (to T.L.W. and A.B.D.) and Department of Defense Grant BC995591 (A.B.D.). T.K.H. was supported by a postdoctoral fellowship from the Dr. Mildred Scheel Stiftung für Krebsforschung.

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[3] Abbreviations used in this paper: OSCC, oral squamous cell carcinoma; APL, altered peptide ligand; CDR, complementarity-determining region; DC, dendritic cell; IVS, in vitro stimulation; wt, wild type.

0022-1767/02/$02.00

tumors indicated no accumulation of p53 or accumulation of mutant p53 with a missense mutation at codon 273, a site known to block processing of the wt $p53_{264-272}$ epitope (16). In contrast, PBMC obtained from patients with tumors considered capable of presenting the wt $p53_{264-272}$ epitope (i.e., tumors that accumulate mutant p53) were nonresponsive to IVS with wt $p53_{264-272}$-pulsed autologous DC. These findings have led us to conclude that CTL specific for the wt $p53_{264-272}$ epitope might play a role in the outgrowth of epitope-loss tumor cells, which are able to escape from the host immune system. This conclusion was further strengthened by the results of a recently completed study in our laboratories that used tetrameric peptide/MHC class I complexes to determine frequencies and characteristics of the $p53_{264-272}$-specific CTL in unstimulated PBMC obtained from 30 OSCC patients and 31 normal donors (data not shown).[4]

Because these observations suggest that it may be possible to accurately predict ex vivo the responsiveness of cancer patients to immunotherapy targeting this epitope, we felt that a means of circumventing the nonresponsiveness of individuals needed to be investigated to proceed with the development of wt p53-based vaccines. One solution is to identify a heteroclitic peptide or, in more precise terms, an altered peptide ligand (APL) with enhanced functional activity relative to the parental wt $p53_{264-272}$ peptide. By substituting amino acids at various positions of an epitope that contact MHC class I and/or TCR, an array of APL with biological potencies higher than those of the parental epitopes has been identified for various antigenic determinants (17–26). In applying this strategy to the wt $p53_{264-272}$ epitope, we anticipated that an APL might induce CTL-mediated responses that cross-react with the parental epitope and that these CTLs also might demonstrate enhanced avidities relative to CTLs induced by the parental peptide. Most importantly, we sought to determine whether an APL would be able to induce anti-wt $p53_{264-272}$ CTL from PBMC that were nonresponsive to the parental peptide, particularly the PBMC obtained from patients whose tumors accumulate mutant p53 and are considered to have the potential to present this epitope.

Materials and Methods

Cell lines and cell culture

The following HLA-$A2^+$ OSCC cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA): SCC-4 and SCC-9. The SCC-4 cell line expresses and accumulates p53 expressing a missense mutation at codon 151 but does not present the wt $p53_{264-272}$ epitope (6). The SCC-9 cell line expresses an altered p53 molecule with a deletion of codons 274–285. It does not accumulate p53 molecules, yet presents the wt $p53_{264-272}$ epitope. In addition, the $p53^{null}$ osteosarcoma cell line, SaOS-2, was obtained from ATCC. The cloned $p53^+$ cell line, SaOS-2Cl3, was derived by transduction of SaOS-2 cells with a p53 cDNA expressing a missense mutation in codon 143 (7). The HLA-$A2^+$ OSCC cell line PCI-13 has been described previously (27). It expresses a p53 missense mutation in codon 286 (Glu to Lys) and presents the wt $p53_{264-272}$ epitope. Tumor cells were cultured in plastic culture flasks (Costar, Cambridge, CA) under standard conditions (37°C, 5% CO_2 in a fully humidified atmosphere) in complete medium consisting of DMEM (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 50 μg/ml streptomycin, and 50 IU/ml penicillin (all from Life Technologies). The T2 cell line was also obtained from ATCC and maintained in RPMI 1640 (Life Technologies) containing 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics. The cultures were routinely tested and found to be free of mycoplasma contamination (Gen-Probe, San Diego, CA).

[4] T. K. Hoffmann, A. Donnenberg, S. Finkelstein, K. Chikamatsu, V. Donnenberg, U. Friebe, E. Appella, A. B. DeLeo, and T. L. Whiteside. Frequencies of tetramer$^+$ T cells specific for the wild type sequence $p53_{264-272}$ peptide in the circulation of patients with head and neck cancer. *Submitted for publication.*

Peptides

The CTL-defined, HLA-A2.1-binding peptide, LLGRNSFEV (1), corresponding to wt $p53_{264-272}$, as well as single amino acid exchange variants of this peptide were synthesized by standard *N*-(9-fluorenyl)methoxycarbonyl methology. Peptides were purified by reversed-phase HPLC, and their amino acid sequence was confirmed by mass spectrometry analysis. All peptides were dissolved in DMSO (Fisher Scientific, Pittsburgh, PA) at 1 mg/ml and diluted with PBS just before use. The 19 variant peptides contain single amino acid exchanges with a bias toward retention of a high degree of similarity to the central region of the parental peptide. The variant peptides are designated 1E, 1F, 1V, 3L, 3F, 3W, 4K, 4L, 5K, 5L, 6G, 6T, 6Y, 7L, 7P, 7Y, 7W, 8A, and 8Y, in which numbers denote the position within the parental sequence and letters refer to exchanged amino acids.

MHC stabilization assay

T2 cells were incubated overnight at room temperature before use in this assay. Cells were washed and incubated at a cell density of 2×10^5/0.2 ml of complete medium with various peptides at final concentrations of 1×10^{-5}–1×10^{-10} M for 3 h at room temperature, followed by a 3-h incubation period at 37°C. After washing with PBS, cells were incubated at 4°C for 30 min with anti-HLA class I mAb, W6/32 (HB95; ATCC), and then with FITC-conjugated goat anti-mouse Ig (Caltag Laboratories, Burlingame, CA) as a secondary Ab. Fluorescence of viable T2 cells was measured at 488 nm in a FACScan flow cytometer (BD Biosciences, San Jose, CA), and the level of MHC class I expression was determined by evaluating the mean fluorescence intensity of stained T2 cells. Cells incubated either at room temperature or 37°C in the absence of peptide served as controls.

Generation of anti-p53 CTL with peptide-pulsed autologous DC

Peripheral blood or leukapheresis products were obtained from previously studied HLA-$A2.1^+$ individuals: seven normal donors and six OSCC patients (12). PBMC were isolated by sedimentation over Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ). The study was approved by the Institutional Review Board at the University of Pittsburgh, and written informed consent was obtained from each individual donating peripheral blood. PBMC were phenotyped for HLA-A2 expression by flow cytometry, using anti-HLA-A2 mAb, BB7.2 (HB82; ATCC), and a mouse IgG isotype as a control. The verification of the A0201 subtype was performed using PCR with sequence-specific primers, as previously described (12).

Human DC were generated from PBMC according to a modification of the method of Sallusto and Lanzavecchia (28), as described by us earlier (7). DC were harvested on day 6, phenotyped by flow cytometry, and then resuspended in AIM-V medium (2×10^6 cells/ml) containing 10 μg/ml peptide and incubated at 37°C for 4 h. The peptide-pulsed DC were then cocultured with autologous PBMC in 24-well tissue culture plates (Costar) in a final volume of 2 ml/well AIM-V medium supplemented with 10% (v/v) human AB serum (Pel-Freez Biologicals, Brown Deer, WI) and 25 ng/ml IL-7 (Genzyme, Cambridge, MA) for the first 72 h and, additionally, with 20 IU/ml IL-2 (Chiron-Cetus, Emeryville, CA) for the remaining time in culture. The lymphocytes were restimulated 1 wk later with peptide-pulsed autologous DC. Irradiated (3000 rad) autologous PBMC were used as APC after the third round of restimulations. Microcultures of CTL lines recognizing the wt $p53_{264-272}$ or 7W peptide were isolated from bulk populations of effectors by limiting dilution (1 cell/well/96-well plates), and the lines were maintained in cytokine-supplemented media plus peptide-pulsed APC, as previously described (7). Specificities of generated T cells were determined using one or more of a panel of assays detailed below. The TCR Vβ expression on T cells in bulk CTL populations and cell lines derived from them was done using the IOTest β Mark TCR Vβ Repertoire kit (Beckman Coulter, San Diego, CA).

ELISPOT assay for IFN-γ

The ELISPOT assay was performed in 96-well plates with nitrocellulose membrane inserts (Millipore, Bedford, MA), as previously described by us (29). The capture and detection anti-IFN-γ mAb were purchased from Mabtech (Nacka, Sweden). The spots were counted by computer-assisted image analysis (ELISPOT 4.14.3; Zeiss, Jena, Germany). For Ab-blocking experiments, target cells were preincubated with anti-HLA class I mAb for 30 min. Cryopreserved aliquots of PBMC obtained from a normal donor were thawed and, after stimulation with PMA (1 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich, St. Louis, MO), were used as a positive control for each assay.

The interassay reproducibility of the assay was acceptable with a coefficient of variation = 15% (n = 30).

Cytotoxicity assay

The 4-h ^{51}Cr release assay was performed at various E:T ratios, as previously described (7). Briefly, sensitized targets were labeled with ^{51}Cr for 45 min at 37°C, washed, and added to wells of 96-well plates (1×10^4 cells/well). Effector T cells were then added to give various E:T ratios. When Ab-blocking experiments were performed, target cells were incubated with anti-HLA class I mAb or the anti-HLA-DR mAb, L243 (HB55; ATCC),for 30 min before adding effector cells. The percentage of specific lysis was calculated according to the formula:

$$\% \text{ specific lysis} = \frac{\text{experimental cpm} - \text{control cpm}}{\text{maximal cpm} - \text{control cpm}} \times 100$$

Flow cytometry analysis using HLA-A2.1/peptide tetrameric complexes (tetramer)

The streptavidin-PE-labeled tetramers used in this study were obtained from the tetramer core facility of the National Institute of Allergy and Infectious Disease (Atlanta, GA). Three-color flow cytometry assays (FACScan; BD Biosciences) were performed with PerCP anti-CD3, FITC anti-CD8, and PE-tetramer. The specificity of the HLA-A2.1/$p53_{264-272}$ tetramer was confirmed by its staining of a CTL line specific for this p53 epitope and by the lack of staining of irrelevant CTL or HLA-A2$^-$ PBMC of healthy donors, as previously described (30). The additional PE-conjugated HLA-A2.1/tetramer used in this study contained the 7W variant peptide. Generally, 75,000 events per sample were collected progressively after live gating on lymphocytes by forward and side scatter.

TCR and CDR3 spectratyping

RNA was extracted from $p53_{264-272}$-specific CTL lines generated using parental or the 7W variant peptide, followed by reverse transcription into cDNA, as previously described (31). Screening for expression of TCR V chains was performed using the primers described by Puisieux et al. (31) for TCR Vβ amplification, followed by a runoff reaction with fluorophore-labeled primers specific for the C region of the TCRβ (5′-TGTGCAC CTCCTTCCCATTCACC) chain. Labeled runoff products were subjected to DNA fragment analysis, as described (32). Finally, amplified products were directly subjected to DNA sequence analysis using ABI 310 sequencer (PerkinElmer, Weiterstadt, Germany).

Statistical analysis

A two-tailed Wilcoxon rank sum test was performed to analyze ELISPOT data. An unpaired two-tailed Student's t test was used to interpret differences in CTL reactivities against different target cells and in the presence of blocking Ab in cytotoxic assays, and differences between the number of spots obtained from T cells incubated with T2 cells pulsed with relevant p53 peptides vs that obtained using T2 cells pulsed with the irrelevant gp100 peptide in ELISPOT assays. Differences were considered significant when $p < 0.05$.

Results

Selection of variant $p53_{264-272}$ peptides recognized by anti-$p53_{264-272}$ CTL

Because the parental peptide binds efficiently to HLA-A2.1 molecules, all of the APL considered in this study represent single amino acid exchanges at nonanchor residues for the purpose of enhancing the interactions of the variant peptides with the TCR rather than MHC class I molecules. Nineteen variants of the wt $p53_{264-272}$ peptide were screened for their recognition by a bulk population of anti-wt $p53_{264-274}$-specific CTL that was maintained in our laboratory (7). T2 cells pulsed with the individual peptides at a fixed concentration of 1×10^{-6} M peptide served as targets for these CTL in a ^{51}Cr release cytotoxic assay. Significant cytotoxic reactivity against T2 cells pulsed with three of the 19 variant peptides, namely, 6T, 7W, and 7P, was detected (data not shown). Therefore, these three variant peptides were selected for further characterization.

Variant peptide binding to HLA-A2.1 molecules

Binding of the 6T, 7W, and 7P variant peptides to HLA-A2.1 molecules was compared with that of the parental peptide in an MHC stabilization assay. The relative mean fluorescence intensity of parental and variant peptide-stabilized HLA-A2 molecules on T2 cells is shown in Fig. 1. All the peptides showed stabilization of HLA-A2 molecules in a dose-dependent manner within the concentration range of 1×10^{-5}–1×10^{-9} M. However, in general, the binding affinities of the variant peptides to HLA-A2.1 molecules on T2 cells were slightly lower than that of the parental wt peptide (wt > 6T ≥ 7W > 7P).

Affinity of $p53_{264-272}$-specific CTL for variant peptides

The affinity of the bulk population of anti-$p53_{264-272}$-specific CTL for the variant peptides was determined in a 4-h ^{51}Cr release assay using T2 cells pulsed with these peptides at concentrations ranging from 1×10^{-5} to 1×10^{-12} M as target cells. As shown in Fig. 2, at concentrations $<1 \times 10^{-8}$ M, the dose-response curves of the three variant peptides were shifted to the left relative to that of the parental wt peptide. Because the increased responsiveness of the CTL for these variant peptides cannot be attributed to enhanced binding to HLA-A2.1 molecules, these results are consistent with an increased affinity of TCR for the variant peptides.

