Univerzita Karlova

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IMUNOTERAPIE NÁDORŮ VYVOLANÝCH VIRY HPV16 A SV40.

Doktorská disertační práce v oboru molekulární a buněčná biologie, genetika a virologie

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Pavel Otáhal

OBSAH

Seznam zkratek použitých v textu

Zkratka	*Anglický termín*	*Český ekvivalent*
APC	antigen-presenting cell	buňka prezentující antigen
CIN	cervical intraepitelial neoplasia	cervikální intraepiteliální neoplázie
CD	cluster of differentiation	diferenciační antigen
CTL	cytotoxic T lymphocyte	cytotoxický T lymfocyt
DC	dendritic cell	dendritická buňka
EBV	Epstein-Barr virus	virus Epsteina a Barrové
ER	endoplazmatic reticulum	endoplazmatické retikulum
HA	hemaglutinin	hemaglutinin
HCV	hepatitis C virus	virus hepatitidy C
HPV	human papilloma virus	lidský papilomavirus
HSV	herpes simplex virus	herpes simplex virus
IFN	interferon	interferon
IL	interleukin	interleukin
LCMV	lymhocytic choriomeningitis virus	virus lymfocytární choriomeningitidy
MHC	major histocompatibility complex	hlavní histokompatibilní komplex
MVA	modified vaccinia virus	modifikovaný virus vakcínie
PAMP	pathogen associated molecular pattern	molekulární znaky specifické pro patogeny
PRR	pathogen recognition receptor	receptor rozpoznávající patogeny
RIP	rat insulin promoter	krysí inzulínový promotor
rVV	recombinant vaccinia virus	rekombinantní virus vakcínie
SV40	simian virus 40	opičí virus 40
T Ag	large tumor antigen	velký nádorový antigen
TAA	tumor associated antigen	antigen asociovaný s nádory
TAP	transporter-associated protein	proteinový transportér
TCR	T-cell receptor	receptor T lymfocytů
TGF	transforming growth factor	transformující růstový faktor
TLR	toll-like receptor	toll-like receptor
TNF	tumor necrosis factor alpha	faktor nekrotizující nádory
Treg	T-regulatory lymphocytes	regulační T lymfocyty
TSA	tumor-specific antigen	nádorově-specifický antigen
VLP	virus –like particles	partikule podobné virům

1. Úvod

1.1 Nádorová imunoterapie

Aplikace poznatků imunologie na prevenci, léčbu a diagnostiku nádorovývch onemocnění je výsledkem řady fundamentálních objevů učiněných před relativně nedávnou dobou. Od identifikace prvního skutečného nádorového antigenu přítomného na lidských nádorových buňkách Boonem a spolupracovníky na počátku 90. let [van der Bruggen et al., 1991] došlo k explozívnímu vývoji celého oboru. Všeobecně akceptované dogma předpokládá, že imunitní reakce navozená specificky na tyto nádorové antigeny bude schopná zničit vyvinuté nádory, bohužel dekáda klinických pokusů ukazuje, že současné vakcinační postupy jsou neúčinné pro navození dostatečně silně protinádorové imunitní odpovědi [Rosenberg, 2001]. Současné poznatky naznačují, že zásadní překážkou v indukci léčebné protinádorové imunity je rozvoj imunologické tolerance na nádorové buňky [Staveley-O'Carroll et al., 1998; Sotomayor et al., 2001]. Studium nádorové imunologie má v současnosti význam nejen pro klinickou terapii nádorů, ale zásadním způsobem přispívá k definování základních imunologických principů jako je například mechanismus rozlišení mezi vlastními a cizími antigeny, nebo mechanismy suprese imunitní opovědi.

Primárním mechanismem protinádorové imunity je zabíjení nádorových buněk prostřednictvím CD8+ cytotoxických lymfocytů, které rozpoznávají nádorové antigeny prezentované ve formě krátkých peptidů navázaných na molekuly MHC I. třídy na povrchu buněk. Nádorové antigeny mohou být produkty normálních buněčných genů, které jsou exprimované v abnormálně zvýšeném množství v nádorových buňkách, ale jsou zároveň fyziologicky exprimované v některých typech buněk. Označují se jako tumor-asociované antigeny (TAA) a patří mezi ně například antigeny z rodiny carcinoembryonálních proteinů (MAGE-3, CEA, P1A a další) nebo diferenciačních antigenů (tyrosináza, Her-2/neu, PSA a další) [Boon et al., 1994; Boon and van der Bruggen, 1996; Wang and Rosenberg, 1996]. Nádorové antigeny mohou být dále produkty mutovaných buněčných genů, které vznikly v průběhu onkogení transformace. Tyto antigeny jsou exprimované pouze v nádorové tkáni a

označují se jako tumor-specifické antigeny (TSA), velmi často se jedná o mutace v genech regulujících buněčný cyklus (například p53, ras, myc, bcr-abl) [Boon et al., 1994; Boon and van der Bruggen, 1996; Wang and Rosenberg, 1996]. V posledním případě mohou být nádorové antigeny produkty genů onkogeních virů které transformovaly somatické buňky [Rapp and Westmoreland, 1976]. Tuto schopnost mají některé malé DNA viry, mezi které patří zejména klinicky významný patogen HPV a některé RNA viry jako je HTLV-1. Dále existuje mnoho dalších onkogenních virů způsobujících nádory na zvířatech, ale nepatogenních pro člověka (např. SV40).

Růst nádorů je závislý na proliferační kapacitě nádorových buněk a na schopnosti těchto buněk invazívního růstu a tvorby metastáz. Nekontrolovaný růst nádorových buněk je esenciální avšak ne jediná podmínka pro vznik makroskopických nádorů. Hypotéza nádorového imunitního dohledu (tumor imunosurveilance) navržená v 50-tých letech Mcfarlane Burnetem tvrdí, že imunitní systém je schopen rozpoznat klony maligních buněk a zničit je před tím, než vytvoří nádor. Tato hypotéza je intenzívně diskutována v současnosti a nejnovější experimentální výsledky zpochybňují schopnost imunitního systému zabránit rozvoji běžných nádorů [Willimsky and Blankenstein, 2005]. Při imunodeficitu buněčné imunity vznikají nádory způsobené zejména onkogeními viry, nedochází však k významně vyšímu rozvoji běžných spontánních nádorů, zvýšená incidence nádorů je tedy připisována poruchám protivirové obrany než neschopnosti organismu zabránit vzniku nádorů . Řada studií u pacientů s imunodeficity buněčné imunity prokázala, že například u pacientů s AIDS dochází k abnormálně zvýšenému rozvoji typických nádorů indukovaných onkogeními viry jako jsou Kaposiho sarkom způsobený lidským herpesvirem typ 8, hepatocelulární karcinom vyvolaný infekcí HCV, lymfomy způsobené EBV a orální či genitální karcinomy vyvolané infekcí HPV [Boshoff and Weiss, 2002]. Tyto údaje jsou podpořeny některými experimentálními pracemi a společně naznačují, že v důsledku rozvoje imunologické tolerance imunitní systém není schopen zabránit vzniku a růstu spontánních nádorů vznikajících v důsledku genetických mutací [Ochsenbein et al., 1999].

Současná léčba nádorů cytostatiky má mnoho vedlejších účinků, protože je toxická pro všechny dělící se buňky – nádorové i nenádorové. Ideální by byla nepochybně taková terapie,

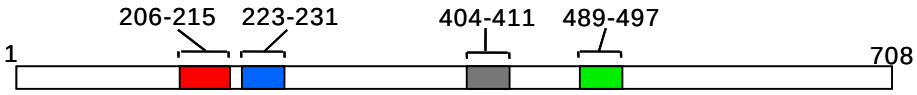
která by působila pouze na nádorovou tkáň aniž by poškodila tkáň zdravou. Nádorová imunoterapie splňuje tato kritéria a má potenciál se stát nejspecifičtější protinádorovou léčbou po překonání překážek, které významně snižují její účinnost. Zejména je nezbytné vyvinout metody, které zabrání rozvoji imunologické tolerance na nádorové antigeny. Podobně důležité je vyvinutí účinných a bezpečných vakcín, které mohou být použity u pacientů. Poslední podmínkou je přesné zmapování antigenů specifických pro individuální nádory. Tyto otázky mohou být experimentálně studovány pouze na zvířecích modelech, pro tento účel je k dispozici vědcům celá řada nádorových modelů které jsou ve velké většině založené na inbredních myších liniích. Žádný z těchto modelů ale není univerzální a nelze ho použít k odpovězení všech otázek. Mnohaleté zkušenosti potvrdily, že mezi nejlepší postupy dostupné v současnosti patří studium myších nádorů indukovaných onkogeními viry SV40 a HPV.

1.2 SV40

Simian virus 40 (opičí virus 40) je malý DNA virus pařící mezi polyomaviry a jeden z nejvíce studovaných virů vůbec. SV40 je blízce příbuzný virus s polyomaviry JC a BK, které jsou patogenní pro člověka a většina jedinců v populaci je trvalými nosiči těchto dvou virů. Infekce SV40 může způsobit vznik nádorů, zcela jednoznačně to bylo prokázáno na hlodavcích (myši, křečci) [Tevethia et al., 1974]. Do současnosti však nebylo přesvědčivě prokázáno či vyvráceno, že SV40 může vyvolat nádory u lidí přestože několik studií prokázalo přítomnost virové DNA ve vzorcích některých lidských nádorů jako mezotheliom, non-Hodgkinský lymfom a některé nádory mozku [Bergsagel et al., 1992; Carbone et al., 1994; Hirvonen et al., 1999; Rizzo et al., 1999]. Pro přirozeného hostitele, kterým je makak rhesus, je SV40 nepatogenní. SV40 kontaminoval živou poliovakcínu, která byla právě vyrobená na buněčných liniích z makak rhesus a tato vakcína byla aplikována v USA mnoha milionům jedinců na začátku šedesátých let 20. století [Shah and Nathanson, 1976]. Tito jedinci byli sledováni po mnoho let a naštěstí se nepotvrdily počáteční obavy, že SV40 bude indukovat tvorbu nádorů [Mortimer et al., 1981]. Popsané případy, kdy byla prokázána přítomnost SV 40 viru v nádorové tkáni jsou raritní případy a někteří z těchto pacientů vůbec nebyli v minulosti očkováni kontaminovanou poliovakcínou. Toto zjištění vyvolalo debatu, že SV40 může přirozeně kolovat v lidské populaci [Tevethia and Schell, 2002]. Genom SV40 kóduje pět hlavních proteinů, tři pozdní VP1, VP2 a VP3, které tvoří kapsidu viru a dva časné proteiny „velký“ T antigen (T Ag) a „malý“ T antigen (t Ag, odvozeno od slova tumor), které jsou odpovědné za onkogenní vlastnosti SV40. T Ag je prototypem virového onkogenu a v průběhu uplynulých let bylo zjištěno, že T Ag váže tumor-supresorový protein p53 a pRb což vysvětluje jeho onkogenní vlastnosti [Levine, 1989]. Je-li T Ag exprimován jako transgen v myších indukuje tvorbu spontánních nádorů v závislosti na lokalizaci exprese [Brinster et al., 1984; Van Dyke et al., 1985; Knowles et al., 1990]. Proces onkogeneze indukované T Ag je velmi podobný přirozenému vzniku lidských nádorů a proto se T Ag-transgenní myši staly výhodným a široce používaným modelovým organismem. T Ag je nejenom typickým onkogenem, ale i velmi dobře charakterizovaným antigenem, který vyvolává tvorbu specifických protilátek a indukuje specifickou buněčnou odpověď nejen u myší [Schell and Tevethia, 2001]. V sekvenci T Ag bylo identifikováno několik epitopů vázajících se na

molekuly MHC I a II v závislosti na MHC haplotypu [Forster et al., 1995; Mylin et al., 1995]. Tento fakt spolu s dostupností T Ag-transgenních myší umožňuje velmi přesně studovat u těchto myší T Ag-specifickou protinádorovou imunitu během onkogeneze.