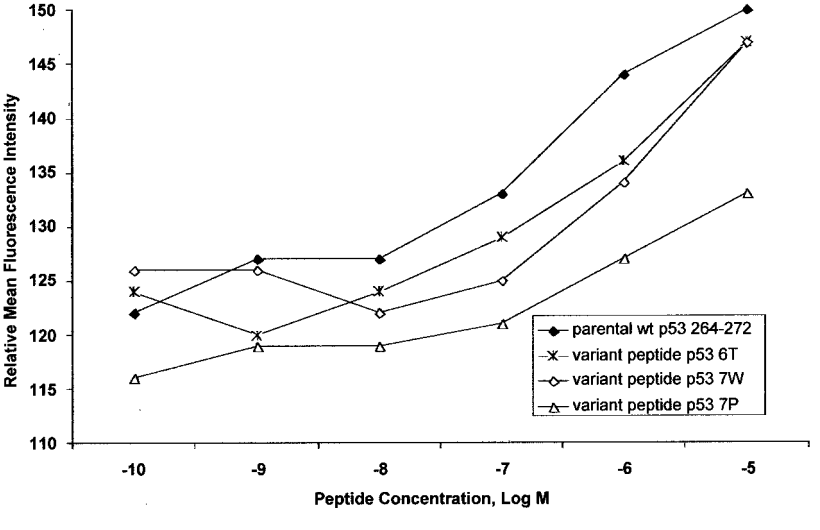

FIGURE 1. Identification of three HLA-A2.1-binding variant peptides of the wt $p53_{264-272}$ epitope. T2 cells were incubated with parental $p53_{264-272}$ peptide (LLGRNSFEV) or 6T, 7P, or 7W variant peptides at final concentrations of 1×10^{-5}–1×10^{-10} M. The relative mean fluorescence intensities of FITC-conjugated anti-MHC class I mAb (W6/32) are indicative of peptide-stabilized MHC class I molecules on T2 cells.

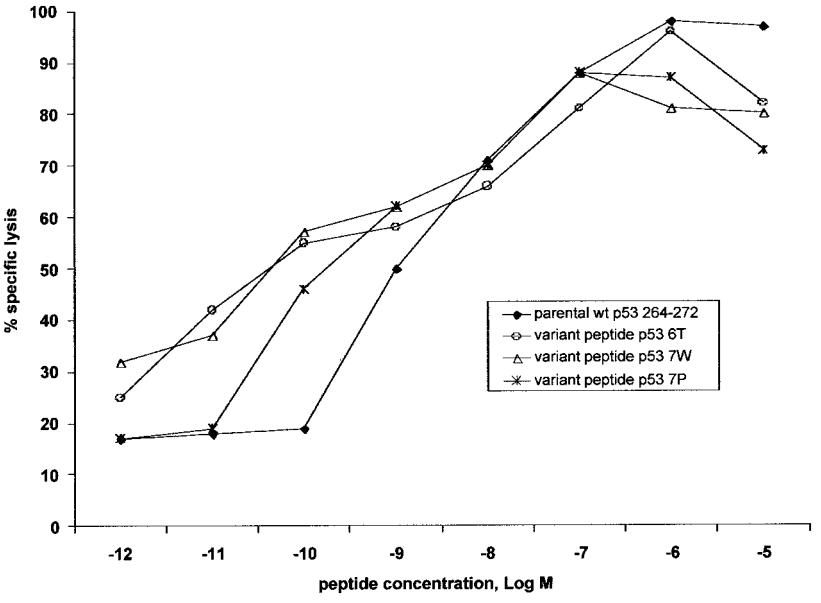

FIGURE 2. Variant peptides are recognized by anti-wt $p53_{264-272}$-specific CTL line. T2 cells were pulsed with different peptide concentrations and tested as targets in a 4-h ^{51}Cr release assay at the E:T ratio of 10:1.

Characterization of wt $p53_{264-272}$-specific CTL generated from PBMC obtained from normal donors using variant peptides

Previously, we reported that CTL reactive against the wt $p53_{264-272}$ epitope could be generated from PBMC obtained from only two of the seven HLA-A2.1$^+$ normal donors tested (12). Analyses involving multiple cryopreserved samples derived from leukopaks obtained from two of the normal donors (a responder and a nonresponder) confirmed the consistency of responses of these donors' PBMC to the parental peptide. In the same experiments in which the seven donors' PBMC were tested for induction using the parental peptide, CTL reactive against this peptide could be generated from five of these seven PBMC using either the 6T or 7W variant peptide. Included in this group were three nonresponsive PBMC (Table I); PBMC obtained from donors 6 and 7 responded to the 7W variant, while PBMC obtained from donor 4 responded to the 6T peptide. None of the seven PBMC tested responded to the 7P variant peptide.

The bulk populations of variant-induced cells generated from PBMC obtained from donors 6 and 7 effectively recognized and lysed T2 cells pulsed with the parental peptide in ELISPOT for IFN-γ and cytotoxicity assays. Fig. 3, *A* and *B*, shows the results obtained with the effectors generated from PBMC obtained from donor 7. Unpulsed T2 target cells or T2 cells pulsed with an irrelevant HLA-A2.1-binding peptide, the melanoma-associated gp100 peptide (33), were not recognized by these CTL in either assay to any noticeable extent. More importantly, these effector cells were also capable of recognizing the naturally presented epitope, as evidenced by their ability to lyse PCI-13 and SCC-9 tumor cells as well as mutant p53-transfected SaOS-2 Cl3 cell lines in a MHC class I-restricted manner (Fig. 3*C*). No significant cytotoxicity was noted against HLA-A2$^+$ tumor cell lines, SCC-4 and SaOS-2, which do not present the epitope.

Table I. *Summary of the anti-p53 CTL responses of PBMC obtained from normal donors following IVS using variant $p53_{264-272}$ peptides*[a]

Donor	Anti-p53 CTL Response After IVS With			
	wt $p53_{264-272}$	Variant 6T	Variant 7P	Variant 7W
1	+	−	−	+
2	+	−	−	+
3	−	−	−	−
4	−	+	−	−
5	−	−	−	−
6	−	−	−	+
7	−	−	−	+

[a] PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; − indicates that no specific reactivity against the wt $p53_{264-272}$ peptide was observed, while + indicates that effectors were reactive against variant and parental peptides. Results using the parental wt $p53_{264-272}$ were reported in a previous publication from this laboratory (12).

The CTL generated from PBMC obtained from donor 4 using the 6T peptide yielded effectors with reactivity comparable with that of the 7W variant-induced CTL. The 6T-induced T cells were responsive to wt $p53_{264-272}$-pulsed T2 cells in the ELISPOT for IFN-γ assay (Fig. 4*A*), and cytolytic against the OSCC lines, SCC-9 and PCI-13, as well as SaOS-2Cl3 (Fig. 4*B*). This response was blocked by anti-HLA class I mAb but not anti-HLA-DR mAb. No significant reactivity was obtained against the tumor cell line SCC-4. The reactivity of these effectors against SaOS-2 cells in the analysis shown in Fig. 4*B* was higher than normally detected against this $p53^{null}$ cell line, using bulk populations of anti-p53 effectors (7, 12). However, the reactivity of the 6T-induced effectors against SaOS-2 targets was not significantly blocked by anti-HLA class I mAb and thus could be attributed to nonspecific effectors present in the bulk population. In summary, variant-induced effector T cells had similar reactivities against the parental epitope as those reported previously for the parental peptide-induced effectors from responsive normal donors as well as OSCC patients (7, 12).

Characterization of wt $p53_{264-272}$-specific CTL generated from PBMC of a nonresponsive OSCC patient using a variant peptide

The critical test of the variant peptides was whether their use could induce CTL capable of recognizing the anti-wt $p53_{264-272}$ epitope from nonresponsive patients whose tumors were considered capable of presenting this epitope (12). The nonresponsiveness of PBMC obtained from at least one of these donors, patient 3, has been repeatedly confirmed during the past 2 years using blood samples obtained at different times, as well as multiple cryopreserved leukapheresis samples obtained from this patient. As shown in Table II, none of the PBMC from three of these patients responded to the 6T or 7P variant peptides. However, the 7W variant

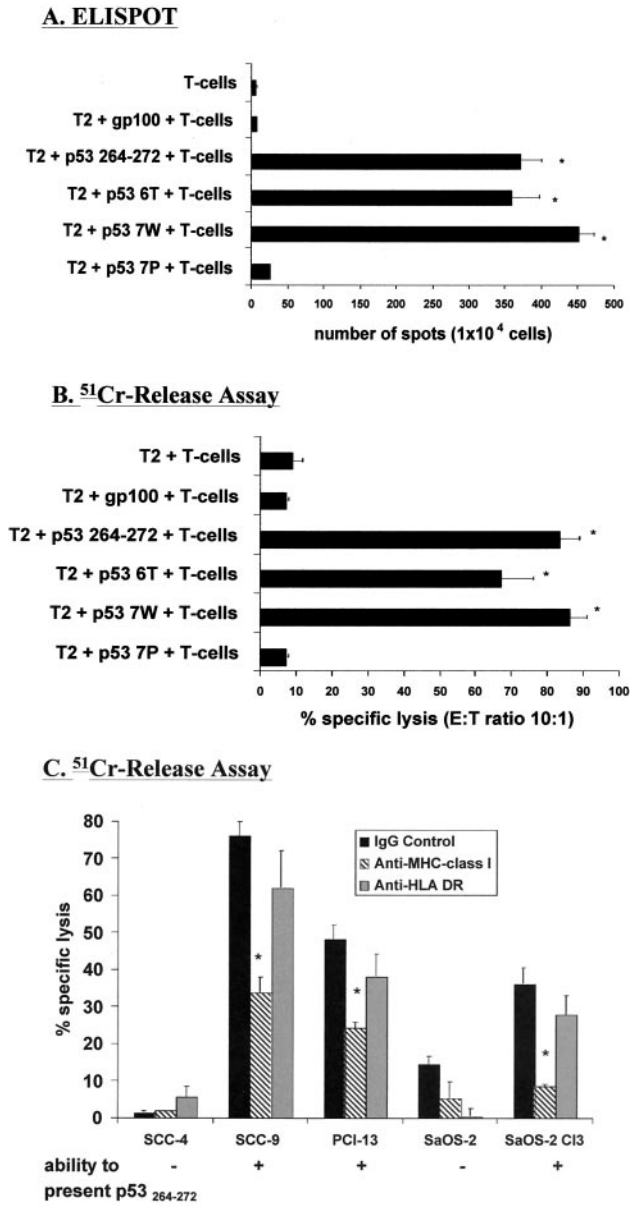

FIGURE 3. The 7W variant peptide-induced effectors induced from PBMC obtained from a nonresponsive healthy donor recognize the parental wt $p53_{264-272}$ peptide pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT for IFN-γ assays. Effectors were tested against T2 cells pulsed with an irrelevant gp100 peptide, the wt $p53_{264-272}$ peptide, or the variant peptides at 10 μg/ml. *B*, Lysis of T2 cells pulsed with various peptides at an E:T of 1:10. *C*, Lysis of tumor targets naturally presenting the epitope at an E:T ratio of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. $*$, A significant ($p < 0.05$) difference relative to IgG controls.

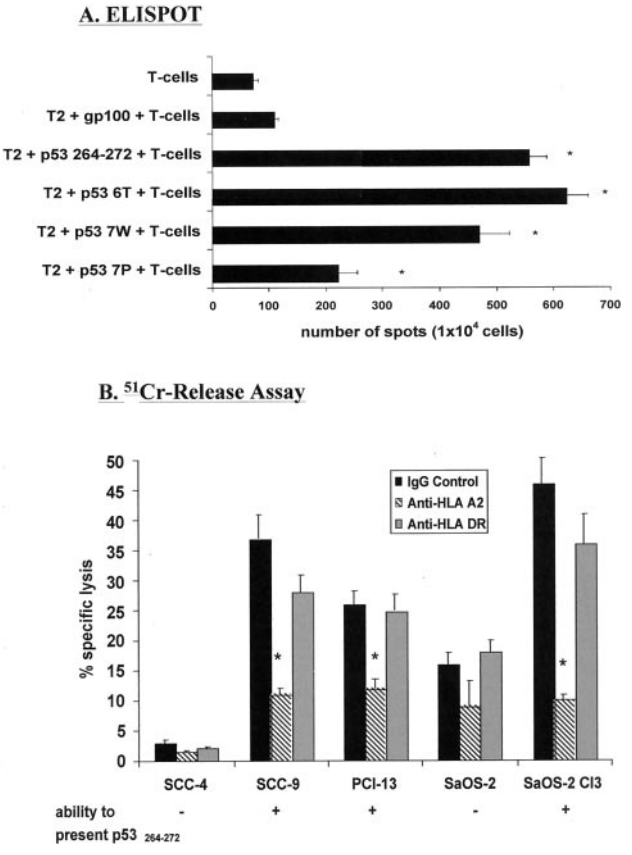

FIGURE 4. The 6T variant peptide-induced effectors induced from PBMC obtained from nonresponsive healthy donor recognize the parental wt $p53_{264-272}$ epitope pulsed onto target cells or naturally presented by tumors. *A*, Recognition of peptide-pulsed T2 cells in ELISPOT assays. Effectors were tested against T2 cells pulsed with either an irrelevant gp100 peptide, the wt $p53_{264-272}$ peptide, or a variant peptide at 10 μg/ml. *B*, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. $*$, A significant ($p < 0.05$) difference relative to IgG controls.

peptide did induce the ex vivo generation of anti-wt $p53_{264-272}$ CTL from PBMC of patient 3, whose autologous tumor, PCI-13, presents this epitope (7, 12). The affinity of these effectors for the parental epitope was comparable with that of a bulk population of CTL induced using the parental epitope, and was in the range of 1×10^{-9} M (Figs. 2 and 5*A*). Furthermore, the 7W-induced CTL were cytotoxic against a panel of tumor cell lines naturally presenting the wt $p53_{264-272}$ epitope, including the autologous PCI-13 cell line, and this reactivity was MHC class I restricted (Fig. 5*B*). This result clearly illustrates the potential value of the 7W variant peptide in immunotherapy targeting the wt $p53_{264-272}$ epitope in individuals like OSCC patient 3.