Obr.1 **CTL H-2^b epitopy v SV40 T antigenu**



epitop	pozice	sekvence	restrikce
I	206-215	SAINNYAQKL	H-2D^b
II/III	223-231	CKGVNKEYL	H-2D^b
IV	404-411	VVYDFLKC	H-2K^b
V	489-497	QGINNLDNL	H-2D^b

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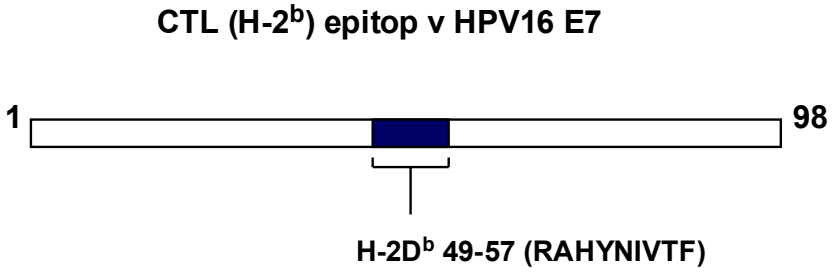
1.3 HPV

Lidský papilomavirus (HPV) je dalším představitelelm malých DNA virů, které způsobují vznik nádorů avšak na rozdíl od SV40 je to typicky lidský patogen. Existuje více než 100 genotypů HPV které se liší mírou onkogenity, některé typy HPV infikují dlaždicový epitel genitálního traktu mužů i žen a sliznice laryngu a způsobují tím vznik karcinomů infikovaných epitelií [Walboomers et al., 1999]. Rozlišují se tzv. vysokorizikové typy (hlavně HPV 16, 18, 31) a nízkorizikové typy (hlavně HPV 6 a 11) [Lorincz et al., 1992]. HPV je závažný zdravotnický problém zejména u žen, neboť bylo prokázáno že 99% případů karcinomu děložního čípku je způsobeno infekcí HPV. Genom HPV kóduje dva pozdní strukturální proteiny L1 a L2 a na rozdíl od SV40 sedm časných genů (E1-E7) nezbytných pro replikaci viru a transformaci buněk. Proteiny E6 a E7 jsou hlavní onkogeny HPV, E6 váže p53 a E7 váže pRB, oba tyto proteiny tedy mají podobnou funkci jako T Ag. Rozdíl mezi low-risk a high-risk typy spočívá právě v účinnosti inhibice p53 a pRB proteiny E6 a E7 [Dyson et al., 1989; Werness et al., 1990; zur Hausen, 1998]. Na rozdíl od T Ag nejsou samotné proteiny E6 a E7 tak silné onkogeny, pro transformaci je nutná aktivita obou proteinů a pro rozvoj

invazívních karcinomů jsou nezbytné další mutace postihující geny regulující buněčný cyklus. Proteiny E6 a E7 jsou specifické nádorové antigeny vůči kterým se nevyvíjí centrální tolerance, protože nejsou exprimované v thymu neboť se jedná o cizí, virové proteiny. CD8+ T lymfocyty specifické na tyto dva proteiny, které jsou indukované vakcinací, velmi specificky rozpoznávají nádorové buňky, protože E6 a E7 jsou trvale exprimované v nádorech [Feltkamp et al., 1993; Ressing et al., 1995].

Zabránění vzniku nádorů v důsledku infekce HPV nastane také v případě, že bude účinně zamezeno virovým partikulím infikovat buňky a nedojde tedy vůbec k jejich onkogenní transformaci. Toho lze docílit vyvoláním imunitní odpovědi při které se tvoří virus-neutralizační protilátky. HVP podléhá stejným zákonitostem jako ostatní viry a proto není překvapující, že po infekci HPV dochází k tvorbě protilátek specifických i na strukturální proteiny L1 a L2 [Hamsikova et al., 1998]. Tvorbu těchto protilátek lze vyvolat vakcinací prostřednictvím tzv. virům podobným partikulím (virus-like particles; VLP) [Zhou et al., 1991]. Tyto VLP jsou uměle připravené v laboratoři a v podstatě se jedná o „prázdné“ virové kapsidy tvořené proteiny L1 anebo L2, které neobsahují virovou DNA a proto jsou i neinfekční. V současné době jsou k dispozici komerční vakcíny na bázi VLP pro klinické použití a jejich účinnost na prevenci HPV-indukovaným násorům se kontinuálně testuje [Koutsky et al., 2002]. Tento způsob vakcinace je účinný před rozvojem nádorového onemocnění a proto se označuje jako preventivní vakcinace. Rozšířená indikace např. u pacientek s prekancerózní lézí cervixu (CIN) je předmětem klinických zkoušek. Imunizace pomocí VLP tedy není protinádorová vakcinace v pravém slova smyslu, neboť je účinná pouze jako prevence proti infekci HPV. Přestože je vývoj vakcín proti karcinomu cervixu nejúspěšnější příklad nádorové imunoterapie v současnosti, stále nejsou k dispozici terapeutické vakcíny, které by navodily regresi invazívních či metastazujících nádorů cervixu, a jejich vývoj je předmětem intenzívního výzkumu.

Obr. 2



1.4 Mechanismus indukce T-buněčné imunitní odpovědi.

Porozumění mechanismům T-buněčné imunitní odpovědi je výsledkem dlouholetého studia, které bylo nejen velmi obtížné, ale bylo také neobyčejně kontroverzní. Existence buněčné imunitní odpovědi je známa mnoho let, již roku 1974 Zinkernagel a Doherty ukázali, že T lymfocyty jsou specifické jak na antigen, tak také na molekuly hlavního histokompatibilního komplexu (MHC) [Zinkernagel and Doherty, 1974]. Po dlouhou dobu však věda zcela tápala v identifikaci mechanismů, které jsou zodpovědné za tuto dvojí specifitu. Rozluštění této vědecké hádanky bylo založeno na několika fundamentálních objevech a řady malých neméně důležitých objevů, které byly společně výsledkem trpělivého bádání mnoha vědeckých týmů. Mezi ty fundamentální objevy patří nepochybně identifikace T-buněčného receptoru (TCR) a odhalení mechanismu rekombinace genů kódujících TCR [Hedrick et al., 1984; Saito et al., 1984; Saito et al., 1984].

Skutečný průlom pro pochopení způsobu jakým CD4+ a CD8+ T lymfocyty rozpoznávají antigeny však byl rok 1989. V tomto roce bylo prokázáno, že TCR přítomný na povrchu CD8+ T lymfocytů rozpoznává krátké peptidové sekvence o délce 8-10 aminokyselin navázané na molekuly MHC I a podobně se ukázalo, že TCR na povrchu CD4+T lymfocytů rozpoznává peptidy asociované s molekulami MHC II [Townsend et al., 1989; Townsend et al., 1990]. Tento poznatek umožnil zcela exaktně určit, která sekvence v proteinovém antigenu je zodpovědná za jeho imunogenitu a odstartoval éru moderní imunologie.

Studium protivirové imunitní odpovědi u myší vyvolané infekcí laboratorními kmeny virů jako je například LCMV nebo influenza je stále klíčovým postupem studia buněčné imunity. CD8+ T lymfocyty jsou nejdůležitější buněčnou populací protivirové imunitě, protože rozpoznávají virové antigeny prezentované molekulami MHC I. třídy, které jsou exprimované na všech buňkách a jsou schopné zabít buňky infikované viry – také se proto označují jako cytotoxické T lymfocyty. Podobně jako v protivirové imunitě, CD8+ T lymfocyty mají zásadní úlohu i v protinádorové imunitě a jsou schopné zabít transformované buňky stejnými mechanismy jako zabíjejí buňky infikované viry.

Naproti tomu CD4+ T lymfocyty rozpoznávají antigeny prezentované molekulami MHC II. třídy, které jsou ale přítomné pouze na specifických buňkách označovaných jakou profesionální antigen-prezentující buňky (APC). CD4+ T lymfocyty poskytují aktivační signály, které jsou nezastupitelné pro navození proliferace a diferenciace CD8+ T lymfocytů a B lymfocytů. Protože ale CD4+ T lymfocyty rozeznávají prostřednictvím TCR antigenní peptidy navázané na molekuly MHC II nemají schopnost přímo zabít nádorové nebo virem-infikované buňky, neboť buňky nepocházející z hematopoetické tkáně zřídka exprimují MHC II. Bylo ale prokázané, že CD4+ lymfocyty v určitých experimentálních systémech byly nezbytné pro navození účinné protinádorové imunity, tyto mechanismy působí nepřímo prostřednictvím secernovaných cytokinů (hlavně IFNγ, TNFα) které aktivují makrofágy infiltrující nádory [Mumberg et al., 1999].

Analýza virově-specifické odpovědi ukázala, že CD8+ T lymfocyty jsou překvapivě specifické pouze na určité peptidy z několika málo virových genů. Například infekce virem LCMV navodí u C57BL/6 kmene myší imunitní odpověď specifickou pouze na tři CD8 epitopy Db/gp_{33-41}, Db/$np_{396-404}$ a Db/$gp_{276-286}$ [Gallimore et al., 1998], které tvoří na vrcholu infekce až 70% všech CD8+ T lymfocytů. Další modelový virus, influenza, po infekci indukuje imunitní odpověď specifickou na dva epitopy Db/$np_{366-374}$ a Db/$pa_{224-233}$ [Belz et al., 2000]. Některé epitopy tedy zcela dominují v celkové CD8+ T-buněčné odpovědi na komplexní viry a pro tento jev se používá označení imunodominance. Důsledkem imunodominance je tedy ustanovení imunologické hierarchie mezi jednotlivými imunogeními epitopy, na jedné straně existují epitopy imunodominantní a na opačné straně jsou epitopy, vůči kterým se tvoří velmi slabá imunitní odpověď a tyto epitopy se označují jako imunorecesívní [Yewdell and Bennink, 1999]. Přestože je odpověď na komplexní antigeny (velké viry, směsi mnoha proteinových antigenů, alogenní buňky, apod.) polarizována ve prospěch imunodominantních determinant, lze zcela spolehlivě detekovat i odpověď vůči imunorecesivním epitopům. Neplatí tedy, že imunorecesívní epitopy nejsou schopné navodit imunitní odpověď. Selekce imunogeních epitopů je složitý proces, kdy imunitní systém vybírá např. při virové infekci z mnoha stovek možných epitopů obsažených v desítkách genů a je výsledkem mnoha faktorů [Kedl et al., 2003], které budou dále diskutovány, ovlivňujících aktivaci naivních CD8+ T lymfocytů.

Na rozdíl od CD8+ T-buněčné imunitní odpovědi, mnohem méně je známo o imunodominanci v CD4+ T-buněčné imunitní odpovědi. Tento nedostatek informací je způsoben hlavně tím, že studium mechanismů CD4+ T-buněčné imunitní odpovědi je podstatně technicky obtížnější. Nicméně se ví, že tato imunodominance na komplexní antigeny také existuje, je však méně vyjádřena. Cílem následujícího textu je úzce zaměřená diskuze na téma imunodominance v CD8+ T-buněčné odpovědi, protože charakter a metody mého experimentálního přístupu nebyly cílené na analýzu CD4+ T-buněčné imunitní odpovědi.

Nejprve je nutné odpovědět na otázku k čemu slouží imunodominance. Imunodominance v CD8+ T-buněčné imunitní odpovědi je výsledkem evolučního procesu, v kterém se organismy naučily maximálně zefektivnit imunitní systém. Neboli s omezenými prostředky a v omezeném čase je nutné co nejrychleji vytvořit co nejvíce cytotoxických lymfocytů, které rozpoznají buňky infikované viry a zahubí je. Příroda rozhodla, že nejúčinějším způsobem bude vybrat několik málo antigenních determinat z mnoha možných a vůči těmto determinantám koncentrovat maximum imunitní odpovědi. Nejimunogenější epitopy aktivují specifické CD8+ T lymfocyty nejdříve [Busch and Pamer, 1998; Mercado et al., 2000]a tato aktivace zahájí proces klonální expanze, fáze exponenciálního množení trvá v řádu několika dnů a asi do pěti-sedmi dnů od momentu infekce (velmi závisí na typu virové infekce) je již detekovatelná antivirová CD8+ odpověď. Expanze antivirových lymfocytů je skutečně masívní, v akutní fázi dosahuje až desítky procent z celkového počtu CD8+ T lymfocytů [Murali-Krishna et al., 1998]. Organismus ale do jisté míry značně riskuje tím, že generuje takto úzce zaměřenou imunitní odpověď. Co by se stalo, kdyby byla imunitní odpověď cílená na nevhodné epitopy? Pravděpodobně by to organismus nepřežil a podlehl virové infekci, zcela spolehlivě lze předpokládat, že v průběhu evoluce k podobným „neúspěchům“ došlo. Tyto „neúspěchy“ se staly podkladem evoluční selekce, kdy organismy s největší rezistencí měly nejefektivnější způsob výběru imunogeních epitopů.

1.5 Pravidla definující imunogení epitopy.

Existuje několik základních pravidel, které musí být splněny, aby daný epitop navodil imunitní odpověď [Yewdell and Bennink, 1999]. Popsané faktory platí pro CD8-specifickou odpověď a v některých případech i pro CD4-specifickou odpověď.

Imunogení epitop:

1. musí být generován v APC v dostatečném množství z prekurzorového polypetidu a dopraven do endoplazmatického retikula, kde dojde k navázání na molekuly MHC I. třídy. Této fázi lze přiřadit termín „Zpracování antigenu“.
2. musí být schopen vazby na molekuly MHC I. třídy v dostatečné afinitě.
3. v organismu musí existovat CD8+ T lymfocyty, které jsou aktivovány komplexy MHC I-peptid.

Nelze přesně určit do jaké míry musí být každá podmínka splněna, neboť výsledná imunogenicita je výsledkem souhry těchto faktorů. Deficit na jedné úrovni může být kompenzován na jiné, neexistuje tedy jeden obecný faktor určující imungenitu. Například epitop, který je velmi málo generován z proteinového antigenu, protože se tvoří v malém množství může být imunogení, váže-li se velmi dobře na MHC I a v organismu je k dospozici velký počet naivních CD8+ T prekurzorů [Chen et al., 2000].

Ad 1 Zpracování antigenu

Většina antigenních peptidů generovaných buňkou pochází z cytoplazmatické frakce proteinů syntetizovaných buňkou [Pamer and Cresswell, 1998]. Na rozdíl od tvorby komplexů peptid-MHC II, ke které dochází pouze v profesionálních APC, všechny somatické buňky mají schopnost tvorby peptidů vázajících se na MHC I. Pochází-li peptid z proteinu syntetizovaného buňkou, která prezentuje tento peptid na svém povrchu, hovoříme o přímé prezentaci antigenů. Tento způsob prezentace antigenů je významný při virových infekcích, neboť viry mohou přímo infikovat APC přítomné buď přímo v místě vstupu viru jako třeba Langerhansovy DC v kůži, ale také APC přítomné ve spádové lymfatické uzlině [Norbury et

al., 2002]. Profesionální APC jsou schopné endocytózy exogenních antigenů – pocházejících třeba z apoptotických či nekrotických buněk. Pohlcený antigenní materiál se následně dostane do cytoplazmy APC a je zpracován na peptidy stejným mechanismem jako proteiny syntetizované v buňce. Vytvořené peptidy jsou pak vystaveny navázané na MHC I na povrchu buňky, tento druhý způsob prezentace antigenů se označuje jako krosprezentace [Bevan, 1976; den Haan et al., 2000].

Proteiny jsou v cytoplazmě naštěpeny proteinázovým komplexem označovaným jako proteazom a vzniklé fragmenty o délce 8-16 aminokyselin jsou po navázání na peptidový transportér TAP přeneseny do endoplazmatického retikula (ER)[Pamer and Cresswell, 1998]. Uvnitř lumen ER dochází k vazbě na MHC I za účasti celé řady proteinů z rodiny molekulárních chaperonů. Je-li sekvence peptidu příliš dlouhá, aby mohlo dojít k vazbě na MHC I, může být zkrácena odštěpením několika amonokyselin z NH_2 konce peptidu prostřednictvím ER-rezidentních aminopeptidáz jako je např. ERAAP [Serwold et al., 2002]. Z těchto oznatků plyne, že kritickým místem je nejprve proteazomální štěpení a následně transport do ER. Ztráta imunogenity může být způsobena tím, že proteasom štěpí sekvenci uprostřed hypotetického epitopu, nebo že generovaný peptid není schopen vazby na TAP a nedostane se tak do ER.

Ad 2 Vazba peptidu na MHC I

Generované peptidy o délce cca 8-10 AMK se váží prostřednictvím nekovalentních vazebných interakcí na povrch MHC I v místě označovaném jako „žlábek“. Každý typ MHC I molekul – ať myší či lidské obsahuje ve vazebném žlábku charakteristické domény, které preferenčně váží typicky 2-3 aminokyseliny označované jako „kotvy“. Tyto kotvící aminokyseliny se nachází na zcela specifických pozicích v peptidu, například peptidy vážící se na myší H2-Kb molekuly často obsahují vazebný motiv xx(Y)x(FY)xx(LMIV)x [Falk et al., 1991]. Tyto vazebné motivy jsou známé pro velký počet aloform molekul MHC I a MHC II a analýzou sekvencí proteinů je možné predikovat na základě těchto motivů možné epitopy. Účinnost vazby peptidu na MHC I je dána primární strukturou peptidu a lze ji fyzikálně-chemicky vyjádřit jako afinitu. Prahová afinita peptidů musí překročit určitou hranici (cca Kd> 500 nM) aby daný peptid navodil imunitní odpověď [Sette et al., 1994]. Navázaný peptid stabilizuje molekuly MHC I přítomné na povrchu buňky a v závislosti na afinitě může být

komplex MHC I-peptid prezentován na povrchu buněk v řádu hodin až dnů. Míra afinity peptidu pro MHC I je asi nejdůležitější faktor určující imunogenitu za fyziologických podmínek, je-li afinita velmi vysoká, není potřeba velké množství antigenu pro generování nezbytného počtu komplexů s MHC I a zároveň tyto komplexy zůstanou po dlouhou dobu na povrchu APC což výrazně zvýší šance, že bude rozpoznán specifickým CD8+ T lymfocytem. Naopak, imunogenní peptidy s nízkou afinitou musí být generovány ve zvýšeném množství a po delší dobu aby bylo dosaženo stejné imunogenity [Vijh et al., 1998].

Ad 3 CD8+ T prekurzory

Přítomnost velkého počtu komplexů na povrchu mnoha APC není zárukou toho, že CD8+ imunitní odpověď bude úspěšně vyvolána. Zcela nezbytným předpokladem je, že v organismu jsou přítomné CD8+ T lymfocyty schopné rozpoznat antigenní komplexy prostřednictvím vazby komplementárního T-buněčného receptoru (TCR). Aby toho bylo možné dosáhnout, T lymfocyty musí generovat dostatečně široké spektrum různých TCR prostřednictvím V-D-J rekombinace. Existuje-li tedy naivní lymfocyt komplementární ke komplexu peptid-MHC I, může dojít k jeho aktivaci a následné klonální expanzi.

Schopnost TCR vazby na komplex MHC-peptid vyjádří afinita resp. avidita je-li posuzována schopnost vazby T lymfocytů na více komplexů MHC-peptid přítomných na povrchu APC. Platí, že pro aktivaci CD8+ T lymfocytů s vyšší aviditou je potřeba menší množství antigenu než pro aktivaci CD8+ T lymfocytů exprimujících nizkoafinitní TCR. Tento poznatek lze demonstrovat analýzou primární a sekundární odpovědi na imunizaci, při primární odpovědi se aktivuje více CD8+ T lymfocytů s TCR majícími různou afinitu a výsledná populace antigen-specifických CD8+ T lymfocytů se skládá ze směsi nízko- a vysko-aviditních CD8+ T lymfocytů Je-li organismus immunizován znovu, tak preferenčně expandují pouze klony vysoko- aviditních CD8+ T lymfocytů a odpověď se postupně mění z polyklonální na oligoklonální[Deng et al., 1997; Chen et al., 2000; Chen et al., 2004].

1.6 Mechanismy imunodominance

V předchozí části textu byly definovány podmínky, které musí být splněny, aby daný antigen byl schopen navodit CD8+ T-buněčnou imunitní odpověď. K jakým jevům ale dochází, obsahuje-li antigen několik imunogenních epitopů současně? Proč jsou některé epitopy immunogenější než jiné? Odpovědi na tyto otázky mají zcela zásadní význam nejen pro porozumění mechanismům indukce buněčné imunity ale také pro vývoj vakcín účinně stimulujících buněčnou imunitu. Podstata imunodominance vychází zjednodušeně z kompetice mezi imunogenními determinantami a lymfocyty o místo a zdroje v organismu. Tato kompetice probíhá na mnoha úrovních, od dějů probíhajících na molekulární úrovni směrem k interakcím mezi jednotlivými buňkami imunitního systému.