Tetramer-binding and TCR Vβ usage by T cell microcultures reactive against parental and/or variant peptides

The ability of the variant peptides to induce the generation of CTL specific for wt $p53_{264-272}$ from nonresponder PBMC raised the question of the relationship between these CTL and those induced by the parental peptide in responder PBMC. The need to investigate this relationship became evident when the cross-reactive bulk population of $CD8^+$ T cells induced with the 7W variant peptide from normal donor 7 was stained with the parental or variant tetramer. Whereas only ~2% $tetramer^{dim}$ cells were detected with the parental tetramer, a cluster of ~40% $tetramer^{bright}$ cells was detected with the 7W tetramer (Fig. 6, *A* and *B*). One possible explanation for this observed difference was that the variant peptide induced a single $CD8^+$ T cell population that bound the variant tetramer with higher avidity/stability than did the parental tetramer. Another possible explanation was that the variant peptide induced two distinct populations of $CD8^+$ T cells; one was cross-reactive and bound both tetramers (most likely with different avidities), while the other was specific for the 7W variant and bound the 7W tetramer with high avidity. The two possibilities could be

Table II. *Summary of the anti-p53 CTL responses of PBMC obtained from nonresponsive OSCC patients following IVS using variant $p53_{264-272}$ peptides*

Patient	Tumor[a]		Anti-p53 CTL Response After IVS[b]		
	p53 genotype	p53 protein	Variant 6T	Variant 7P	Variant 7W
1	Mutant R248W	+	−	−	−
2	Mutant V 157 F	+	−	−	−
3	Mutant E286K	+	−	−	+

[a] Patients' tumors were analyzed for genetic alterations in p53 exons 5–8, and the identified codon and missense mutations are denoted. The level of p53 expression in tumors was determined by immunohistochemistry, using anti-p53 mAb, and + denotes accumulation of p53 (12).

[b] PBMC were stimulated with the peptide-pulsed autologous DC or PBMC in two to four IVS cycles. Effector cell reactivity was tested in ELISPOT and cytotoxicity assays; − indicates that no specific reactivity against the wt $p53_{264-272}$ peptide was observed, while + indicates that effectors were reactive against variant and parental peptides.

distinguished based on TCR usage of the T cells involved in recognition of these peptides. To accomplish this, T cell microcultures were established by limiting dilution from bulk CTL populations induced with either parental or variant peptide. Several T cell clones from each type of microculture were expanded for further analysis. Based in part on their rates of proliferation as well as peptide specificities (Fig. 7), four oligoclonal T cell lines, designated 2, 4, 53, and 68, were selected for TCR analysis by complementarity-determining region (CDR)3 spectratyping.

Two of the cell lines analyzed, 53 and 68, were derived from the bulk population of 7W-induced CTL that was described above and shown to exhibit differential staining with the parental and 7W

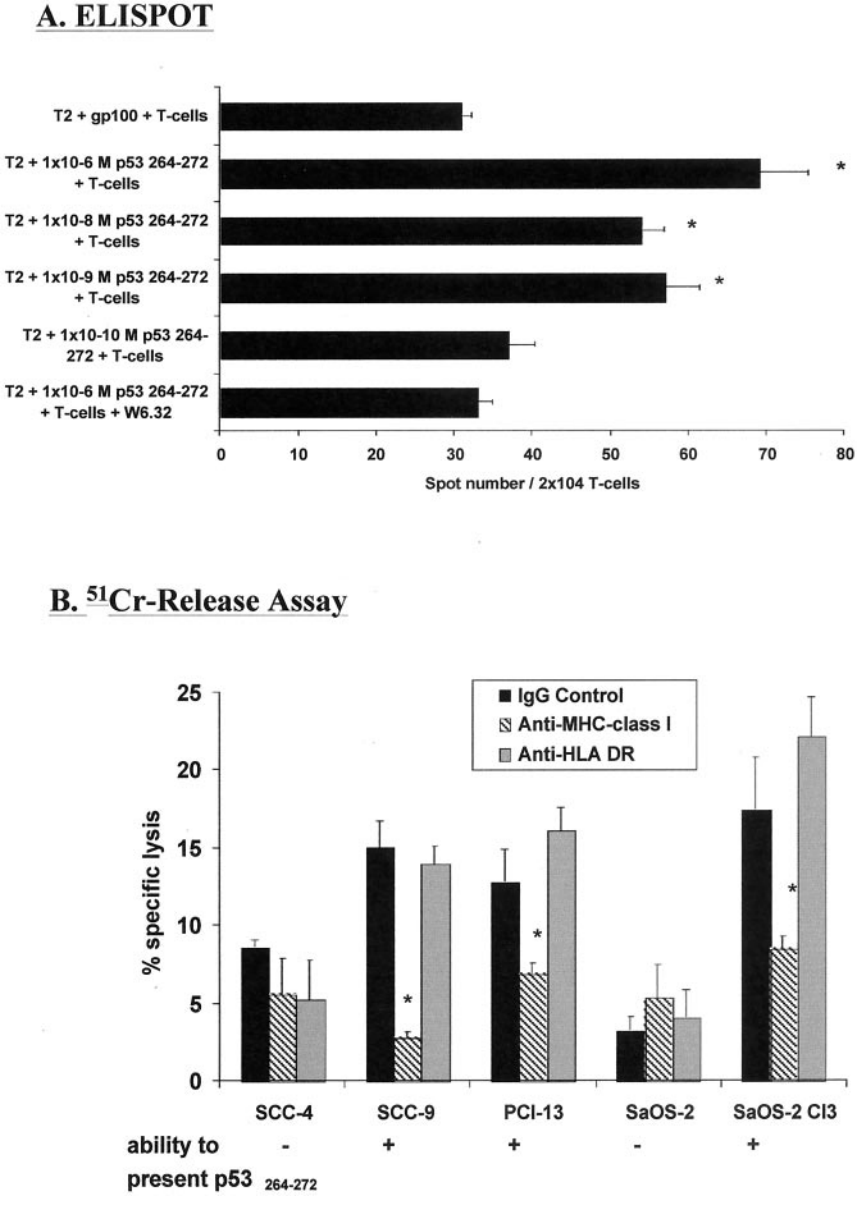

FIGURE 5. The 7W variant peptide-induced effectors obtained from a nonresponsive OSCC patient recognize the parental wt $p53_{264-272}$ peptide pulsed onto target cells or naturally presented by tumors, including PCI-13, the autologous tumor cell line. *A*, Affinity of effectors for the parental wt $p53_{264-272}$ peptide as determined in ELISPOT assay. T2 cells were incubated with parental peptide at concentrations of 1×10^{-6}–1×10^{-10} M. T2 cells pulsed with an irrelevant gp100 peptide served as a control. *B*, Lysis of tumor targets naturally presenting the epitope at an E:T of 40:1 in the presence or absence of anti-MHC class I or anti-HLA-DR mAb. ∗, A significant ($p < 0.05$) difference relative to IgG controls.

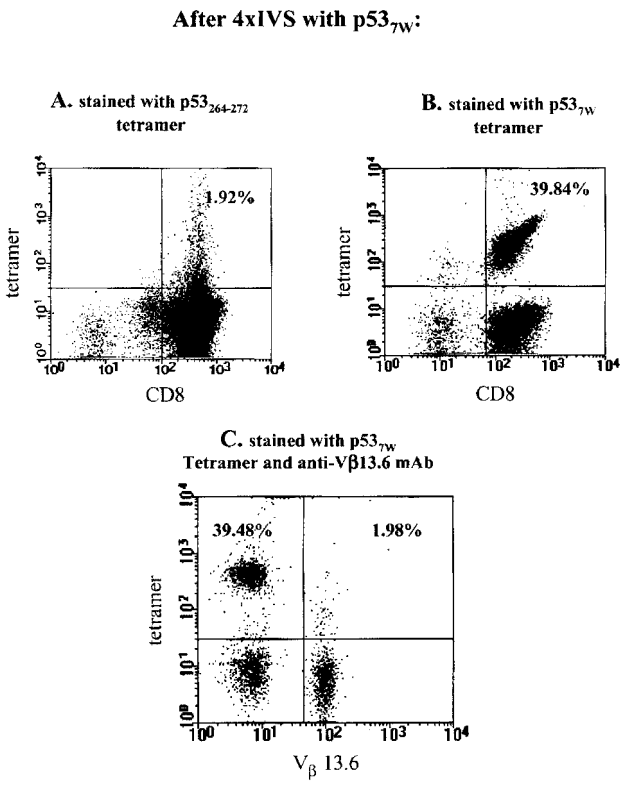

FIGURE 6. $CD8^+$ cells induced from PBMC of a nonresponsive normal donor using the 7W variant peptide that recognizes the parental peptide express TCR Vβ13.6. Three-color flow cytometry analysis of $CD8^+$ cells stained with HLA-A2.1 tetramers containing either the parental peptide (*A*), 7W variant peptide (*B*), or the 7W tetramer and anti-Vβ13.6 mAb (*C*). The numbers in the *upper right quadrants* indicate the percentage of $tetramer^+$ cells. The analyses shown in *A* and *B* involved 75,000 events, while in *C* 10,000 events were analyzed.

tetramers (Fig. 6). Although tetramer analysis of the bulk population suggested that the vast majority of CTL were 7W specific, one of the several T cell clones isolated was cross-reactive. The 53 cell line, which was specific for the 7W variant, was found to express Vβ9. The 68 cell line, which recognized the variant as well as the parental peptide, was found to express Vβ13.6 with completely different CDR3 and J regions from those expressed by line 53 (Table III). Consistent with these findings was the result of a combined tetramer/Vβ expression flow analysis of the bulk population from which the 53 and 68 cell lines were derived. We observed that the ~40% cells that stained as a distinct cluster with the 7W tetramer were $V\beta13.6^-$, whereas the ~2% cells that stained weakly with this tetramer were $V\beta13.6^+$ (Fig. 6*C*).

The other two cell lines analyzed, 2 and 4, were derived from bulk populations of effectors induced with the parental peptide from PBMC obtained from a patient (patient 2 in Ref. 12) and a normal donor (donor 2, Table I), respectively. The 2 cell line was found to express Vβ13.6, with identical motifs for the CDR3 and J regions as the 68 cell line, which was induced from a different individual using the 7W peptide (Table III). The observation that cross-reactive T cells induced by the variant peptide from PBMC obtained from one donor express the identical TCR/CDR3/J region as that expressed by T cells induced with the parental peptide from another donor illustrates the heteroclitic nature of the 7W variant peptide.

The 4 cell line was shown to express Vβ1 with a CDR3 and J region sequence distinct from those of any of the other cell lines analyzed (Table III). Interestingly, Vβ analysis of the bulk population of effectors from which the 4 cell line was isolated detected mainly Vβ1 and Vβ13 $CD8^+$ cells. Another bulk population of parental peptide-induced CTL, which was obtained from a different normal donor (7) and used to identify 7W and 6T peptides as potential APLs (Table II), was found to consist of >90% $V\beta1^+$ cells (data not shown). These results, summarized in Table IV, are strongly suggestive of a relatively limited TCR Vβ usage being involved in recognition by CTL of the HLA-A2.1-restricted, wt $p53_{264-272}$ epitope, regardless of whether these cells are induced by the parental or variant peptide.

Discussion

Most studies of APL of tumor Ags involve amino acid exchanges at anchor residue positions of the peptide to enhance its binding to class I MHC molecules. The recent modification of the HLA-A2.1-restricted wt $p53_{149-157}$ peptide at anchor position 2 to improve its binding to the restriction element and immunogenicity is one example of this approach (26). Particularly relevant to this study are variants designed to enhance TCR/peptide interactions rather than increase MHC binding, such as the HLA-A2.1-restricted, melanoma-associated MART1/Melan A_{27-35} and carcinoembryonic Ag, CAP1, peptides, which involve amino acid exchanges in residues other than anchor positions (18–21). While the binding affinities of these variant and parental peptides to HLA-A2.1 molecules are comparable, amino acid exchanges of these peptides at nonanchor positions yielded variant peptides that were more immunogenic than the parental peptides.

Since the parental wt $p53_{264-272}$ peptide has a reasonable affinity for HLA-A2.1 molecules ($>1 \times 10^{-9}$ M), the 19 $p53_{264-272}$ variants designed for this study had unmodified anchor positions. Among the amino acid exchanges tested, those at position 6 (6T) and position 7 (7W) appeared to be promising. Since both variants have lower affinities than the parental peptide for HLA-A2.1 molecules, their ability to increase the frequency of anti-$p53_{264-272}$ CTL responses generated from nonresponsive PBMC does not appear to be due to their enhanced binding to HLA-A2.1 molecules. Instead, their increased immunogenicity might be due to the replacement or counterbalancing of residues causing adverse TCR-peptide interactions. Such a replacement could result in an improved interaction of the peptide/MHC complex with TCR and a subsequent expansion of T cells capable of recognizing the parental epitope (23, 24). Two lines of evidence support this conclusion. First, using the parental tetramer to determine the frequency of $tetramer^+$ precursor T cells in unstimulated PBMC obtained from normal donors and patients with cancer, we found that most of the nonresponsive individuals had markedly lower frequencies of these cells in their peripheral circulation than did the responders (data not shown).[4] Second, the parental and variant peptides were found to engage and expand T cells expressing the same TCR in PBMC obtained from responsive and nonresponsive donors (see Tables III and IV). These findings support the concept that increased stability of interaction with the TCR is the basis for the enhanced functional activity of the 7W variant peptide.

Although the use of variant peptides did reverse the nonresponsiveness in IVS of PBMC obtained from some donors, their use did not yield high-affinity CTL. The persistence of low-affinity CTL against self tumor peptides, such as wt p53 epitopes, which is considered a true consequence of tolerance (15), might be due to a limited TCR repertoire being available for recognition of these epitopes. Our analyses detected the predominant use of only two TCR Vβ families, Vβ1 and Vβ13.6, being involved in CTL recognition of the wt $p53_{264-272}$ epitope in four different donors. Furthermore, in two different donors, identical usage by the parental

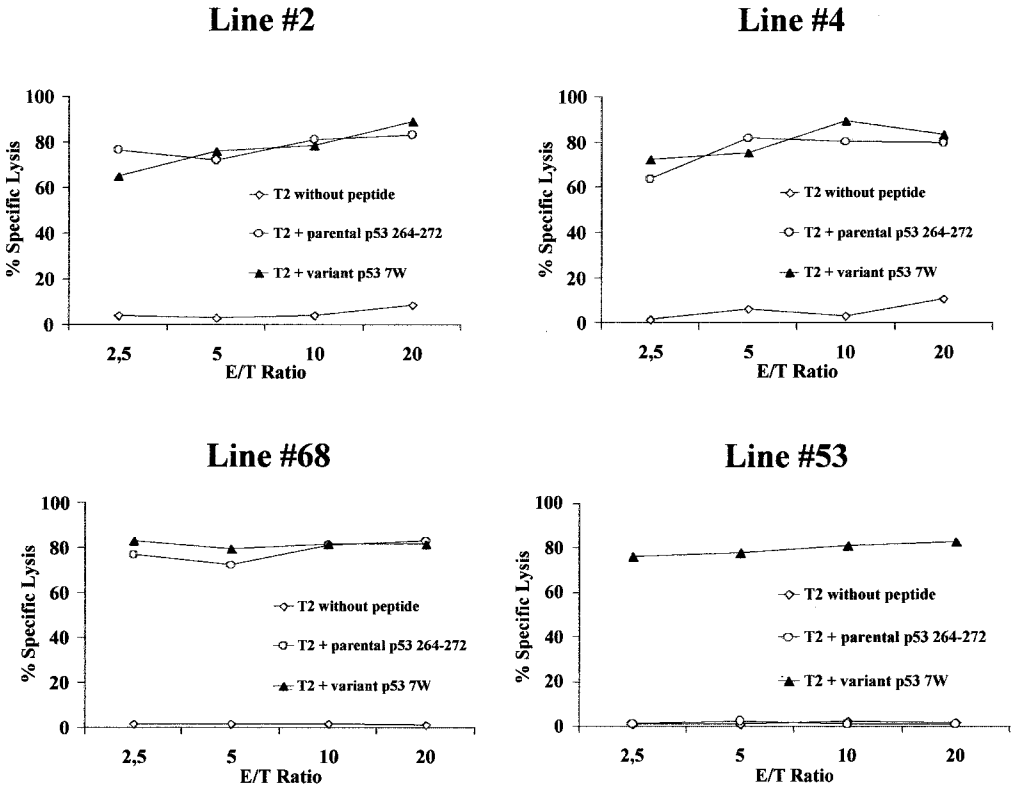

FIGURE 7. Specificity of T cell lines analyzed for TCR Vβ usage. Lines 2 and 4 were derived from bulk populations of effectors induced from an OSCC patient and a normal control, respectively, using the parental peptide. Both are cross-reactive against the variant peptide. Lines 53 and 68 were derived from a bulk population of effectors induced from a nonresponsive healthy donor with the 7W variant peptide. The 53 cell line is specific for the 7W variant, while the 68 cell line is cross-reactive.

and variant peptide was detected. In contrast, an analysis of responses in HLA-A2.1$^+$ patients to repeated immunizations with an anchor position-variant peptide of the melanoma-associated $gp100_{209-217}$ epitope demonstrated that the appearance of higher-affinity T cells was associated with an expansion of the TCR repertoire rather than an increased oligoclonal response (33). In the future, additional data on TCR usage of cross-reactive and variant-specific CTL cell could allow for extensive molecular modeling of the interactions within the trimeric complexes and, perhaps, the design of APL with more enhancing properties than those of the 6T and 7W variants. These variants might engage more diverse populations of T cells that are capable of cross-recognition of the parental epitope with, perhaps, higher avidity. However, the apparent outgrowth of epitope-loss tumors in OSCC patients responsive to this epitope suggests that even intermediate-affinity CTL recognizing wt $p53_{264-272}$ might be effective in tumor eradication (12).

To fully estimate the potential of p53-based vaccines in immunotherapy of cancer, it is becoming increasingly apparent that an array of T cell-defined wt p53 epitopes needs to be analyzed, and strategies for optimal induction of T cells recognizing these epitopes need to be further evaluated. In this regard, the use of genetically modified DC expressing intact wt p53 appears to enhance the generation and increase the frequency of antitumor effectors from PBMC of normal donors and cancer patients (34). The p53-based immunotherapy also might be critically dependent on targeting the right epitopes and matching a patient's ability to respond ex vivo to wt p53 epitopes with the potential of his/her tumor to present these epitopes for immune recognition. Again, of course, it is necessary to be aware that a patient's ex vivo responsiveness to these epitopes does not guarantee a successful in vivo response to immunization with them. In this study, HLA-A2.1$^+$ patient 3 with OSCC, for whom the tumor cell line and tumor-specific CTL are available in the laboratory, has been

Table III. *Amino acid sequences of monoclonal TCR transcripts expressed in four parental and/or variant $p53_{264-272}$-specific CTL lines*[a]

Line	IVS	Specificity	Vβ Family	Sequences		
				Vβ	CDR3 region	J region
2	wt	wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3
4	wt	wt + 7W	Vβ1	LELGDSALYFCA	SSEGGL	ETQYFGPGTRL/BJ2-5
53	7W	7W	Vβ9	LGDSAVYFCA	SSAGTNT	YEQYFGPGTRLTVT/BJ2-7
68	7W	wt + 7W	Vβ13.6	RLELAAPSQTSVYFCA	SSQTPLG	DTQYFGPGTRLT/BJ2-3

[a] Lines 2 and 4 were stimulated with the parental wt $p53_{264-272}$ peptide (wt) and were reactive against the parental and 7W variant peptides. Lines 53 and 68 were stimulated with the 7W variant peptide. Line 53 was reactive against the 7W variant peptide only, while line 68 was reactive against the parental and 7W variant peptides. Single peaks in individual TCR variable chain families, suggesting clonality, were analyzed by direct sequencing of the PCR products.

Table IV. *Summary of evidence of limited TCR Vβ usage for CTL recognition of the wt $p53_{264-272}$ epitope*[a]

PBMC Donors[b]	Induced with $p53_{264-272}$ Peptide	Bulk T Cell Population Vβ Usage	Derived T Cell Lines[c]	
			Vβ usage	p53 peptide specificity
Normal donor 2 (R)	wt	Vβ1, 13.6	4 Vβ 1	wt/7W
Normal donor 4[d] (R)	wt	Vβ1	ND	
Normal donor 7 (NR)	7W	Vβ 9, 13.6	53 Vβ 9	7W only
			68 Vβ 13.6[e]	wt/7W
OSCC patient 2[f] (R)	wt	Vβ 13.6	2 Vβ 13.6[e]	wt/7W

[a] See Table III for the details on TCR Vβ usage.
[b] Normal donors and patients identified in Tables I and II; R, responsive to IVS of PBMC to the wt $p53_{264-272}$ peptide; NR, nonresponsiveness.
[c] T cell lines derived by limiting dilution.
[d] Normal donor used as source of PBMC for induction of a bulk population of CTL specific for wt $p53_{264-272}$. Generation and characterization of this cell line was detailed in Ref. 7.
[e] These T cell lines express identical Vβ, CDR3, and J region sequences (see Table III).
[f] The OSCC patient 2 identified in Table I in Ref. 12.

of particular interest. The tumor cell line established from this patient and designated PCI-13 accumulates p53 molecules expressing a missense mutation at codon 286 and naturally presents the $p53_{264-272}$ epitope, albeit following pretreatment with IFN-γ (7, 12). The ability to generate anti-$p53_{264-272}$ CTL with the 7W variant from this patient's PBMC, which were nonresponsive to the parental peptide, provides a basis for the potential use of the 7W variant peptide in immunotherapy of this patient and, perhaps, other nonresponsive OSCC patients with tumors expressing similar characteristics. Concurrently, it needs to be determined whether the trends observed in OSCC patients regarding their responsiveness to wt p53 epitopes and the potential of their tumors to present these epitopes are also apparent in patients with other types of cancers.

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[CANCER RESEARCH 62, 3521–3529, June 15, 2002]

Frequencies of Tetramer$^+$ T Cells Specific for the Wild-Type Sequence p53$_{264–272}$ Peptide in the Circulation of Patients with Head and Neck Cancer[1]

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ABSTRACT

Immunization with wild-type sequence (wt) p53 epitopes represents a novel therapeutic strategy for cancer patients with tumors accumulating mutant p53. To evaluate usefulness of p53-derived peptides as future cancer vaccines, frequencies of wt p53$_{264–272}$ peptide-specific CD8$^+$ T cells were determined in the peripheral circulation of patients with squamous cell carcinoma of the head and neck (SCCHN). T cells of 30 HLA-A2.1$^+$ patients and 31 HLA-A2.1$^+$ healthy individuals were evaluated by multicolor flow cytometry analysis using peptide-HLA-A2.1 complexes (tetramers). T cells specific for an influenza matrix peptide (a model recall antigen) or an HIV reverse transcriptase peptide (a model novel antigen) were studied in parallel. Patients with SCCHN had a significantly higher mean frequency of CD8$^+$ T cells specific for wt p53$_{264–272}$ than normal donors ($P = 0.0041$). Surprisingly, the frequency of epitope-specific T cells in the circulation of patients did not correlate with p53 accumulation in the tumor. In patients whose tumors had normal p53 expression or had p53 gene mutations preventing presentation of this epitope, high frequencies of wt p53$_{264–272}$-specific CD8$^+$ T cells were found, of which many were memory T cells. In contrast, patients whose tumors accumulated p53 had low frequencies of wt p53$_{264–272}$-specific CD8$^+$ T cells, which predominantly had a naive phenotype and were unable to proliferate *ex vivo* in response to the epitope, as reported by us previously (T. K. Hoffmann, J. Immunol., *165:* 5938–5944, 2000). This seemingly contradictory relationship between the high frequency of epitope-specific T cells and wt p53 expression in the tumor suggests that other factors may contribute to the observed anti-p53 responses. Human papillomavirus-16 E6/E7 expression is common in SCCHN, and E6 is known to promote presentation of wt p53 epitopes. Although human papillomavirus-16 E6/E7 expression was detected in 46% of the tumors, it did not correlate with the frequency of wt p53$_{264–272}$-specific CD8$^+$ T cells or with p53 expression in the tumor. These findings emphasize the complexity of interactions between the tumor and the host immune system, and, thus, have particularly important implications for future p53-based immunization strategies.

INTRODUCTION

The gene encoding p53 protein is frequently mutated in many human cancers, including SCCHN,[3] which generally results in accumulation (overexpression) of p53 molecules in these tumors (1–4). As most of these mutations involve an alteration of a single amino acid in p53 molecules, the majority of the accumulating mutant protein resembles the wt p53 (4). Therefore, enhanced presentation of wt epitopes derived from p53 accumulating in tumors is possible, and might lead to their recognition by the immune system and the development of antitumor CTLs (5–9). For this reason, wt p53 epitopes are considered attractive targets for immunotherapy of cancer.

We reported previously on the generation of CTLs recognizing the HLA-A2.1-restricted wt p53$_{264–272}$ epitope from PBMCs obtained from SCCHN patients using autologous peptide-pulsed dendritic cells as antigen-presenting cells (9). Surprisingly, we observed that CTLs reactive against this wt p53 epitope could only be generated from T-cell precursors in PBMCs of patients whose tumors either did not accumulate p53 or accumulated mutant p53 molecules that could not present this epitope (9). We hypothesized that *in vivo*, the presence of expandable CTL precursors specific for the wt p53$_{264–272}$ epitope led to immunoselection, resulting in the elimination of tumors expressing the epitope and favored the outgrowth of "epitope-loss" tumor cells able to evade the host immune system. On the other hand, it was also possible that HPV infection, known to occur in a substantial proportion of SCCHN, could lead to inactivation of wt p53 and enhanced processing of p53 epitopes (10). The consequence of either phenomenon would be the presence in patients with wt p53 tumors of relatively high frequencies of T cells specific for the wt p53$_{264–272}$ epitope. To test these hypotheses, we investigated the frequency of p53$_{264–272}$-specific precursor T cells in the peripheral circulation of 30 HLA-A2.1$^+$ patients with SCCHN and 31 HLA-A2.1$^+$ healthy controls, using multimeric peptide-MHC complexes. We also performed PCR analyses of genomic DNA isolated from the patient tumors for p53 and HPV E6/E7.

The availability of multimeric peptide-MHC complexes, which are generically referred to as tetramers, allows for direct identification and phenotyping of antigen-specific T cells in the peripheral circulation. Tetramers bind to more than one TCR on a specific T cell and, therefore, have a relatively slow dissociation rate (11, 12). However, the specificity of tetramer binding to the TCR has to be carefully controlled, particularly when the frequency of peptide-specific precursor T cells in the peripheral circulation is expected to be very low. Anticipating that discrimination between nonspecific and specific tetramer binding to p53$_{264–272}$-specific T cells might be difficult, we used a novel four-color flow cytometry assay that simultaneously measures tetramer, CD3, CD8, and CD14 binding (13). Furthermore, binding of the p53 tetramer was compared with that of the influenza virus matrix peptide GILGFVFTL (FLU, a model for recall responses) and the HIV reverse transcriptase peptide ILKEPVHGV (HIV, a model for responses to a new antigen).

In this study, the frequency of wt p53$_{264–272}$ peptide-specific CD8$^+$ T cells in the circulation of patients with SCCHN was correlated with the p53 expression in each patient tumor, its HPV status, and the presence of p53 antibodies in the serum. Our results provide significant insights into the *in vivo* interactions that might occur between the developing tumor and immune system in these patients.

MATERIALS AND METHODS

PBMCs. Peripheral blood samples or leukapheresis products were obtained from 30 HLA-A2.1$^+$ SCCHN patients, 31 HLA-A2.1$^+$ healthy donors, and 10

Received 3/6/01; accepted 4/16/02.