1. Generování epitopů.

Aby bylo možné posoudit význam účinnosti zpracování antigenů (antigen processing) na ustanovení imunologické hierarchie je nezbytné mít k dispozici metody, které umožní kvantifikovat tvorbu jednotlivých epitopů z proteinového antigenu. Tyto postupy prošly historickým vývojem a v podstatě existují tři základní metody. Technicky nejméně obtížné je stanovit, jestli buňky exprimující antigen jsou rozpoznány v cytotoxickém testu a porovnat je s referenčními buňkami jako jsou například RMA/s, které byly inkubovány v přítomnosti syntetického peptidu o stejné sekvenci [Lippolis et al., 1995]. Tato metoda je hrubě orientační a v žádném případě neposkytne exaktní informaci, kterou je počet epitopů vytvořených z definovaného množství anigenu za jednotku času. Druhá metoda je naopak velmi přesná, ale značně nepraktická a nákladná. Peptidy navázané na molekuly MHC I lze extrahovat z velkého počtu buněk a pomocí HPLC určit jejich koncetraci porovnáním se syntetickým analogem [Gallimore et al., 1998]. Poslední metoda je v současné době nejvýhodnější, ale lze ji aplikovat pouze na velmi omezený počet antigenních epitopů. Tato metoda je založena na detekování komplexů MHC-peptid pomocí monoklonální protilátky, která specificky reaguje pouze s těmi MHC, které mají na povrchu navázaný specifický peptid [Germain and Jenkins, 2004]. Tato protilátka tedy reaguje analogicky jako TCR rozpoznávající komplexy peptid-MHC. Například pomocí protilátky rozpoznávající komplex Kb-SIINFEKL bylo určeno, že buňky infikované virem vakcinie exprimující ovalbumin jako modelový antigen mají na povrchu zhruba 3500

komplexů Kb-SIINFEKL [Princiotta et al., 2003]. Příčiny, proč některé epitopy mohou být generovány účiněji než jiné mohou být zejména určeny účinností proteazomální degradace [Villanueva et al., 1994], t.j. je-li COOH konec peptidu štěpen ve správném místě, protože zkracování peptidu ER-rezidentními proteázami probíhá pouze na NH_2 konci. Účinnost vazby peptidů na transportní komplex TAP a jejich transport do ER není obvykle limitující faktor. Ovšem za jistých okolností, např. při infekci viry, které inhibují prezentaci vlastních antigenů pomocí proteinů blokujících TAP (například protein ICP47 exprimovaný virem HSV[Hill et al., 1995]) může být tento krok limitující.

2. Vazba peptidů na MHC I.

Vytvoření hierarchie mezi několika imunogeními epitopy podstatně závisí na účinosti vazby těchto peptidů na příslušné molekuly MHC I. Buněčné proteiny jsou kontinuálně degradovány v proteazomu a vytvořené peptidy jsou následně transportovány do ER. Zatímco přísun generovaných peptidů je obrovský, v ER je však k dispozici limitovaný počet MHC I molekul schopných navázat peptid. Mezi jednotlivými pepidy probíhá kompetice o dostupné MHC I molekuly a znovu je pravidlem, že peptidy s vyší afinitou pro MHC I preferenčně obsadí vyší počet dostupných MHC I molekul v ER . Tato kompetice probíhá ale pouze o stejné molekuly MHC I, t.j. vazba peptidů na H2-K^b probíhá nezávisle na vazbě peptidů na H2-D^b. Některé publikace naznačují, že neplatí lineární vztah mezi množstvím dostupných komplexů MHC I-peptid a množstvím aktivovaných CD8+ T lymfocytů. Naopak se ukazuje, že existuje učitý minimální počet komplexů prezentovaných na povrchu APC, které jsou nezbytné pro generování efektivní CD8+ T- imunitní odpovědi a další zvýšení prezentace antigenu nemá vliv na zvýšení expanze CD8+ T lymfocytů [Vijh et al., 1998]. Proto i slaběji se vázající peptidy mohou navodit dobrou imunitu, jsou-li prezentované na povrchu APC v určitém minimálním množství a to i v případě, že jiné kompetující peptidy ze stejného antigenu tvoří komplexy s MHC I v několikanásobně větším množství.

3. Dostupnost CD8+ T prekurzorů.

Tento parametr určuje kolik naivních CD8+ T lymfocytů v organismu je specifických pro danný epitop. Bohužel není snadné toto číslo zjistit a musíme se spoléhat pouze na odhady,

které ještě navíc platí pouze pro určité antigeny studované na myších. Je zcela nemožné zjistit současnými metodami, jaká je prekurzorová frekvence pro imunodominantní epitopy z běžných virových antigenů u lidí. Je-li prekurzorová frekvence vysoká, tak může organismus generovat rychleji dostatečné množství efektorových lymfocytů, zatímco při malém počtu prekurzorů je nutná vetší proliferace, aby bylo dosaženo stejného počtu efektorů [Choi et al., 2002]. Liší-li se významně počet prekurzorů pro dva jinak stejně imunogenní epitopy, dojde po vyvolání imunitní odpovědi ke generování nestejně velké populace efektorů.

4. Kompetice mezi jednotlivými CD8+ T lymfocyty o antigen a prostor k expanzi.

V průběhu indukce buněčné imunitní odpovědi dochází k interakci APC s mnoha CD8+ T lymfocyty specifickými pro prezentované antigeny. Bylo ukázáno, že APC mohou prezentovat antigeny současně většímu počtu T lymfocytů, ev. mohou aktivovat více T lymfocytů prostřednitvím mnoha sériových kontaktů [Mempel et al., 2004]. Přestože ale APC mohou prezentovat mnoho antigenních CD8 epitopů v komplexu s MHC I a tyto komplexy jsou rozpoznány specifickými CD8 lymfocyty, dojde k efektivní aktivaci pouze u malého počtu CD8+ T lymfocytů. Populace naivních CD8+ T lymfocytů specifických na stejný antigen může být tvořena různými klony, které ale mají různé TCR a proto i odlišnou aviditu pro antigen. V takto polyklonálním spektru CD8+ T lymfocytů dojde k prefererenční aktivaci CD8+ T lymfocytů o vyšší aviditě.

Kompetice na úrovni APC se odehrává nejen mezi CD8+ T lymfocyty specifickými pro jeden epitop, ale také mezi CD8+ T lymfocyty specifickými pro různé komplexy MHC-peptid, které jsou generované ze stejného antigenu. Také mohou vzájemně kompetovat CD8+ T lymfocyty specifické pro různé antigeny prezentované současně. Dosud není k dispozici jasná odpověď na otázku, jestli mohou mezi sebou soutěžit CD8+ T lymfocyty a CD4+ T lymfocyty, také nebyla dosud popsaná kompetice mezi CD4+ T lymfocyty.

Aktivované T lymfocyty mohou snížit množství antigenu prezentovaného na povrchu APC po rozpoznání komplexů MHC-peptid při interakci s APC [Kedl et al., 2002]. Toto snížení dostupnosti antigenu účinně inhibuje aktivaci ostatních naivních CD8+ T lymfocytů o stejné specifitě, zejména těch CD8+ T lymfocytů, které mají nižší aviditu. Nedochází ale ke ztrátě jiných komplexů MHC-peptid z povrchu APC a bylo ukázáno, že kompetice o stejný antigen je mnohem více účinná než kompetice mezi CD8+ T lymfocyty specifickými pro různé

antigeny [Kedl et al., 2002] (tato kompetice se také nazývá kros-kompetice). Kros-kompetice byla ale popsána a lze ji vysvětlit pomocí dalších mechanismů [Kedl et al., 2003]. Proliferující klon CD8+ T lymfocytů vytváří okolo APC fyzickou bariéru, která limituje přístup jiných CD8+ T lymfocytů k APC [Kedl et al., 2000]. Fyzická exkluze omezuje opět preferenčně CD8+ T lymfocyty, které mají nižší aviditu pro antigen a tedy vyžadují delší dobu kontaktu s APC aby došlo k jejich plné aktivaci. Dojde-li rozpoznání antigenu na povrchu APC, aktivované CD8+ T lymfocyty mohou zabít APC analogicky jako zabíjejí terčové buňky a tento jev dále nespecificky omezuje dostupnost antigenů pro další dosud neaktivované CD8+ T lymfocyty [Loyer et al., 1999].

1.7 Imunologická tolerance

Regulační nástroje imunitního systému velmi účinně suprimují lymfocyty specifické pro vlastní antigeny a zabraňují tak rozvoji autoimunitních onemocnění [Kedl et al., 2000]. Navození imunologické tolerance probíhá dvěma hlavními procesy. První, centrální tolerance, vzniká při ontogenezi v thymu. V tomto procesu dochází ke tvorbě prekurzorového repertoáru naivních T lymfocytů, které opouštějí thymus a přežívají v periferii organismu desítky let. Tato selekce probíhá jednak pozitivní selekcí, kdy rozpoznání vlastních molekul MHC exprimovaných na thymických stromálních buňkách inhibuje apoptózu CD4+CD8+ thymocytů. Při opačném procesu, označovaném jako negativní selekce je apotóza maturujících thymocytů indukována nerozpoznáním MHC molekul prezentujících peptidy derivované z buněčných proteinů. Tyto procesy nelze chápat odděleně, realitě se nejspíše přibližuje představa, že pozitivní i negativní selekce probíhá zároveň při kontaktu thymocytů s DC, a síla interakce TCR-MHC určí, jestli thymocyty buď nerozpoznávají MHC molekuly (negativní selekce), nebo naopak reagují s MHC příliš silně (pozitívní selekce) [Hogquist et al., 2005]. Centrální tolerance je tedy děj jednorázový a závislý na thymu, po ukončení vývoje imunitního systému a involuci thymu je ukončen.

Druhý mechanismus imunologické tolerance se označuje periferní tolerance a jak plyne z názvu, probíha v periferii organismu [Kedl et al., 2000]. Tento proces probíhá kontinuálně po

celý život a zjednodušeně si ho lze představit jako určité „vylaďování“ imunitního systému, při kterém se kontinuálně odstraňují autoreaktivní lymfocyty, které nebyly odstraněné centrální tolerancí, nebo takové, které jsou specifické pro antigeny neexprimované v thymu. Poruchy imunologické tolerance se projevují rozvojem autoimunitních onemocnění, existují ale i stavy, při kterých naopak dochází k navození nežádoucí imunologické a mezi tyto stavy typicky patří nádorová onemocnění. Nádorové antigeny jsou ve své podstatě auto-antigeny (pokud se nejedná o nádory vyvolané onkogeními viry) a periferní tolerance nečiní rozdíly mezi supresí lymfocytů specifických pro běžné proteiny či specifických pro nádorové antigeny [Heath and Carbone, 2001].

Jak k tomu dochází? Nejprve je nutné krátce popsat experimentální systémy, které odpoví na tyto otázky. Interakce naivních lymfocytů se self-antigeny je studována převážně na myších modelech, musí být totiž definován jednak antigen indukující toleranci a pak musí být definovány T lymfocyty v kterých je tolerance indukována. Prakticky se využívá následujícího postupu: první kmen myší-označíme si ho jako příjemce- je transgenní pro definovaný antigen, který je exprimován pod nějakým tkáňově specifickým promotorem, jako jsou inzulínový, keratinový, nebo alfa-amylázový, atd.[Hanahan, 1985; Knowles et al., 1990; Doan et al., 1998]. Mezi často používané modelové antigeny exprimované pod těmito promotory patří například LCMV glykoprotein, LCMV nukleoprotein, SV40 T antigen, chřipkový hemaglutinin, ovalbumin. Druhý syngenní kmen myší -označíme si ho jako dárce- je transgenní pro TCR. Tyto TCR transgenní myši mají často více než 90% všech lymfocytů (buď CD4+ nebo CD8+) specifických pro tento definovaný epitop v důsledku exprese transgenu, který kóduje cDNA pro TCR [Kouskoff et al., 1995]. V současnosti je k dispozici mnoho linií TCR-transgeních myší specifických pro hlavní dominantní epitopy z výše zmíněných antigenů. V následném experimentu se vpraví T lymfocyty dárce do organismu příjemce a sleduje se reakce naivních lymfocytů dárce na přítomný self-antigen. Jaké poznatky tyto experimenty přinesly?