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[1] Supported in part by the NIH Grant PO-1 DE-12321 (to T. L. W. and A. B. D.), RO1 AI41408, RO1 AI14870, 1PO1 A143664 (to A. D. D.), and by Grant D/99/08916 of the Dr. Mildred Scheel Stiftung für Krebsforschung (to T. K. H.).

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[3] The abbreviations used are: SCCHN, squamous cell carcinoma of the head and neck; Ab, antibody; CTL, cytotoxic T lymphocyte; HPV, human papillomavirus; HLA, human leukocyte antigen; IL, interleukin; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; ECD, electron capture detection; TCR, T-cell receptor; wt, wild-type sequence; FLU, influenza.

HLA-A2.1$^-$ healthy donors. PBMCs were isolated by centrifugation over Ficoll-Hypaque gradients (Amersham Pharmacia Biotech, Piscataway, NJ). Leukapheresis products were obtained from the Institute of Transfusion Medicine, Pittsburgh, PA. The study was approved by the Institutional Review Board at the University of Pittsburgh, and written informed consent was obtained from each participating individual. All of the products were tested and found to be negative for HIV-I antigens and antibodies to HIV. PBMCs were phenotyped for expression of HLA-A2 molecules by flow cytometry using the anti-HLA-A2 mAb BB7.2 (American Type Culture Collection, Manassas, VA) and an IgG isotype as a control. The verification of the A2.1 subtype was performed using polymerase chain reaction with sequence-specific primers as described previously (9, 14). PBMCs were either used fresh or were frozen at a concentration of 50×10^6 cells/ml in the freezing medium consisting of human AB serum (Pel-Freeze, Brown Deer, WI) plus 10% DMSO (Fisher Scientific, Pittsburgh, PA).

In 30 patients, histologically verified squamous cell carcinomas originated in one of the following sites: the nose ($n = 1$), oral cavity ($n = 7$), oropharynx ($n = 4$), larynx ($n = 16$), and hypopharynx ($n = 2$). Tumors were classified for tumor stage ($T_1 = 10$; $T_2 = 9$; $T_3 = 3$; $T_4 = 8$; and $T_x = 1$), nodal stage ($N^0 = 22$; $N_1 = 2$; $N_2 = 5$; and $N_3 = 1$), and the presence of distant metastases ($n = 0$ of 30).

Tetrameric Peptide-MHC Class I Complex (Tetramer) Assay. Tetramers were obtained through the National Institute of Allergy and Infectious Diseases Tetramer Facility and the NIH AIDS Research and Reference Reagent Program. Stock solutions contained 0.5 μg monomer/ml. Peptides provided to the National Institute of Allergy and Infectious Diseases Tetramer Facility were either GILGFVFTL, an influenza matrix immunodominant peptide (residues 58–66), the HIV-1 reverse transcriptase peptide (pol 476–484) ILKEPVHGV, or the HLA-A2.1-binding peptide LLGRNSFEV (15, 16), corresponding to wt $p53_{264-272}$ peptide. The specificity of the LLGRNSFEV tetramer was confirmed by staining against the anti-p53-specific CTL line as described previously (9) and by the lack of staining with irrelevant CTLs, as well as HLA-A2-negative PBMCs of healthy donors.

To minimize background staining each tetramer was titered and used at the lowest concentration that still gave a clearly discernible positive population in a donor vaccinated for influenza (for FLU_{58-66} tetramer) as well as in an HIV-infected individual (for $pol_{476-484}$). The final dilution of both preparations during staining, relative to the stock reagent supplied by the NIH, was 1/300. Within a 2-fold range of tetramer concentrations bracketing the concentrations used here, the frequency of tetramer-positive events and competition of CD3 binding (13) were stable, and tetramer fluorescence intensity was within 80% of that obtained at saturating concentrations. At saturating concentrations, CD3 competition decreased, fluorescence intensity of tetramer positive cells increased, and tetramer frequency increased, the latter attributable chiefly to a greater number of tetramer-dim events.

Antibodies. The default panel of antibodies used for these studies was CD14-FITC (RMO52; Immunotech, Miami, FL), Tetramer-PE, CD3 ECD (HIT3a; Beckman Coulter, Miami, FL), and CD8-PC5 [SFCI21Thy2D3(T8); Beckman Coulter]. Additionally, antihuman CD45RA FITC (Immunotech) and anti-CD45RO ECD (Beckman Coulter) were used for the characterization of the CD45 phenotype.

Flow Cytometry Analysis. Immediately before staining, cells were washed twice with the staining medium, consisting of PBS +0.1% (w/v) BSA +0.1% (w/v) sodium-azide, and resuspended at a concentration of 5×10^6/ml in a volume of 150 μl. Tetramer (5 μl of 1:10 dilution of stock solution) was added at room temperature for 30 min, followed by a 30 min incubation with antibodies (7.5 μL of each) at 4°C. After two additional washes, the cells were resuspended in ~1 ml of 0.5% methanol-free formaldehyde in PBS. At least 1×10^6 events were collected using a four-color Coulter Epics XL cytometer set on low or medium flow rate at a maximum of 1000 events/sec. Flow cytometry data were analyzed in real time using Beckman-Coulter System II software. In initial experiments, the region defining tetramer-positive events was determined by evaluating PBMCs stained with the Ab panel but without tetramer. This region was held constant throughout the analysis. Data were saved as FCS 2.0 Listmode files for subsequent reanalysis in System II or WinList (Verity Software House, Topsham, ME).

Confocal Microscopy. A wt $p53_{264-272}$-specific CTL line (9) was used as a positive control for the flow cytometric evaluation and additionally to visualize tetramer binding to the specific T cells by confocal microscopy. The CTL line was incubated with the $p53_{264-272}$ peptide containing tetramer in azide-free PBS followed by incubation with FITC-conjugated anti-CD8 Ab. The cells were then fixed with 1% (w/v) paraformaldehyde, placed on a slide, and analyzed by confocal laser scanning microscopy at ×600 original magnification (Leica TCS NT confocal LSM; Leica Lasertechnik, Heidelberg, Germany). Images were edited using the Adobe PhotoShop software program (Adobe Systems, Mountain View, CA).

p53 Mutation Analysis, Immunohistochemistry, and Detection of p53 Antibodies. Tumors of 30 SCCHN patients included in this study were available as paraffin blocks archived at the University of Pittsburgh Medical Center. The histology of each case was reviewed by a pathologist (S. D. F.), and representative tissue sections containing areas of invasive SCCHN were selected for microdissection. Normal-appearing salivary gland tissue or skeletal muscle was microdissected separately to serve as an internal nontumor control. Using 4-μm thick recut, unstained histological sections, normal and malignant tissue samples were removed under stereomicroscopic observation. Sufficient material was collected from a single histological section to afford replicate analysis. Samples were treated with proteinase K at a final concentration of 100 μg/ml for 2 h and then boiled for 5 min to remove protease activity. PCR used sets of amplification primers flanking exons 5–8 of the *p53* gene in four separate PCRs (17). Amplified DNA from microdissected tissues also included splice sites. PCR products were electrophoresed in 4% agarose, and the ethidium bromide-stained bands were excised and then isolated with glassmilk. DNA sequencing used antisense PCR primers for each exon with [^{33}P]dATP as the reporter molecule, and sequence analysis was read from overnight exposed autoradiograms of 6% polyacrylamide gels.

For p53 immunohistochemistry, formalin-fixed, paraffin-embedded tumor tissues were sectioned (3–5 μm), air-dried overnight at 37°C, deparaffinized, and dehydrated and stained with a mAb against p53, D0–7 (Dako, Carpinteria, CA), which recognizes an epitope in the NH_2 terminus between amino acids 35–45, and reacts with wild-type and most mutant forms of the p53 protein. The avidin-biotin-peroxidase method was used to visualize the p53, according to the instructions supplied by the manufacturer (Dako). The immunostained slides were evaluated by light microscopy for p53 accumulation. The tumor was considered p53-positive when >25% of the tumor cells showed staining intensity of 2+ and higher on the scale of 0–4+. IgG isotype mAb used at the same concentration as the primary mAb served as a negative control.

Ab to p53 in the patient and control sera were detected by an Enzyme ELISA purchased from PharmaCell Immunotech Coulter, Miami, FL, using microtiter plates coated with recombinant human wt p53 protein. Peroxidase-conjugated goat antihuman IgG was used for detection of human anti-p53 Ab by a colorimetric reaction. Staining intensity was compared with a standard curve, and anti-p53 levels ≥1.1 units/ml were considered to be positive. Assays were performed twice in triplicates and included sera obtained from seropositive as well as seronegative individuals as internal positive/negative controls.

PCR Analysis for HPV-16. PCR analysis was performed using amplification primers for HPV-16 E6/E7 (ATGCACCAAAAGAGAACTGC and TGCCCATTAACAGGTCTTCC) and β-actin (GCGAGAAGATGACCCAG and GCCTGGATAGCAACGTA) as control, using tumor DNA isolated as described above. DNA aliquots obtained from 25 of 30 specimens were screened in four separate PCR reactions. A solution (50 μl) consisting of 25 mM $MgCl_2$, 1.5 μM of each primer, 1.25U of Taq DNA-polymerase (Promega), 2 mM of deoxynucleotide triphosphate, and double-distilled H_2O was added to each amplification tube. Amplification was performed with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 2 min. The process was repeated for the total of 40 cycles. In all of the PCR reactions, DNA obtained from HPV-16 E6/E7+ Caski and C33 cell lines were included as positive controls. PCR products (700-bp for HPV-16 E6/E7 and 500-bp for β-actin) were electrophoresed in 4% agarose and the bands visualized in the presence of ethidium bromide. Twelve of 25 tumors tested were positive for HPV-16 E6/E7.

Statistical Analysis. Tetramer-positive cells were quantified by flow cytometry and expressed as frequencies (*e.g.*, 1/1000) or reciprocal frequencies (*e.g.*, 1000). We examined raw reciprocal frequency data and log-transformed reciprocal frequency data using normal probability plots (13). For all three tetramers, the log-transformed data were better modeled by the normal distribution. Accordingly, descriptive statistics (means, SDs, and confidence intervals) and statistical analyses (Student's *t* test, two-tailed), were performed on

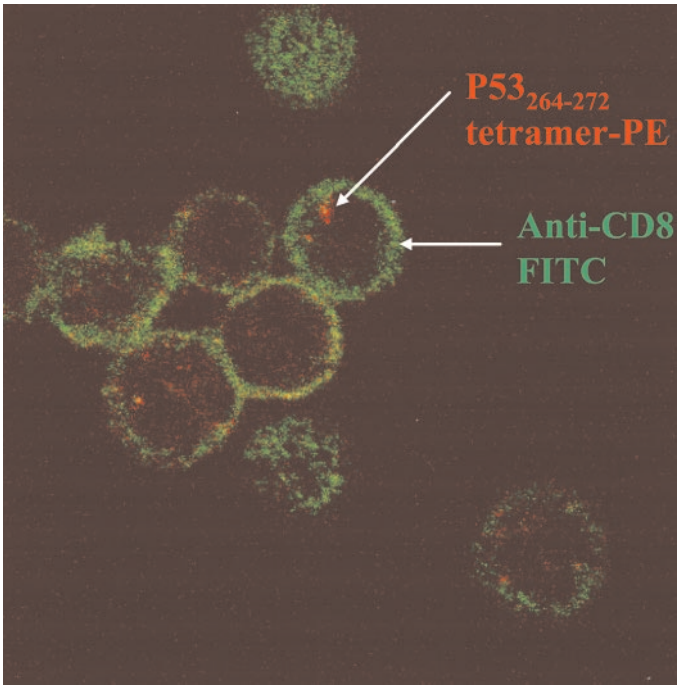

Fig. 1. Confocal microscopy demonstrating internalization of the tetrameric $p53_{264-272}$-MHC-class I complexes by Ag-specific CD8 T cells. A T-cell bulk line specific for the HLA-A2.1- restricted p53 epitope LLGRNSFEV was stained with PE-labeled tetrameric $p53_{264-272}$-MHC-class I complexes at room temperature for 30 min, washed, and incubated with anti-CD8 (FITC) for 30 min on ice. After fixation, the cells were examined in a confocal microscope (*midplane image*).

log-transformed reciprocal frequencies. For the comparison of multiple parameters, ANOVA (Tukey-Kramer multiple comparison test) was applied. The specificity of tetramer binding to the TCR was measured by competition of CD3 binding (13). Competition of CD3 binding was expressed according to the formula:

$$\% \text{ Competition} = \frac{[(\text{CD3Mnl}_{\text{CD3}^+\text{CD8}^+\text{tmer}^-}) - (\text{CD3Mnl}_{\text{CD3}^+\text{CD8}^+\text{tmer}^+})]}{(\text{CD3Mnl}_{\text{CD3}^+\text{CD8}^+\text{tmer}^-})} \times 100$$

where CD3Mnl is the log MFI of CD3 staining of the population designated by the subscript. For tetramer frequencies as well as competition of Ab binding, lower limits of detection were established by estimating the 99th percentile (geometric mean plus 2.6 SDs) of responses measured in PBMCs from HLA-A2$^-$ subjects. Linear regression was performed by the method of least squares. In regression plots the line of best fit and the 95% confidence intervals about the line of best fit are shown. The coefficient of correlation (r^2), the slope of the regression line, and the *P* associated with the slope are reported as regression summary statistics. Multivariate analysis was performed to determine the relationship between the frequency of p53-specific CTL, accumulation of p53 in the tumor, and the HPV status of the tumor. Statistical analysis and statistical graphics were performed using Systat Version 9 (SPSS Inc, Chicago, IL).

RESULTS

Binding of the Tetramer to wt $p53_{264-272}$-specific T Cells. A wt $p53_{264-272}$-specific CTL line established earlier (9) was stained with the FITC-conjugated anti-CD8 (green) and PE-conjugated tetramer (red). Its confocal microscopy mid-plane image is shown in Fig. 1. On binding to the TCR, tetrameric $p53_{264-272}$-MHC class I complexes were internalized in the absence of sodium azide. This $p53_{264-272}$-specific T-cell line served as positive control in subsequent flow cytometry analyses of precursor T cells in the peripheral circulation of human subjects.

Gating Strategy for Tetramer Analysis. For the detection of unstimulated precursor T cells specific for the wt $p53_{264-272}$ peptide, PBMCs of SCCHN patients and healthy donors were directly stained with the tetramer and analyzed by flow cytometry. To assure the specificity of tetramer binding, we developed previously a gating strategy to eliminate CD14$^+$ monocytes as well as apoptotic and necrotic cells, all of which could bind tetramer and/or Ab nonspecifically (13). Furthermore, CD3-negative cells were eliminated by compound gating, which finally resulted in discriminative dot plots showing CD8$^+$ tetramer$^+$ T cells. Fig. 2 shows the representative dot plots of stained PBMCs obtained from a healthy individual and from 2 patients with SCCHN. The cases shown are representative of relatively low (Fig. 2, *left*; 1 of 5757), intermediate (Fig. 2, *middle*; 1 of 3063) and high (Fig. 2, *right*; 1 of 1140) $p53_{264-272}$ tetramer binding frequencies, respectively.

Definition of the Lower Limit of Detection of Tetramer-positive T Cells. To establish the lower detection limit for tetramer binding in HLA-A2.1$^+$ individuals, we stained PBMCs obtained from 10 HLA-A2$^-$ individuals. Despite the application of the gating strategy described above, low levels of $p53_{264-272}$ tetramer$^+$ CD3$^+$ CD8$^+$ T cells were detected in PBMCs of HLA-A2$^-$ donors (geometric mean = 1/23,397). Because these events were nonspecific by definition, we established a cutoff for the lower detection limit of this assay at the upper 99th percentile of tetramer$^+$ CD8$^+$ T cells in HLA-A2$^-$ individuals. This cutoff frequency of 1 of 7,805 was applied to all of the data obtained from testing of the 10 HLA-A2$^-$ subjects, 30 HLA-A2.1$^+$ SCCHN patients, and 31 HLA-A2.1$^+$ healthy controls. As shown in Fig. 3, none of the HLA-A2$^-$ individuals had frequencies of $p53_{264-272}$ tetramer$^+$ CD8$^+$ T cells exceeding the established cutoff. On the other hand, 23 of 30 HLA-A2.1$^+$ SCCHN patients and 25 of 31 healthy controls had frequencies of $p53_{264-272}$ tetramer$^+$ CD8$^+$ T cells above the cutoff point (geometric means = 1/3,533 and 1/5,207, respectively).

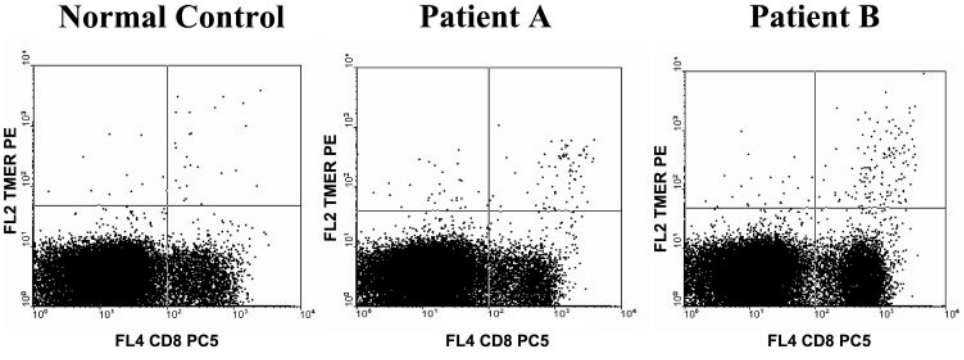

Fig. 2. Dot plots showing the frequency of wt $p53_{264-272}$-specific CD8$^+$ T cells in a normal control (*left*) and two SCCHN patients (*middle* and *right*). Compound gating strategy was used to identify tetramer$^+$ CD8$^+$ T cells. PBMC from an HLA-A2.1$^+$ individuals were stained with $p53_{264-272}$ tetramer followed by anti-CD14, anti-CD3, and anti-CD8 mAbs, using four-color flow cytometry. Tetramer-positive cells were evaluated within a Boolean gate, excluding CD14$^+$ events and including cells within an extended lymphoid light scatter gate and a generous CD3$^+$ gate. In the example shown, CD3$^+$ CD8$^+$ tetramer$^+$ events were present at a frequency of 1/5757 CD3$^+$ CD8$^+$ T cells for the healthy individual, and 1/3063 and 1/1140 for 2 patients with SCCHN.

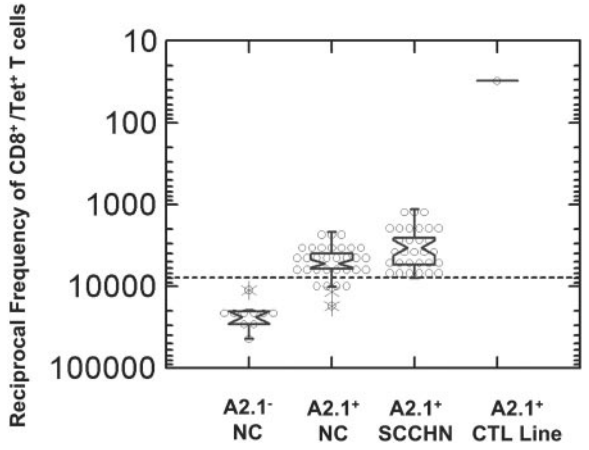

Fig. 3. Reciprocal frequencies of $p53_{264-272}$ tetramer$^+$ CD8$^+$ CD3$^+$ T cells. ○ show individual data points. *Superimposed notched box plots* display nonparametric descriptive statistics. The waist indicates the group median, the hinges (*upper and lower boundaries of the box*) indicate interquartile distances. The notches show simultaneous 95% confidence intervals about the median. The whiskers (*bars*) give the ranges, exclusive of outliers. Outliers (>1.5 times the hingespread from the median) are shown by ∗ and far outliers (more than three times the hingespread from the median) are shown by *double circles*. Reciprocal frequencies of $p53_{264-272}$ tetramer$^+$ CD8$^+$ T cells in 10 HLA-A2.1$^-$ and 30 HLA-A2.1$^+$ healthy donors, 30 HLA-A2.1$^+$ patients with SCCHN, and the HLA-A2.1$^+$ $p53_{264-272}$-specific T cell line. The *dashed line* represents the cutoff for the lower detection limit as determined in HLA-A2.1$^-$ donors (geometric mean +2.6); *bars*, ± SD.

Confirmation of the Specificity of Tetramer Binding to TCR. We have reported previously that tetramer-positive T cells stained dimmer for CD3 than did tetramer-negative T cells in PBMCs obtained from HLA-A2.1$^+$ subjects. However, this is not the case for the spurious tetramer-positive events seen in PBMCs obtained from HLA-A2$^-$ subjects (13). We demonstrated that this phenomenon results from a competition between tetramer and anti-CD3 Abs binding to TCR. This competition was subsequently quantified and introduced as a marker for the specificity of tetramer binding to the TCR complex (13). Because there was no detectable competition between tetramer and anti-CD3 Ab binding for CD8$^+$ T cells in 9 of 10 HLA-A2.1$^-$ individuals, we were able to define a cutoff based on the 99th percentile of CD3 competition in these HLA-A2$^-$ subjects (3.2%). Competition by anti-CD3 Ab in excess of this cutoff was considered to be significant. The mean of competition for the wt $p53_{264-272}$ tetramer was 10.0 ± 1.4% (mean ± SE) in T cells obtained from patients and 5.3 ± 1.5% in normal controls. PBMCs of 23 of 30 SCCHN patients but only 10 of 31 normal controls exceeded both the cutoff for competition as well as the cutoff for frequency (see above), and, thus, were considered to exhibit specific binding of the $p53_{264-272}$ tetramer (Fig. 4).

Comparison of Frequencies of wt $p53_{264-272}$-specific *versus* HIV- or FLU-specific T Cells. Next, frequencies of wt $p53_{264-272}$-specific CD8$^+$ T cells were compared with those obtained with the HIV tetramer or the FLU tetramer. These comparisons were performed to evaluate p53-specific responses in the context of those to known novel and recall antigenic peptides. The frequencies of FLU- and HIV-specific T cells are displayed in Fig. 5 as normal distribution curves. The frequencies of wt $p53_{264-272}$-specific T cells are shown as individual circles. For healthy individuals (Fig. 5, *left*), the majority of p53 frequencies fall within the HIV normal distribution curve. In contrast to PBMCs from the healthy control group, PBMCs from SCCHN patients seem to have a bimodal distribution, with a majority of the frequencies located within the normal HIV distribution curve, and six frequencies shifted to the right (Fig. 5, note the log scale) toward FLU frequencies.

Frequencies of wt $p53_{264-272}$-specific T Cells in Patients and Controls. On more careful examination, when the frequencies of wt $p53_{264-272}$ peptide-specific CD8$^+$ T cells in the circulation of patients (see Table 1) were compared with those obtained in normal controls, it appeared that the patients could be divided into two different groups. The first subset consists of patients with wt p53 tumors that do not accumulate p53 or those with tumors unlikely to present the wt $p53_{264-272}$ epitope because of the type of mutation they harbor. These patients have significantly higher frequencies ($P \leq 0.0005$) of wt $p53_{264-272}$-specific CD8$^+$ T cells in the peripheral circulation than the patients in the second subset, whose tumors accumulate p53 and, in theory, could have a higher potential for presentation of this epitope (Fig. 6). The SCCHN patients in the second subset, those with tumors accumulating p53, have lower frequencies of CD8$^+$ T cells specific for this p53 epitope, which do not significantly differ from those obtained for normal controls (Fig. 6). Confirming our initial observations, this result suggests that accumulation of p53 in the tumor does not positively correlate with the frequency of the epitope-specific CD8$^+$ T cells detectable in the circulation of patients with SCCHN.

Analysis of wt $p53_{264-272}$-specific Memory *versus* Naive T Cells. Expression of CD45 isoforms on the surface of T cells is routinely used to discriminate between naive (CD45RA$^+$) and memory (CD45RO$^+$) T-cell subsets (18). To determine whether tetramer$^+$ CD8$^+$ T cells detected in the circulation of patients with SCCHN belong to the memory or naive T-cell subsets, multicolor flow analysis including anti-CD45 Abs was performed. The gates for CD45RO and CD45RA expression were set on CD8$^+$ tetramer$^-$ T cells, as shown in the left panel of Fig. 7. We have shown previously that the majority of CD8$^+$ tetramer$^+$ cells for the recall antigen, FLU, were CD45RO$^+$/CD45RA$^-$ memory T cells, whereas those recognizing the novel HIV antigen were predominantly CD45RO$^-$/CD45RA$^+$ naive T cells (13). This analysis was performed on samples obtained from two groups of representative patients: one with relatively high frequencies (average ~1/2700), the other with low frequencies (average ~1/5500) of wt $p53_{264-272}$-specific CD8$^+$ T cells as well as from normal donors (see Table 2). Normal donors (Fig. 7, *middle panel*) and patients with low frequencies of $p53_{264-272}$ tetramer$^+$ T cells had similar percentages of memory (~10%) and naive (~70%) T cells in the peripheral circulation. In contrast, a significantly higher percentage of memory cells (36.5%) was found in patients with high frequencies of wt $p53_{264-272}$-specific T cells (Fig. 7, *right panel*).

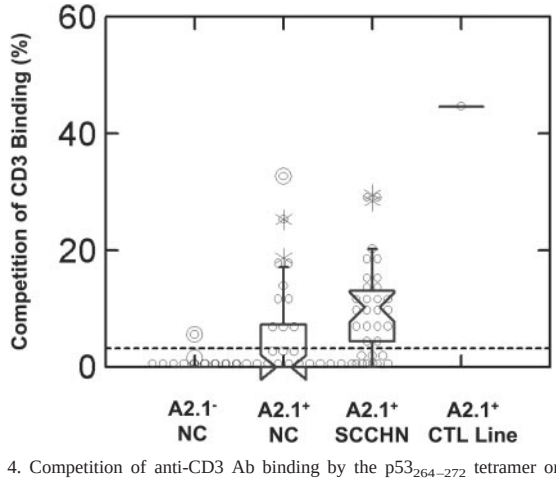

Fig. 4. Competition of anti-CD3 Ab binding by the $p53_{264-272}$ tetramer on CD8$^+$ CD3$^+$ T cells. *Notched box plots* show individual data points and nonparametric statistics, as described in Fig. 3. Competition in percentage is shown for $p53_{264-272}$ tetramer$^+$ CD8$^+$ T cells obtained from 10 HLA-A2.1$^-$ and 30 HLA-A2.1$^+$ healthy donors, 30 HLA-A2.1$^+$ patients with SCCHN, and the HLA-A2.1$^+$ $p53_{264-272}$-specific T cell line. The *dashed line* represents the cutoff for the lower limit of competition as determined in HLA-A2.1$^-$ normal controls; *bars*, ± SD.

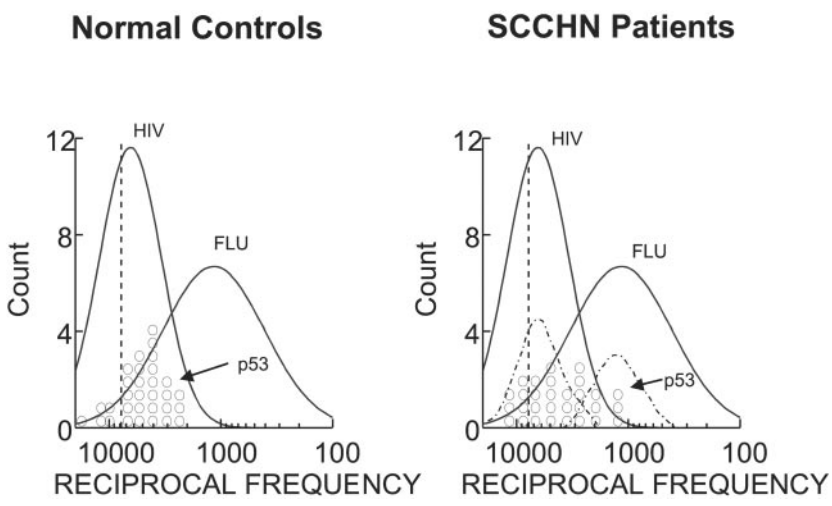

Fig. 5. Reciprocal frequency of $p53_{264-272}$ tetramer$^+$ CD8$^+$ CD3$^+$ T cells in the peripheral circulation of patients with SCCHN. ○ show individual data points. Superimposed normal distribution curves were calculated from sample means, and SDs of HIV and FLU tetramer data obtained in the normal control group. The *dashed line* represents the cutoff for the lower detection limit of tetramer frequency.