Zcela jednoznačně bylo prokázáno, že v okamžiku exprese self-antigenu dojde k rozpoznání přítomného antigenu specifickými T lymfocyty a jejich následné aktivaci. Tato aktivace je ale odlišná, než ke které dojde při aktivaci virově specifických lymfocytů při infekci. Nastane krátkodobé proliferace aktivovaných T lymfocytů, ale tato proliferace je abortivní, nedojde k navození klonální expanze autospecifických T lymfocytů, ale naopak

dojde ke jejich apoptóze po prodělání několika cyklů buněčného dělení [Marzo et al., 1999; Belz et al., 2002; Nguyen et al., 2002]. Vědecká komunita se shoduje na těchto základních dějích, nejisté informace jsou ale k dispozici na následující otázky. Zaprvé, s jakým typem buňky interaguje T lymfocyt specifický pro autoantigen, jsou to buňky exprimující antigen (např. beta buňky pankreatu u RIP-transgenních myší) nebo se tato interakce odehrává v uzlině spádové pro místo exprese autoantigenu a interagujícím typem buněk jsou APC které krosprezentovaly antigenní materiál pocházející s místa exprese autoantigenu? Dále, jsou-li to APC ve spádové uzlině, jak se tato interakce liší od normální interakce jejímž výsledkem je navození imunity? Současné poznatky naznačují, že odpovědi na tyto otázky se liší podle použitého experimentálního modelu (typ antigenu, typ promotoru, MHC haplotyp myší). Lze se přiklonit ke stanovisku některých předních vědeckých skupin které je následující: Self-specifické lymfocyty (platí pro CD4+ i CD8+) rozpoznávají primárně antigeny krosprezentované dendritickými buňkami a tato interakce se odehrává ve spádové lymfatické uzlině [Sotomayor et al., 2001; Belz et al., 2002]. Pro navození tolerance je zásadní fenotyp buněk prezentujících antigeny, data ukazují, že jsou to CD8+ dendritické buňky (označované jako lymfoidní DC) které indukují toleranci CD8+ T lymfocytů [Albert et al., 2001; Hawiger et al., 2001; Belz et al., 2002]. Tolerogenní DC musí mít nezralý fenotyp, t.j. sníženou expresi kostimulačních molekul CD80, CD86, CD40. Exprese těchto kostimulačních molekul je pravděpodobně zásadní pro rozhodnutí, jestli výsledkem interakce T lymfocyt-DC bude klonální expanze nebo apoptóza [Spiotto et al., 2003].

Jakým způsobem jsou odlišeny autoantigeny od cizích antigenů? Na tuto otázku neexistuje obecně akceptovaná odpověď, v současnosti lze pouze diskutovat o hypotézách. První a klasická hypotéza spočívá na principu centrální tolerance. Lymfocyty které jsou specifické na autoantigeny exprimované v thymu jsou eliminovány pozitivní selekcí, k další editaci repertoáru prekurzorů již nedochází. Bylo nepochybně prokázáno, že genová exprese v thymu je unikátní. Řada proteinů exprimovaných ve specifických tkáních je také exprimována v thymu, určité transkripční faktory, jako je AIRE [Anderson et al., 2005] , jsou aktivní v thymických buňkách a navozují expresi genů, které se normálně exprimují pouze v určitých tkáních. Thymus je tedy schopen edukovat T lymfocyty na neobyčejně široké spektrum antigenů. Na druhou stranu bylo také prokázáno, že některé proteiny skutečně nejsou v thymu exprimovány a přesto indukují toleranci [Ye et al., 1994]. Předpokládá se, že

tolerance vůči těmto proteinům vzniká mechanismem periferní tolerance. Nejasný je ale způsob, jakým rozliší APC v lymfatické tkáni původ antigenů – musí existovat nějaký „signál“, který označí krosprezentované antigeny jako cizí a odliší je tak od krosprezentovaných autoantigenů.

Ukazuje se, že funkci tohoto „signálu“ přebírá systém vrozené imunity, který rozpoznává patogeny prostřednictvím skupiny receptorů označovaných jako PRR (pattern recognition receptors), tyto receptory rozpoznávají specifické molekulární struktury přítomné v mikroorganismech (PAMP – pathogen associated molecular patterns) . Podle modelu navrženého Janewayem, aktivace PRR přítomných na povrchu APC vede k indukci povrchové exprese kostimulačních molekul (CD80, CD86, CD40) a navození „maturace“ APC [Janeway and Medzhitov, 2002]. Teprve po této aktivaci jsou APC schopné plně aktivovat T lymfocyty a navodit imunitu, nezralé APC jsou namísto toho tolerogenní. Objev Toll-like receptorů, které specificky rozpoznávají PAMP je důkazem platnosti této teorie a ukazuje, že systémy vrozené a adaptivní imunity jsou neobyčejně těsně propojeny.

Profesionální APC, termín navržený Polly Matzinger [Matzinger, 1994], mezi které patří dendritické buňky jsou primárním typem buněk, který integruje adaptivní a vrozenou imunitu. Za normálního ustáleného stavu DC fagocytují tkáňové autoantigeny pocházející z apoptotických buněk. Narozdíl od nekrotických buněk nebo obecně buněk odumřelých v důsledku infekce, tyto apoptotické buňky neaktivují DC a proces krosprezentace tkáňových autoantigenů za této situace probíhá nezánětlivě bez aktivace systémů vrozené imunity [Steinman et al., 2000; Heath and Carbone, 2001]. DC prezentující tkáňové autoantigeny mají zvýšenou produkci inhibičních cytokinů jako jsou TGFβ, IL-10 a PGE_2 . Indukce těchto protizánětlivých cytokinů se uskutečňuje prostředictvím aktivace receptorů jako jsou CD36, trombospondin, integriny vβ3 a vβ5, pentraxiny, scavenger receptors a řada dalších, které specificky rozpoznávají produkty apoptotických buněk [Savill et al., 1992; Albert et al., 1998].

DC mají schopnost velmi citlivě detekovat přítomnost nekrotických buněk. Nekróza nebo apoptóza indukovaná virovou infekcí je známka porušení integrity organismu a představuje pro imunitní systém jakýsi „Danger signal“ který je detekován na povrchu DC specifickými receptory. Tyto receptory rozpoznávají proteiny tepelného šoku (gp96, hsp90, hsp70, kalretikulin), které se v průběhu smrti buňky dostávají do extracelulárního prostředí a aktivují např. receptor CD91 na povrchu DC [Basu et al., 2001]. Mezi další produkty

uvolňující se z nekrotických buněk s podobnou funkcí patří kyselina močová [Shi et al., 2003]. Podobně jako aktivace PRR mikrobiálními produkty, má tedy nekróza tkáně za následek maturaci APC. Původ antigenů (apototické vs. nekrotické buňky) je primární faktor určující rozvoj imunity nebo tolerance [Sauter et al., 2000]. Jsou-li T lymfocyty aktivovány bez účasti signálů vrozené imunity nastane tolerance a k tomu právě dochází při periferní toleranci, tkáňové autoantigeny jsou kontinuálně krosprezentovány a vystaveny na povrchu APC aniž by došlo k aktivaci systémů vrozené imunity, protože tento proces probíhá přirozeně a nezánětlivě.

Jsou DC jediným typem buněk zodpovědným za periferní toleranci? Intenzivně diskutovaným tématem je specifický typ lymfocytů označovaný jako T-regulační lymfocyty(T_{reg}), které mají schopnost tlumit T-buněčnou imunitní odpověď. Tyto T_{reg} lymfocyty jsou charakterizovány přítomností receptorů CD4, CD25 a expresí transkripčního faktoru FoxP3, u myší tvoří cca 5-8 % celkové populace T lymfocytů [Shevach, 2000]. Přesný mechanismus suprese těmito buňkami není znám, diskutuje se exprese supresívních cytokinů TGFβ a IL-10 [Chen et al., 2005]. Nelze vyloučit ani kontaktní inhibici, nicméně není definováno prostřednictvím jakých interakcí dochází k této kontaktní inhibici. Dále není známo, jestli suprese prostřednictvím T_{reg} je antigen-specifická či polyklonální. Role T_{reg} v indukci suprese se dá prokázat na myších modelech, kde bylo pozorováno, že specifické odstranění T_{reg} vede k indukci buněčné imunity, která byla dříve suprimována. Podobně byla popsána role T_{reg} v protinádorové imunitě, řada experimentálních prací dokládá, že deplece T_{reg} vyvolaná aplikací anti-CD25 protilátky inhibuje růst nádorů [Ko et al., 2005].

1.8 Nádorová tolerance

Identifikace definovaných nádorových antigenů na počátku 90-tých let u pacientů s nádory dodala do té doby chybějící vědecký argument, který potvrdil smysl vakcinace proti nádorům. Teoreticky by navození imunity proti těmto antigenům mělo vést k destrukci nádorů a dlouhodobé protekci proti rekurenci nádorového obnemocnění. Bohužel realita v tomto případě neobyčejně spolehlivě odporuje teorii a přes nesmírné úsilí nebylo dosud dosaženo úspěšné léčby nádorů tímto způsobem. Více než desetileté zkušenosti s nádorovými vakcínami ukazují, že se nedaří dosáhnout konzistentně vysoké T-buněčné imunitní odpovědi po vakcinaci a nedaří se navodit regrese nádorů [Rosenberg, 2004; Rosenberg et al., 2004]. Pravděpodobné příčiny selhání protinádorové imunity spočívají v navození imunologické tolerance pro nádorové buňky a v současnosti lze formulovat několik specifických úrovní na kterých se tato suprese odehrává.

V předchozím textu byly popsány mechanismy suprese buněčné imunity za fyziologických stavů, pro porozumění mechanismům nádorové tolerance je nutné si uvědomit, že stejné mechanismy které fungují za fyziologických stavů se stejně projevují i v supresi specifické pro nádorové antigeny, neboť principielně se nádorový antigen neliší od běžného self-antigenu. Dále působí mechanismy, které se odehrávají v nádorovém stromatu a způsobují snížení protinádorové aktivity infiltrujících lymfocytů *in situ* buď přímou inhibicí jejich funkce nebo snížením exprese a prezentace nádorových antigenů nádorovou buňkou.

1.9 Mechanismy suprese protinádorové imunitní odpovědi.

1. Nedostatek vysokoaviditních protinádorových T lymfocytů.
2. Nedostatečný priming protinádorových T lymfocytů.
3. Suprese/delece aktivovaných protinádorových T lymfocytů.
4. Antigenní selekce nádorových buněk.

Ad 1 Nedostatek vysokoaviditních protinádorových T lymfocytů.

Jak bylo zmíněno výše, v thymu probíhá selekce T lymfocytů (centrální tolerance) které nejsou specifické pro self-antigeny. Vzhledem k tomu, že řada běžných nádorových antigenů jsou běžné, nemutované proteiny, dochází mechanismem centrální tolerance k odstranění vysokoaviditních T lymfocytů specifických právě pro tyto nádorové antigeny [Schell, 2004]. V repertoáru T-prekurzorů zbývají pouze T lymfocyty s nízkou aviditou, které mají vysoký aktivační práh a jejich aktivace imunizací je obtížná. Bohužel velká většina nádorově-specifických lymfocytů pravděpodobně patří do této kategorie a má-li být protinádorová vakcinace úspěšná musí být navržena tak, aby specificky aktivovala nízkoaviditní T lymfocyty. Byly vyvinuty postupy jak specificky zvýšit odpověď na tyto slabé antigenní epitopy. Změny některých aminokyselin v sekvenci epitopu mohou vést k velmi silnému zvýšení imunogenity [Slansky et al., 2000]. V takto mutovaných epitopech je provedena změna v kotvících aminokyselinách, která zvýší afinitu peptidu pro MHC molekuly aniž by došlo ke změně struktury komplexu MHC-peptid a tedy i změně specifity pro TCR. Bylo prokázáno, že imunizace pomocí těchto „modifikovaných peptidových ligandů“ může efektivně indukovat CD8+ T lymfocyty, které rozpoznají původní nádorový antigen [Slansky et al., 2000]. Dále je možné takto mutovaný epitop exprimovat jako krátký „minigen“ ve virovém vektoru [Overwijk et al., 1998]. Při imunizaci minigeny dochází k expresi pouze velmi krátkého peptidu cca 10 aminokyselin dlouhého (minigen), který je identický s imunogenním epitopem a přímo se váže na MHC I molekuly bez nutnosti být zpracován proteasomem. Nevýhodou tohoto postupu je, že každá vakcína je specifická pouze na určitý MHC haplotyp a při použití v klinice musí být navržena specificky pro jednotlivé pacienty.

Ad 2 Nedostatečná aktivace naivních protinádorových T lymfocytů.

T lymfocyty, které unikly selekci v thymu jsou v periferii vystaveny přítomnosti antigenu jednak prezentovaného na povrchu nádorových buněk a pak na povrchu APC, které krosprezentovaly nádorové buňky. Vzhledem k tomu, že tyto T lymfocyty mají nízkou aviditu, nejpravděpodobnějším výsledkem této interakce je nedostatečná signalizace přes TCR bez navození aktivace T lymfocytů [Lyman et al., 2005]. Tento stav se označuje jako imunologická ignorance [Soldevila et al., 1995] a primárně závisí na dvou parametrech: aviditě T buněk a množství prezentovaného antigenu. Avidita T buněk je neměnná a závislá na thymické selekci, ale velmi snadno může dojít ke změnám v množství antigenu, který je krosprezentován pomocí APC. K tomu typicky dochází při růstu nádorů a bylo opakovaně prokázáno na myších modelech, že ignorance nádorových antigenů může být překonána růstem nádoru [Kurts et al., 1999]. Od určité velikosti tak nádor jednoduše přestane být „neviditelný“ pro imunitní systém. Rostoucí nádory tedy mohou aktivovat nádorově-specifické T lymfocyty překonáním imunologické ignorance, avšak tato aktivace nemá za následek klonální expanzi, ale je abortivní v důsledku rozvoje periferní tolerance [Lyman et al., 2004]. Až na diskutabilní vyjímky, rostoucí nádory spontánně neindukují specifické T lymfocyty. Pravděpodobně také nedochází k aktivaci nádorově-specifických CD8+ T lymfocytů antigeny prezentovanými na povrchu nádorových buněk (přímá prezentace), protože naivní T lymfocyty potřebují být aktivovány profesionálními APC, které mají na povrchu kostimulační molekuly. Nádorové buňky tyto kostimulační molekuly nemají a je tedy nepravděpodobné, že by mohlo dojít k iniciaci imunitní odpovědi na úrovni nádorových buněk [Huang et al., 1994].

Ad 3 Suprese/delece aktivovaných protinádorových T lymfocytů.

Dojde-li k aktivaci T lymfocytů specifických pro nádorové antigeny následkem vakcinace, okamžitě se stanou tyto aktivované T lymfocyty vnímavé k homeostatickým imunitním mechanismům zabraňujícím rozvoji autoimunity. K supresi dochází nejen periferní tolerancí jak bylo popsáno v detailu výše ale také přímou supresí v nádorovém stromatu. Mechanismus této nádorové suprese (v užším smyslu) lze rozdělit na parakrinní působení inhibičních cytokinů (např. TGFβ, IL-10) a na inhibici v důsledku přímých buněčných interakcí. Studium nádorů v minulosti se klasicky zaměřovalo na analýzu pouze nádorových buněk a studium nenádorových buněk přítomných v nádorové tkáni bylo opomíjeno. Podle

současného pohledu je ale nezbytné pro pochopení interakce nádoru s okolní zdravou tkání porozuměr roli infiltrujících buněk.

V určité fázi nádorového bujení začnou nádorové buňky prostřednictvím zánětlivé reakce ovlivňovat okolní tkáně a začnou atrahovat mesenchymální buňky [Blankenstein, 2005]. Nádorové stroma je směs těchto infiltrujících buněk vytvářejících podporu pro růst nádoru zejména indukcí angiogeneze. Ve stromatu nádorů jsou dále obsaženy buňky leukocytárního původu, které fagocytují nekrotickou nádorovou tkáň (makrofágy, dendritické buňky) a zároveň mají schopnost tyto antigeny prezentovat T lymfocytům. Většina nádorů také obsahuje infiltrující CD4+ a CD8+ T lymfocyty, NK buňky a T-regulační lymfocyty. Buňky navzájem interagují prostřednictvým přímých buněčných spojů a mají schopnost produkce mnoha cytokinů, toto komplexní buněčné zastoupení vytváří tzv. nádorový mikroenvironment [Bissell and Radisky, 2001]. Studujeme-li tedy interakci T lymfocytů specifických pro nádorové antigeny s nádorovou tkání, tak musíme vzít v úvahu, že T lymfocyty v nádorech jsou ovlivněny jednak přímo maligními buňkami prezentující své nádorove antigeny na povrchu ve vazbě na MHC I a interakcemi s buňkami nádorového stromatu.

Nádorové buňky mohou mít na svém povrchu atypické kostimulační molekuly jako je např. PD-L1, která inhibuje aktivaci CD8+T lymfocytů [Dong et al., 2002]. Podobným způsobem funguje galektin-1 přítomný na povrchu nádorů [Rubinstein et al., 2004], nebo produkce enzymů arginasy či indoleamin dioxygenasy , které způsobí lokální depleci argininu a tryptofanu, respektive, což má také negativní vliv na proliferační schopnosti aktivovaných T lymfocytů [Bronte and Zanovello, 2005; Muller et al., 2005]. U mnoha nádorů bylo dále prokázáno, že mohou exprimovat molekuly Fas, které indukují apoptózu aktivovaných T lymfocytů [Whiteside, 2002]. Nedávno bylo popsáno, že nádorové buňky exprimují konstitutivně aktivovaný protein STAT3 [Wang et al., 2004], který dosud ne zcela přesně známým mechanismem inhibuje maturaci DC a produkci prozánětlivých chemokinů a cytokinů.

Aktivované T lymfocyty mohou být tlumeny v nádorech působením supresorových lymfocytů – nyní přejmenovaných na T regulační lymfocyty [Chen et al., 2005]. Řada studií přesvědčivě ukázala, že lidské nádory jsou infiltrovány T_{reg} a na myších modelech byla prokázaná jejich role v nádorové supresi [Woo et al., 2002]. Inhibice T lymfocytů může být

indukována jednak přímými buněčnými interakcemi s výše popsanými typy buněk, dále ale může být suprese vyvolána působením cytokinů a chemokinů. Na prvním místě z těchto inhibičních faktorů je nepochybně TGFβ. Tento velmi komplexně působící cytokin může být produkován inhibičními DC, T-regulačními lymfocyty, nebo přímo nádorovými buňkami. Studie ukazují, že je-li v nádorově-specifických lymfocytech přerušena siganlizace přes TGFβ receptor (např. expresí dominantně-negativního receptoru), je velmi silně inhibována vnímavost T lymocytů k nádorové supresi [Gorelik and Flavell, 2001; Chen et al., 2005].

Několik studií naznačilo, že T-lymfocyty infiltrující nádory mohou být inhibovány specifickým subtypem buněk označovaných jako myeloidní supresorové buňky. Jedná se o heterogení skupinu buněk, která je charakterizována expresí povrchových znaků CD11b a Gr-1[Bronte et al., 2000; Bronte et al., 2001]. Je prokázané, že tato populace způsobuje dysfunkci T-buněčné imunity jak v experimentáních myších modelech, tak i u pacientů s nádory. Mezi popsané mechanismy vyvolávající supresi T lymfocytů patří produkce enzymu arginasy nebo navození suprese přímým buněčným kontaktem. Kromě výše zmíněných myeloidních supresorových buněk je v současnoti popsána řada subtypů myeloidních buněk s inhibičními vlastnosmi jako jsou M2-polarizované makrofágy, či nezralé dendritické buňky [Kusmartsev and Gabrilovich, 2002; Mantovani et al., 2002]. Je diskutabilní, jestli se jedná o různé typy buněk či to jsou naopak různá diferenciační stadia či různé fenotypy jednoho „univerzálního“ typu buněk myeloidního původu.

T lymfocyty specifické pro nádorové antigeny působí nejen na maligní buňky ale i na buňky stromatu. Na základě vakcinačních experimentů na myších bylo prokázáno, že regrese nádorů vyvolaná T lymfocyty, které byly aktivované imunizací je výsledkem nejen přímé cytotoxické aktivity CD8+ T lymfocytů vůči nádorovým buňkám. Ukázal velmi zajímavý fakt, že T lymfocyty (CD4+ a CD8+) působí na nádorové stroma prostřednictvím IFN-γ, který aktivuje infiltrující makrofágy krospresentující nádorové antigeny [Qin and Blankenstein, 2000; Schuler and Blankenstein, 2003]. Podobnou úlohu jako má IFN-γ se připisuje i cytokinu TNFα, který je také produkován aktivovanými CD8+ T lymfocyty. Nádorové stroma tvořené nemaligními buňkami je tedy zcela esenciální pro růst nádoru a zároveň je to i místo, na které velmi účinně působí imunoterapie.

Ad 4 Antigenní selekce nádorových buněk.

Proliferující nádorové buňky dynamicky reagují na okolní podmínky a v nádoru se tak selektuje populace buněk, které mají největší schopnost růstu a tvorby metastáz. Jedním z těchto selekčních tlaků je i protinádorová imunita. Je logické, že čím méně bude nádor imunogenní, tím méně bude i vnímavý na možnou imunitní reakci. Nádory mají schopnost unikat imunitnímu systému v důsledku změn exprese nádorových antigenů nebo snížením jejich prezentace. CD8+ T lymfocyty indukované na nádorové antigeny rozpoznávají pouze několik hlavních imunodominantních epitopů prezentovaných nádorovou buňkou. Změny exprese antigenů ale i bodové mutace v imunodominantních epitopech mají za následek vznik rezistence nádorových buněk na CD8+ T-buněčnou imunitu. Původní klon nádorových buněk tedy může dát vzniku tzv. ztrátovým variantám, které neexprimují nádorový antigen (antigen-loss) nebo exprimují variantu antigenu, který obsahuje mutaci v imunogenních epitopech (epitope-loss) [Lill et al., 1992].