Immunohistochemistry and Genomic PCR Analysis of p53 in Patient Tumors. Immunohistochemistry of p53 protein and sequencing of genomic PCR products of p53 exons 5–8 in patient tumors were done to evaluate the potential of these tumors to present the wt $p53_{264-272}$ epitope, and ultimately relate this information to the frequencies of tetramer$^+$ CD8$^+$ T cells detected in the peripheral circulation of these patients (Table 1). Although exceptions have been noted in the literature, most tumor cells lines sensitive to CTL recognizing this epitope have been shown to accumulate p53. Of the 30 primary tumors analyzed, more then half (17 of 30) showed accumulation of p53. The primary tumor of one patient, #28, scored negative, but a lymph node metastasis was positive. From this total of 31 tumors (18 of 31 with p53 accumulation), 28 underwent sequencing of genomic PCR products of exons 5–8 (3 cases were not available). Of the 16 available tumors (2 of 18 not available) showing p53 accumulation, 13 were found to express missense mutations within p53 exons 5–8. In two tumors (patients #7 and #10), p53 missense mutations were located within or directly next to the wt $p53_{264-272}$ peptide sequence. Such a mutation (R273H) was shown previously to prevent presentation of the epitope (19). For subsequent analysis, these tumors were considered as unlikely to be presenting the wt $p53_{264-272}$ epitope, and they are designated by brackets for p53 accumulation in Table 1.

A lack of agreement between the mutated p53 exon 5–8 genotype and p53 accumulation was reported for patients with SCCHN (20). In general, the results of our study indicated a good correlation between

Table 1 *Summary of wt $p53_{264-272}$-specific T-cell frequencies in patients with SCCHN and the p53 status in their tumors, as well as the presence of p53 autoantibodies*[a]

Patient	Tumor site	Tumor-Node-Metastasis status	Frequencies of wt $p53_{264-272}$-specific T cells among CD8$^+$ T cells	Tumor p53 genotype	Tumor p53 protein accumulation	Serum p53 autoantibodies	HPV16 status
1	Larynx	T2N0M0	1/1140	Wild type	−	−	+
2	Oral cavity	T3N2M0	1/1264	Wild type	−	−	+
3	Larynx	T1N0M0	1/1450	Wild type	−	−	+
4	Oral cavity	T1N0M0	1/1798	Wild type	−	−	−
5	Larynx	T2N0M0	1/1992	Wild type	−	−	−
6	Larynx	T2N0M0	1/2099	Wild type	−	−	ND
7	Oral cavity	T2N0M0	1/2566	E8 R273H	+[b]	−	ND
8	Larynx	T4N2M0	1/2707	E6 213 Stop	−	−	−
9	Oropharynx	T1N2M0	1/2746	Wild type	−	+	+
10	Hypopharynx	T4N0M0	1/3063	E8 E271K	+[b]	+	−
11	Larynx	T4N0M0	1/3126	Wild type	−	−	+
12	Larynx	T2N0M0	1/3151	E5 V157F	+	−	ND
13	Larynx	T4N0M0	1/3237	n.a.	+	−	−
14	Lip	T1N0M0	1/4527	E7 R248W	+	−	+
15	Nose	TxN0M0	1/4782	E6 Y220C	+	−	−
16	Larynx	T4N1M0	1/4933	Wild type	−	−	+
17	Oral cavity	T1N0M0	1/4958	n.a.	+	−	−
18	Larynx	T4N0M0	1/5025	E 6 deletion	−	−	+
19	Oropharynx	T1N0M0	1/6219	Wild type	+	−	−
20	Larynx	T3N0M0	1/6333	Wild type	+	−	−
21	Larynx	T1N0M0	1/6916	E6 G226R	+	−	−
22	Oral cavity	T2N0M0	1/7097	E6 Y220C	+	−	−
23	Oropharynx	T4N1M0	1/7236	E8 E286K	+	−	−
24	Hypopharynx	T4N2M0	1/7805[c]	E5 S149C	+	+	+
25	Oral cavity	T2N0M0	<1/7805[c]	n.a.	−	−	−
26	Larynx	T2N0M0	<1/7805[c]	E5 H168Y	+	−	−
27	Larynx	T1N0M0	<1/7805[c]	Wild type	+	−	+
28	Oropharynx	T2	<1/7805[c]	Wild type	−	−	+
		N3 M0		E7 G245V	+		
29	Larynx	T1N0M0	<1/7805[c]	E5 T150R	+	−	+
30	Larynx	T3N2M0	<1/7805[c]	E8 C278T	+	−	ND

[a] The table is arranged in descending order according to detected frequencies of $p53_{264-272}$-specific CD8$^+$ T cells; n.a., not available; Breaks are cutpoints as explained in the legend to Figure 8; ND, not done.

[b] The R 273 H mutation has been shown to prevent presentation of $p53_{264-272}$ epitope (19). The E271K mutation occurs within this epitope.

[c] These values were below the limit of detection or did not meet criteria of specific tetramer binding because competition with CD3 was lower than the cutoff.

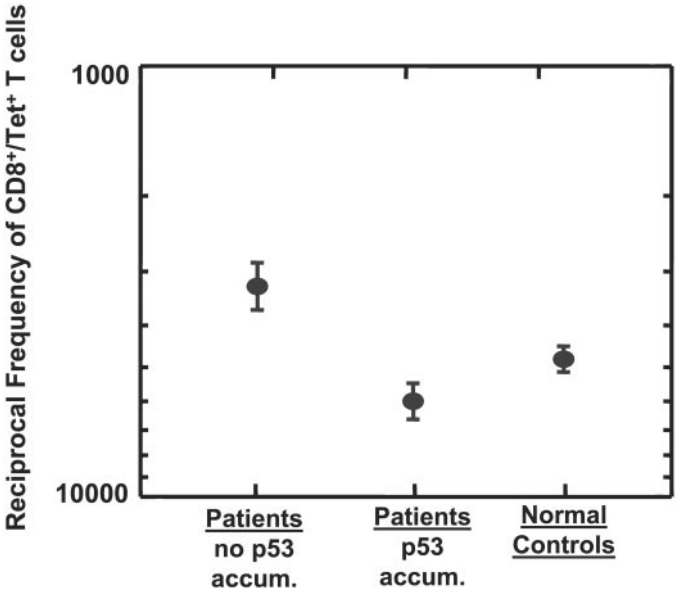

Fig. 6. Reciprocal frequency of $p53_{264-272}$-specific $CD8^+$ T cells in patients with SCCHN and normal controls. The mean frequencies for normal controls and for patients with SCCHN with tumors found to accumulate p53 or not to accumulate p53 (see Table 1) were determined. Paired tumors and PBMC samples of 30 HLA-A2.1$^+$ patients with SCCHN were evaluated. Tumors showed either normal p53 protein expression, accumulated p53 protein, or had a mutation within or next to the $p53_{264-272}$ epitope, most likely preventing presentation of the epitope (18). Such tumors were considered to have normal p53 expression; *bars*, ± SD.

p53 missense mutations and accumulation of the altered protein in tumors (Table 1). In 3 cases (patients #19, #20, and #27) a discrepancy between the p53 genotype and p53 expression was observed (Table 1). All three represented tumors with a wt p53 genotype and with p53 accumulation, which could possibly be because of a mutation outside the exons 5–8 or in genes impacting on the stability of p53, such as mdm2 (21).

Association between p53 in Tumors and Frequency of wt $p53_{264-272}$-specific T Cells. Stratification of the frequencies of wt $p53_{264-272}$-specific T cells detected in the circulation of patients with SCCHN identified three groups of patients, those displaying high, intermediate, or low frequencies of these cells (Table 1). The cutoff for the high frequency designation was based on the upper 95th percentile (>1/2128) frequency of tetramer$^+$CD8$^+$ T cells established in HLA-A2$^+$ healthy controls. The tumors obtained from the 6 patients with the highest frequencies of these T cells were wt p53 and did not accumulate p53 (Table 1; Fig. 8).

In the second patient group, the intermediate reciprocal frequencies of wt $p53_{264-272}$-specific $CD8^+$ T cells were lower than 1/2128, yet they exceeded the geometric mean of the patient group as a whole (1/4767). This group of 7 patients was heterogeneous in respect to p53 expression in the tumor: 3 tumors were p53 wt, 2 had p53 mutations and accumulation, and 2 tumors (#7 and #10) expressed missense mutations within or next to the $p53_{264-272}$ epitope (positions 273 and 271, respectively) and were probably unable to present this epitope to T cells (Fig. 8).

The third and largest group of the patients ($n = 17$) had the lowest frequencies of wt $p53_{264-272}$-specific T cells. In this group, 14 of 17 primary tumors (>80%) accumulated p53 and had the potential to present this epitope. The frequencies of wt $p53_{264-272}$-specific T cells exceeded the lower limit of detection (1/7805) in 7 patients. The mean frequency for the group was significantly lower than that for the other two groups of patients. As indicated in Table 2, wt $p53_{264-272}$-specific $CD8^+$ T cells present in low frequencies in the peripheral circulation of these patients predominantly expressed a naive phenotype ($CD45RA^+/CD45RO^-$). On the other hand, in patients with high frequencies of wt $p53_{264-272}$ epitope-specific T cells, memory T cells ($CD45RA^-/CD45RO^+$) significantly increased in proportion (Fig. 7). Therefore, it would appear that in the peripheral circulation of patients whose tumors have a low potential for presenting the epitope, the frequency of wt $p53_{264-272}$-specific T cells with a memory phenotype is high. Therefore, it is likely that these T cells had a previous interaction with targets capable of presenting the $p53_{264-272}$ epitope.

HPV-16 Positivity and Frequency of wt $p53_{264-272}$-specific T Cells. PCR analysis indicated that 12 of 26 (46%) tumors we examined contained HPV-16 E6/E7 DNA (Table 1). Among 12 tumors with p53 mutations, 4 (33%) were E6/E7$^+$, whereas 8 of 12 (66%) wt p53 tumors were E6/E7$^+$. One tumor (#28) was heterogeneous, with cells expressing either wt or mutated p53, and it was also E6/E7$^+$. The p53 genotype was not available for 3 tumors analyzed for HPV. We found that 3 of 5 patients with the highest frequencies of wt $p53_{264-272}$-specific T cells and wt p53 in the tumor were HPV-16$^+$. The patient group with the intermediate frequencies (Fig. 8), contained 7 patients of whom 2 could not be tested for HPV and 1 was not genotyped for p53. Of the remaining 4, 2 were wt p53 and HPV$^+$, whereas the other 2 had mutated p53 and were HPV negative. The cohort of 17 patients with low CTL frequencies contained 13 informative cases (tumor #28 was excluded from analysis), with 4 of 9 mutated p53 tumors and 2 of 4 wt p53 tumors positive for HPV-16. By fitting loglinear models to the frequencies of each variable in the $3 \times 2 \times 2$ contingency table, we determined that the frequency of $p53_{264-272}$-specific CTL was significantly correlated with the p53 status of the tumor ($P = 0.016$). In contrast, both the frequency of CTL and p53 status of the tumor were found to be independent of HPV E6/E7 positivity ($P = 0.9260$ and $P = 0.2924$, respectively).

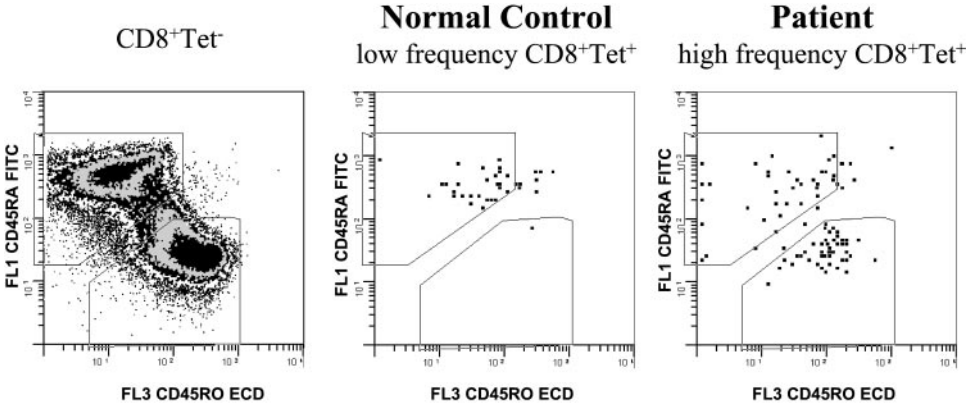

Fig. 7. Representative data (patient #1 in Table 1) for CD45 isoform expression on $p53_{264-272}$-specific $CD8^+$ T cells. Cells were stained with the tetramer followed by CD45RO-FITC, CD8-ECD, and CD45RA-PC5. Monocytes and high side scatter natural killer cells were eliminated from the analysis by using a tight lymphocyte light scatter gate. In normal controls as well as patients with low frequencies of $p53_{264-272}$-specific T cells (not shown), these cells were predominantly $CD45RO^-/CD45RA^+$. In SCCHN patients with high frequencies of $p53_{264-272}$-specific T cells (*right panel*), a significant number of $CD8^+$ tetramer$^+$ cells were $CD45RO^+/CD45RA^-$.