Další mechanismus, který snižuje imunogenitu nádorových buněk jsou mutace a změny exprese genů důležitých pro prezentaci nádorových antigenů. Opakovaně bylo popsáno, že nádorové klony se mohou velmi výrazně lišit množstvím povrchových molekul MHC I [Marincola et al., 2000]. Tyto MHC I-deficientní klony mají opět selektívní výhodu a preferenčně mohou dát vzniku nádorům se sníženou expresí MHC I molekul. Podobným způsobem jako je nízká exprese MHC I molekul fungují alterace v genech pro zpracování a prezentaci antigenů. Velmi často lze detekovat mutace v peptidových transportérech TAP [Cromme et al., 1994], které dopravují peptidy generované proteazomem do ER, kde dojde k vazbě na MHC I. Snížená exprese TAP 1 a 2 proteinů má dále za následek i snížení celkové exprese MHC I neboť, MHC I na povrchu buněk jsou nestabilní bez navázaných peptidů a spontánně denaturují.

2. Výsledky

2.1 Komplexní pohled na indukci CD8+ T lymfocytů na modelu SV40 T Ag.

Publikace:

Inefficient Cross-Presentation Limits the CD8+ T Cell Response to a Subdominant Tumor Antigen Epitope.

Pavel Otahal, Sandra C. Hutchinson, Lawrence M. Mylin, M. Judith Tevethia, Satvir S. Tevethia, and Todd D. Schell.

Journal of Immunology, 2005, 175: 700–712.

Definování principů ustanovení hierarchie v CD8 buněčné imunitní odpovědi je nezbytné pro porozumění principům rozvoje nádorové tolerance a pro vývoj účinných protinádorových vakcín. Přestože je tato oblast imunologie velmi intenzívně studována, mnoho otázek je stále nezodpovězených. Jednou z takovýchto otázek je, jaký význam má krosprezentace antigenů pro indukci CD8+ T lymfocytů specifických pro imunorecesívní epitopy. Respektive, lze vysvětlit příčiny imunorecesivity na úrovni krosprezentace antigenů. Pochopení příčin imunorecesivity umožní vývoj vakcinačních strategii, které specificky aktivují tyto slabě reagující CD8+ T lymfocyty, které jediné zbývají v repertoáru prekurzorů a mohou být využity pro imunoterapii nádorů.

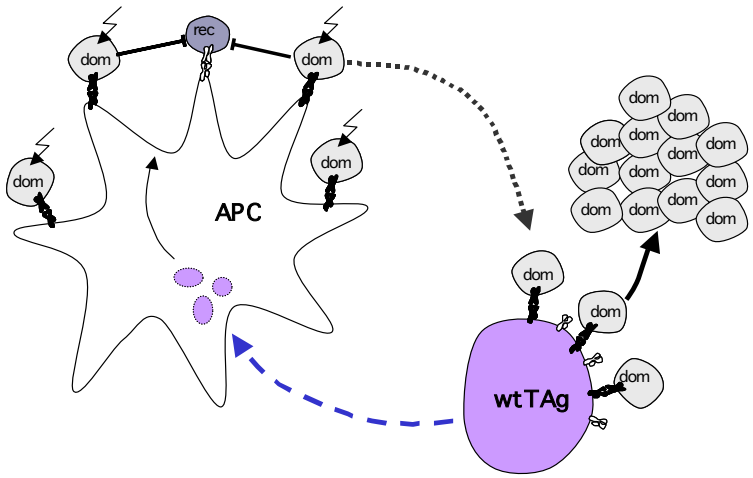
V současné experimentální imunologii jsou nezbytným nástrojem pro studium definované antigenní modely. Z ryze praktických důvodů se jako modelový organismus používají myší inbrední linie, které mají definovaný MHC haplotyp a modelové antigeny, v kterých byly identifikovány hlavní CD8 a CD4 epitopy. V našem modelu jsme analyzovali odpověď C57BL/6 myší (H2-b) na imunizaci syngenními buňkami transformovanými SV40 T Antigenem (T Ag). Specificky nás zajímalo, jestli imunorecesivita T Ag epitopu V může být vysvětlena nedostatečnou krosprezentací a na základě získaných výsledků jsme formulovali

následující model aktivace CD8+ T lymfocytů, který vysvětluje příčiny dominantními a recesívní imunitní odpověď na SV40 T antigen.

Imunizace buňkami transformovanými T-antigenem je specifická v tom, že T Ag je velmi silně imunogenní, takže přesto že se imunizuje syngenními nádorovými buňkami, nedojde k tvorbě nádorů, protože T Ag-specifické CD8+ T lymfocyty eliminují injektované buňky. Pro porovnání účinnosti krosperezentace na aktivaci T Ag-specifických lymfocytů jsme využili faktu, že B6-derivované myší buňky, které nemají TAP1 protein nejsou schopné generovat komplexy MHC I-peptid a tyto buňky tedy nejsou schopné přímé aktivace CD8+ T lymfocytů (direct priming), veškerá zjištěná aktivace je výsledkem krosprezentace [Yewdell et al., 1993]. Účinnost imunizace T Ag-transformovanými buňkami jsme zjišťovali prostřednictvím TCR transgenních myší, které jsou specifické pro epitop I (TCR-I) nebo epitop V (TCR-V) z T Ag. Epitop V je imunorecesívní, což bylo zjištěno po imunizaci mutovaným T Ag z kterého byly odstraněny dominantní epitopy (I, II/III, IV), pouze tento mutovaný T Ag, dále označovaný ΔT Ag, navodí účinnou expanzi CD8+ T lymfocytů specifických pro epitop V. V naší publikaci jsem tedy posuzoval, proč wild-type T Ag (wt T Ag) slabě aktivuje TCR-V zatímco ΔT Ag je aktivuje dostatečně.

A. Aktivace imunodominantních CD8+ T lymfocytů.

Obr. 3



Dom - CD8+ T lymfocyty specifické na dominantní MHC I epitopy.

Rec - CD8+ T lymfocyty specifické na recesívní MHC I epitopy.

APC - antigen prezentující buňka.

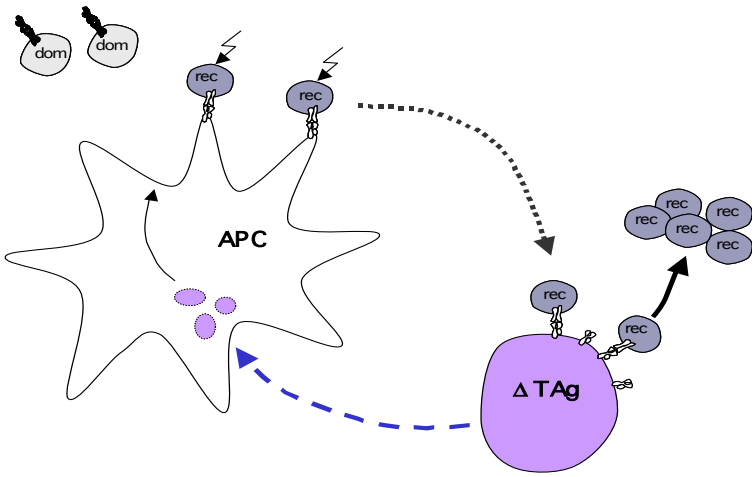
wt T Ag - nádorová buňka transformovaná wt T Ag .

Po imunizaci buňkami transformovanými wt T Ag dojde k jejich rychlé migraci do spádových lymfatických uzlin během cca 1-2 dnů, kde mohou být snadno detekovány (nepublikovaná data). Následně je antigenní substrát fagocytován profesionálními APC a zpracován na antigenní peptidy, které se objeví na buněčném povrchu ve vazbě na molekuly H2-Db a H2-Kb. Tyto komplexy jsou rozpoznány CD8+ T lymfocyty specifickými buď pro dominantní epitopy Db/I a Kb/IV nebo pro recesívní epitop Db/V. Výsledkem této interakce je buněčná aktivace a v tomto okamžiku dojde k první kompetici mezi dominantními (dom) a recesívními (rec) CD8+ T lymfocyty. Preferenčně nastane aktivace dominantních CD8+ T lymfocytů, kvůli většímu množství přítomných komplexů MHC-peptid, neboť komplexy Db/V jsou méně stabilní a rychleji mizí z buněčného povrchu. Aktivované dominantní CD8+ T lymfocyty následně inhibují recesívní CD8+ T lymfocyty aktivované stejnou APC – dojke ke kompetici o antigen na úrovni APC. Aktivované dominantní CD8+ T lymfocyty dále omezí schopnost APC aktivovat další CD8, neboli tzv. „vypnou“ schopnost prezentovan antigeny (používá se také termín „APC shut-off“) [van Stipdonk et al., 2003]. Pro aktivaci CD8+ T lymfocytů je tedy k dispozici relativně úzké časové období, které účiněji využijí dominantní CD8+ T lymfocyty. Další proliferace již nevyžaduje přítomnost antigenu, neboť plně aktivované CD8+ T lymfocyty iniciují program tzv. antigen independentní proliferace.

Kromě kompetice na úrovni APC muže dojít ke kompetici na úrovni nádorových buněk, které přímo prezentují T Ag. Aktivované dominantní CD8+ T lymfocyty rozpoznají T Ag-transformované buňky přítomné v lymfatických uzlinách a tento kontakt je zdrojem další aktivace, která výsledně amplifikuje celkovou imunitní odpověď na dominantní epitopy. Tyto děje se mohou odehrávat takřka současně, neboť všechny zůčastněné buňky se nacházejí v těsném kontaktu ve spádových lymfatických uzlinách a nelze vyloučit tvorbu vícebuněčných klastrů tvořených právě CD8+ lymfocyty, APC, a T Ag-transformovanými buňkami. Příčinou, proč při imunizaci s wt T Ag nedojde k účinné aktivaci recesivních CD8+ T lymfocytů specifických pro T Ag epitop V je tedy kompetice s T Ag-specifickými dominantními CD8+ T lymfocyty na úrovní APC a na úrovni nádorové buňky.

B. Aktivace imunorecesívních CD8+ T lymfocytů.

Obr. 4



Dom - CD8+ T lymfocyty specifické na dominantní MHC I epitopy.

Rec - CD8+ T lymfocyty specifické na recesívní MHC I epitopy.

APC - antigen prezentující buňka.

Δ T Ag - nádorová buňka transformovaná mutovaným T Ag obsahujícím pouze recesívní epitop Db/V.

Proč ale dojde k účinné expanzi recesívních CD8+ T lymfocytů specifických pro epitop V při imunizaci mutovaným ΔT Ag z kterého byly odstraněny dominantní epitopy I, II/III a IV ? Předchozí publikace opakovaně prokázaly, že vlastnosti epitopu V generovaného z ΔT Ag jsou totožné jako vlastnosti epitopu V generovaného z wt T Ag, a že epitop V je účinně generován jak z tak z wt T Ag. Buňky transformované wt T Ag jsou rozpoznány v cytotoxickém testu CTL klony specifickými jak pro dominantní epitopy I II/III a IV tak CTL klonem specifickým pro epitop V. Významným rozdílem mezi epitopem V a dominantními epitopy I a IV však je, že komplexy Db/V jsou významně méně stabilní než komplexy Db/I a Kb/IV. Je-li limitující množství antigenu, bude k dispozici méně komplexů Db/V než komplexů Db/I a Kb/IV.