Table 2 *Frequencies of wt $p53_{264-272}$-specific memory and naive T cells in healthy controls and patients with SCCHN*

Peptide	$CD45RA^+/CD45RO^-$ (naive)	$CD45RA^-/CD45RO^+$ (memory)	Transitional cells
	% of $CD8^+$ $tetramer^+$ cells		
Healthy controls			
HIV (novel; $n = 5$)	71.9 ± 9.4	16.6 ± 5.7	11.5 ± 3.9
FLU (recall; $n = 5$)	11.2 ± 1.3	76.7 ± 2.7	12.1 ± 2.3
wt $p53_{264-272}$ ($n = 4$)	74.7 ± 8.4	7.5 ± 5.6	17.8 ± 4.3
SCCHN patients			
wt $p53_{264-272}$ ($n = 3$)[a]	50.3 ± 8.5	36.5 ± 12.8	13.2 ± 4.3
wt $p53_{264-272}$ ($n = 3$)[b]	72.7 ± 2.5	10.0 ± 6.6	17.3 ± 6.3

[a] High frequencies of wt $p53_{264-272}$-specific T cells (~1/2700).
[b] Low frequencies of wt $p53_{264-272}$-specific T cells (~1/5500).

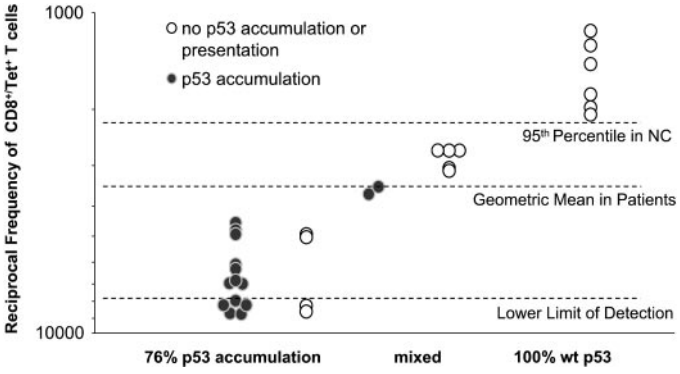

Fig. 8. The summary of associations between the frequency of $p53_{264-272}$-specific $CD8^+$ T cells in patients with SCCHN and p53 accumulation in the patient tumors. Paired PBMCs and primary tumors from 30 HLA-A2.1$^+$ patients with SCCHN were evaluated. The *bottom dashed line* represents the cutoff for the lower detection limit of tetramer frequency, the *intermediate line* shows the geometric mean of the patient group as a whole, and the *top dashed line* indicates the upper 95th percentile (>1/2128) of $tetramer^+CD8^+$ T cells in 30 HLA-A2.1$^+$ normal controls. According to the T-cell frequency and p53 tumor status, 3 groups of patients were identified. The evaluated tumors either showed normal p53 protein expression, had mutations within or next to the $p53_{264-272}$ epitope, shown previously to prevent epitope presentations (18), or displayed p53 protein accumulation.

Association of p53 Antibodies and Frequency of wt $p53_{264-272}$-specific T Cells. An analysis of IgG antibodies to p53 in the sera of SCCHN patients by Bourhis *et al.* (22) identified nearly 20% as seropositive. Because the presence of IgG antibodies to p53 implies a T-cell mediated response, it was of interest to determine whether the frequencies of wt $p53_{264-272}$-specific T cells present in the peripheral circulation of our seropositive patients were higher than the mean for all of the SCCHN patients. As indicated in Table 1, 3 of the 30 patients (#9, #10, and #24) scored p53 seropositive. The mean frequency (1/4538) of wt $p53_{264-272}$-specific T cells in these 3 patients was not significantly higher than the geometric mean frequency for all of the SCCHN patients (1/4767). In 2 of these patients the tumor accumulated mutant p53, whereas in the third (#9), it did not. Interestingly, the tumor of this patient contained ~2% of cells positive for p53 (Fig. 9), and it was HPV-16 E6/E7 positive. The presence of p53 autoantibodies in the serum, which is usually associated with p53 accumulation (22), and the relatively high frequency of wt $p53_{264-272}$-specific T cells detected in this patient's circulation (1/2746), suggest that the virus-related enhanced processing of p53 might contribute to effective CTL generation *in vivo*.

DISCUSSION

SCCHN, which arise at or in close proximity to mucosal surfaces, interact closely with the host immune cells during tumor initiation, promotion, and progression. As a result of this interaction, tumor cells, which are recognized by immune effector cells, can be eliminated, whereas tumor cells able to evade immune recognition can grow and become resistant to the host immune cells. Tumors can evade immunodetection by a general down-regulation or loss of antigen-presenting molecules, or, more specifically, the loss of immunogenic epitopes (23–25). An outgrowth of epitope-loss tumor variants, which are resistant to immune effector cells, gives the tumor a "competitive edge" for growth in a hostile environment. Another general mechanism of tumor evasion may involve tumor-associated factors, which can cause dysfunction or even death of immune effector cells (26).

It has been generally assumed that p53 accumulation provides an opportunity for presentation to T cells of immunogenic wt p53 epitopes (7, 27) and generation of wt p53 epitope-specific T cells in tumor-bearing hosts. The expected result would be the presence of relatively high frequencies of wt p53 epitope-specific T cells in the circulation of patients with tumors accumulating p53. However, contrary to expectations, the results of our earlier study indicated that CTL could be generated only from PBMCs of the patients whose tumors either did not accumulate p53 or accumulated, but could not present, the $p53_{264-272}$ epitope (9). To confirm these unexpected results, we recruited a larger group of HLA-A2.1$^+$ patients with SCCHN and using tetramer technology, determined frequencies and phenotypic characteristics of T cells specific for the wt $p53_{264-272}$ peptide in the peripheral circulation of these patients and a group of healthy controls.

We found the highest frequencies of wt $p53_{264-272}$-specific $CD8^+$ T cells in a subset of patients with SCCHN whose tumors did not accumulate p53 protein and had a wt p53 genotype. Furthermore, in a subset of patients with tumors accumulating mutant p53, the mean frequency of $p53_{264-272}$-specific T cells did not differ significantly from that in healthy controls. In principle, p53 accumulating in the tumor has an increased opportunity to be presented to immune cells (28). However, it is known that some tumors with mutated p53 are unable to process the wt $p53_{264-272}$ epitope. The precedent for blocking of the epitope processing by tumor with a missense mutation at the hotspot codon 273, flanking wt $p53_{264-272}$ epitope, has been described by Theobald *et al.* (19). It is possible that additional instances of blocked, altered, or incomplete processing of this as well as other p53 epitopes exist. Another plausible explanation for the observed low

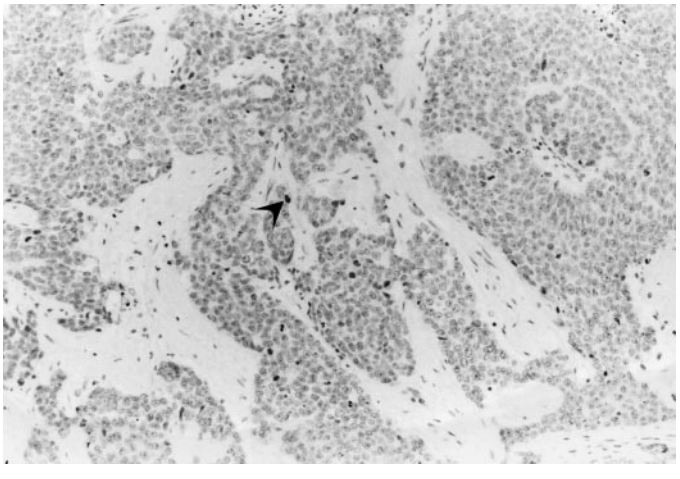

Fig. 9. Immunostaining for p53 on a section of SCCHN in patient #9 (see Table 1). The tumor was HPV-positive. The *arrow* indicates a tumor cell nucleus stained for p53. Original magnification, ×250. Note that <2% of tumor cells were p53-positive. The features characterizing the immune response of this patient to the tumor are as follows: Antibodies to p53 present in serum. Frequency of the $wtp53_{264-272}$ epitope-specific $CD8^+$ T cells in the peripheral circulation = 1/2746. HPV-16 E6/E7+

frequency of the epitope-specific T cells in patients with mutated p53 could be that recognition of tumors by CTL depends not on p53 accumulation alone but on its turnover and processing by malignant cells, as recently reported by Vierboom *et al.* (29). Thus, it is possible that processing of mutated p53 by the tumor cell proteasome may not lead to optimal presentation of the wt $p53_{264-272}$ epitope and effective generation of specific CTL. On the other hand, it has been shown that even when no accumulation of p53 is evident, wt p53 epitope presentation can occur, rendering the tumor susceptible to wt p53-specific CTL (8, 29–31). Thus, accumulation of mutated p53 is not the only criterion associated with the presentation of wt p53 epitope by the tumor and generation of CTL with p53 specificity.

The presence of HPV E6 in tumor cells could also influence p53 processing and CTL generation (29). HPV-16 E6/E7 expression has been reported in a substantial proportion of oral SCCHN (32). Expression of wt p53 in HPV E6-transformed cells is compatible with p53 inactivation, its proteolytic degradation, and enhanced processing, as well as presentation of its epitopes to T cells (29). For this reason, we examined the tumors studied for HPV-16 E6/E7 expression, and sought to correlate it with p53 expression and the frequency of CTL specific for wt $p53_{264-272}$ epitope. Multivariate analysis indicated that the frequency of wt p53-specific CTL depended on the p53 status of the tumor and not on its positivity for HPV. Nevertheless, it is interesting to note that in 5 of 7 patients with a relatively high frequency of wt p53-specific CTL, who did not accumulate p53, HPV-16 DNA was detected.

Another explanation for the observed low frequency of wt $p53_{264-272}$-specific T cells in patients with tumors accumulating p53 is that wt p53 epitopes are "self" determinants, and, thus, tolerance to them has to be overcome to induce an immune response. Studies by Theobald *et al.* (33) and Hernández *et al.* (34) demonstrated that tolerance to "self" p53 epitopes in mice is associated with deletion of high avidity T cells and retention of low to intermediate affinity T cells. We have consistently generated comparable anti-wt p53 CTL in humans following *in vitro* sensitization in the presence of epitope-pulsed dendritic cells (8, 9). Others have also reported generation of such CTL (35, 36). Furthermore, the current study shows that precursors of tetramer-positive anti-$p53_{264-272}$ T cells exist, albeit with low frequencies, in PBMCs of patients with tumors accumulating p53. Therefore, it is unlikely that CTL precursors of wt $p53_{264-272}$-specific T cells are deleted in cancer patients, as suggested previously. The mechanisms responsible for the failure of these precursor cells to expand *ex vivo* are presently unknown. It is possible that a certain threshold frequency of these precursor cells is needed to overcome anergy to self or immunosuppressive effects of the tumor microenvironment.

This study emphasizes the complexity of tumor-host interactions relevant to anti-wt p53 responses and to the development of wt p53-based vaccines. Our findings suggest that immune precursor cells of the wt $p53_{264-272}$ epitope are present in the circulation of HLA-$A2^+$ patients with SCCHN and that in a subset of these patients, this epitope is immunogenic, results in CTL development, and contributes to shaping immunological memory. In this subset of SCCHN patients, CTL specific for wt $p53_{264-272}$ epitope might well have been responsible for elimination of tumors presenting the epitope and the outgrowth of epitope-loss tumor cells able to avoid these effector cells. On the other hand, it is also possible that tumors accumulating mutant p53, which are associated with poor prognosis (37), actively participate in elimination of tumor-specific effector cells, as suggested by studies reported from our laboratories.[4] Tumor-associated apoptosis of such epitope-specific T cells might account for low frequencies of tetramer-positive $CD8^+$ T cells in patients with tumors accumulating p53.

A complex interplay of factors, which might determine tumor survival or regression, is best illustrated in patient #9 (Table 1; Fig. 9). A relatively high frequency of the epitope-specific T cells in the circulation, the presence of anti-p53 Abs, accumulation of p53 in a small proportion (~2%) of tumor cells, and tumor positivity for HPV in this patient, suggest that the patient's immune system is actively modulating tumor growth. Delivery of wt p53-based vaccines to patients such as this one could result in a rapid expansion of CTL, which might drive the selection of epitope-loss tumor variants. On the other hand, patients with tumors harboring p53 mutations and a low frequency of wt p53-specific CTL are unlikely to benefit from wt p53-based vaccines, because the expected postvaccine expansion of CTL is unlikely. Strategies that might help to overcome these difficulties include the use of an altered peptide ligand of the $p53_{264-272}$ epitope (38), identification of other wt p53 epitopes, which might be more immunogenic than $p53_{264-272}$, as well as MHC-class II restricted p53 epitopes to provide help for generation of antitumor effector cells. We and others are in the process of evaluating several other known HLA-A2.1-restricted wt p53 epitopes in hope of identifying those that may be able to support generation of high-affinity CTL (39). In planning for future p53 vaccines, the use of individualized or personalized vaccines targeting mutant p53 also needs to be revisited, particularly in light of newer evidence that a considerable number of p53 mutations occur within known CTL-defined epitopes in HLA-$A2^+$ SCCHN patients. However, the efficiency of such vaccines depends on the demonstration that a given mutated p53 epitope can be processed and is immunogenic. Therefore, the selection of an optimal wt p53 peptide for vaccination must await additional studies to define characteristics of other available p53 epitopes.

[4] T. K. Hoffmann, G. Dworacki, T. Tsukihiro, N. Meidenbauer, W. Gooding, J. T. Johnson, and T. L. Whiteside. Spontaneous apoptosis of circulating T lymphocytes in patients with head and neck cancer and its clinical importance, submitted for publication.

ACKNOWLEDGMENTS

We thank Dr. Saleem Khan, University of Pittsburgh School of Medicine, for performing HPV-16 E6/E7 analysis and William Gooding, Biostatistics Center, University of Pittsburgh Cancer Institute, for providing statistical support.

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