Po imunizaci buňkami transformovanými ΔT Ag dojde také k jejich migraci do lymfatických uzlin a následně jsou krosprezentované prostřednictvím APC v uzlinách. Počáteční krosprezentace antigenu je zásadní pro aktivaci naivních CD8 lymfocytů, naše výsledky i data jiných autorů pracujících s obdobnými modely ukazují, že jsou to pouze profesionální APC které mohou aktivovat naivní CD8+ T lymfocyty.

Zjistili jsme, že tato fáze indukce T Ag-specifické imunitní odpovědi se velmi liší pro dominantní epitop Db/I a recesivní epitop Db/V. Zatímco epitop Db/I byl krosprezentován

velmi efektivně, tak epitop Db/V naopak velmi slabě. Jak ale tedy může dojít k aktivaci CD8+ T lymfocytů specifických pro epitop Db/V je-li krosperezntace velmi slabá a zároveň bylo jasně prokázáno, že bez účinné krosprezentace nedojde k indukci imunitní odpovědi? Je-li imunizace provedena mutovaným ΔT Ag, který neopbsahuje dominantní epitopy, tak i výsledná velmi neúčiná krosprezentace epitopu Db/V může navodit částečnou aktivaci CD8+ T lymfocytů specifických pro epitop Db/V, neboť recesívní DC8+ T lymfocyty nejsou inhibovány dominantními CD8+ T lymfocyty a mají dostatečný prostor na expanzi. Zároveň tyto částečně aktivované CD8+ T lymfocyty specifické pro epitop Db/V rozpoznávají buňky transformované T Ag, které jsou přítomné v těsné blízkosti. Tento kontak, který se odehrává pravděpodobně bez iniciace kostimulačních signalizačních kaskád poskytne další aktivační signály, které navodí klonální expanzi. Přestože je tedy krosprezentace epitopu Db/V neúčinná, absence kompetujících dominantních CD8+ T lymfocytů neihibuje aktivaci recesívních CD8+ T lymfocytů je-li imunizováno mutovaným ΔT Ag.

Naše práce identifikovala nedostatečnou krosprezentaci recesívních epitopů jako nový faktor způsobující imunorecesivitu v CD8 buněčné imunitní odpovědi. Toto zjištění může mít velký význam pro vývoj vakcín, které mají za úkol aktivovat málo imunogenní či imunorecesívní epitopy obsažené v nádorových antigenech. Analýzy imunitní odpovědi specifické na nádorové antigeny ukazují, že slabě krosprezentované epitopy mohou být méně citlivé k mechanismům imunologické tolerance a proto je imunitní odpověď specifická na tyto epitopy považována za výhodný cíl protinádorové imunoterapie. Naše data dále naznačují, že pro aktivaci imunorecesívních epitopů je výhodné použít epitop-specifickou imunizaci například pomocí minigenů, která redukuje ustanovení imunologické hierarchie jejímž důsledkem by byla suprese slabě krosprezentovaných imunorecesívních epitopů.

2.2 Rozvoj periferní tolerance CD8+ T lymfocytů během T Ag-indukované spontánní karcinogeneze.

Publikace:

Early Immunization Induces Persistent Tumor-Infiltrating CD8+ T Cells against an Immunodominant Epitope and Promotes Life-long Control of Pancreatic Tumor Progression in SV40 T Antigen Transgenic Mice.

Pavel Otahal, Todd D. Schell, Sandra C. Hutchinson, Barbara B. Knowles, and Satvir S.Tevethia.

Publikace v recenzním řízení (odesláno do tisku 10.1. 2006)

SV 40 nádorový antigen (T Ag) velmi spolehlivě indukuje tvorbu nádorů v myších je-li exprimován jako transgen. V uplynulých dvou desetiletích bylo zkonstruováno mnoho T Ag-transgenních myší u kterých došlo k tvorbě spontánních nádorů v závislosti na tkáňové expresi T Ag. Jednou z těchto linií je myší kmen Rip1-TAg4 (RT4). Tato linie exprimuje T Ag pod kontrolou inzulínového promotoru, který směřuje produkci T Ag do beta buněk v pankreatu. V průběhu vývoje dochází k postupnému nárůstu exprese T Ag což indukuje postupnou transformaci beta buněk a tvorbu nádorů u 100% myší homozygotních pro T Ag. Hyperporodukce inzulínu vede k rozvoji fatální hypoglykemie, průměrná doba přežití RT4 myší je 200 dnů. Tento proces onkogeneze je postupný a velmi podobný přirozené onkogenezi pozorované u lidí.

Exprese T Ag začíná u RT4 myší asi okolo 4 týdnů po narození a protože T Ag není exprimován v průběhu časné ontogeneze v thymu, nedochází k rozvoji centrální tolerance. RT4 myši jsou tedy schopné navodit CD8 odpověď po imunizaci specifické na T Ag. Podobný kmen myší Rip1-Tag1(RT1) je zcela tolerantní na T Ag, protože T Ag je exprimovaný v thymu. Příčina rozdílné exprese T Ag mezi RT4 a RT1 myšmi je dána pravděpodobně odlišnou integrací transgenu (který je stejný) a která má za následek časnější nástup exprese T Ag u RT1 myší. U RT4 myší nedochází k rozvoji centrální tolerance na T Ag, ale CD8+ T

lymfocyty specifické na dominantní epitopy Db/I a Kb/IV jsou suprimovány mechanismy periferní tolerance v důsledku exprese T Ag v periferních tkáních.

Naší primární otázkou bylo, jestli v ustanovení T Ag-specifické periferní tolerance lze pozorovat hierarchii mezi jednotlivými T Ag epitopy a dále jsme studovali, jestli lze inhibovat růst spontánních nádorů imunizací proti T Ag. Naše výsledky ukázaly, že CD8+ T lymfocyty specifické pro imunodominantní epitopy v T-antigenu jsou rozdílně citlivé k periferní toleranci. Imunizace RT4 myší pomocí T Ag-transformovaných buněk vedla k indukci CD8+ T lymfocytů specifických pro epitop Kb/IV a tato odpověď se snižovala s věkem a postupnou progresí nádorů, ale i u myší s nádory se podařilo izolovat plně funkční CD8+ T lymfocytů specifické na epitop Kb/IV přímo z nádorů. To naznačuje, že tyto CD8+ T lymfocyty nebyly suprimovány periferní tolerancí, ale nádory se místo toho staly rezistentní na imunitní odpověď. V kontrastu s imunitní odpovědí specifickou na epitop Kb/IV je schopnost RT4 myší navodit imunitní odpověď na epitop Db/I. Imunizace pomocí T Ag-transformovaných buněk nevede k indukci CD8+ T lymfocytů specifických na epitop Db/I u myší, které jinak odpovídají na epitop Kb/IV. Dokonce i velmi mladé RT4 myši bez vytvořených nádorů mají téměř nedetekovatelnou imunitní odpověď specifickou na epitop Db/I. Tento poznatek naznačuje, že v důsledku exprese T Ag v periferii se tlumí velmi výrazně CD8+ T lymfocyty specifické na epitop Db/I aniž by došlo k supresi CD8+ T lymfocytů specifických na epitop Kb/IV.

Současně naše data ukázala, že pro inhibici růstu spontánních nádorů je nezbytné, aby T Ag-specifická imunizace byla provedena před nástupem exprese T Ag v periferii – ke které dochází okolo 4 týdnů věku RT4 myší. Imunizace v pozdějším období nevedla ke zvýšení přežití RT4 myší, přestože imunizace navodila dostatek CD8+ T lymfocytů specifických pro T Ag. Tento fakt lze vysvětlit kombinací několika mechanismů. Primární bude pravděpodobně rozvoj rezistence nádorů k cytotoxickým CD8+ T lymfocytům specifickým na T Ag, což koresponduje s obecnou hypotézou úniku nádorových buněk imunitnímu systému. T Ag-specifické CD8+ T lymfocyty mají schopnost zabránit vývoji nádoru ale pouze ve velmi časné fázi, je-li nádor již vytvořen, imunizace je neúčinná. Proti této hypotéze ale částečně stojí fakt, že účinnost časné imunizace nekoreluje s objevením mikroskopických nádorů (cca ve 3 měsících věku RT4 myší), ale s nástupem exprese T Ag v periferii (cca ve 4 týdnech věku).

Imunizace např. v 9 týdnech nemá protektivní účinek přestože RT4 myši v tomto věku mají minimum T Ag-pozitivních buněk v pankreatu.

Jaký to má význam pro imunoterapii lidských nádorů? Popsanou situaci si lze velmi názorně představit při skutečné imunoterapii lidských nádorů. Stejně tak jako u T Ag, většina lidských nádorových antigenů pravděpodobně obsahuje více než jeden imunogenní epitop. Variabilita mezi individuálními pacienty bude navíc umocněna rozdíly v HLA alelách, s tím, že pro každý haplotyp bude specifická naprosto odlišná hierarchie mnoha různých epitopů. Do tohoto scénáře vstupuje další proměnná a tou je variabilita mezi nádorovými antigeny. Nádory obvykle exprimují několik nádorových antigenů zároveň a exprese těchto antigenů se může významně lišit mezi individuálními pacienty postiženými stejným typem nádoru. Lze očekávat, že v důsledku této variability někteří pacienti mohou indukovat velmi efektivní imunitní odpověď zatímco u jiných pacientů bude odpověď na stejný antigen nízká právě z důvodů odlišné hierarchie. Dále lze velmi obtížně predikovat do jaké míry bude navozená odpověď suprimována periferní tolerancí, může se vyskytovat situace ekvivalentní epitopu I anebo epitopu IV. S jistotou lze tvrdit, že vakcinací pacientů lze obecně indukovat nádorově-specifické T lymfocyty. Nevyřešeným problémem však zůstává, zda-li jsou tyto nádorově-specifické CD8+ T lymfocyty cytotoxické in situ a mají dostatečnou schopnost navodit regresi nádorů.

2.3 Vakcíny proti nádorům vyvolaným HPV.

Publikace:

Immune response to E7 protein of human papillomavirus type 16 anchored on the cell surface.

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Cancer Immunol Immunother, 2002, 51: 111–119

Účinná protinádorová vakcinace musí navodit dostatečnou expanzi endogenních protinádorových CD8+ T lymfocytů, které pro trvalou protekci musí vytvořit paměťové buňky. Nicméně jak bylo popsáno výše, dosažení tohoto stavu je obtížné, ne-li nemožné současnými postupy. Nejjednodušeji ovlivnitelným faktorem, který má zásadní úlohu v indukci protinádorové imunity je charakter a způsob vakcinace. Ačkoliv je známé již od 70tých let, že lze u křečků a myší vyvolat imunizací efektivní protekci růstu nádorů, skutečně přelomovým poznatkem byla identifikace nádorových antigenů na počátku 90tých let. Známe-li protein, který terčem imunitní odpovědi při rozvoji nádorového onemocnění, lze tento protein (=nádorový antigen) využít pro imunizaci. Alternativou k tomuto přístupu je imunizace prostřednictvím směsi necharakterizovaných nádorových antigenů, tak jak se to provádí např. při imunizaci nádorovými lyzáty. Není snadné posoudit, která forma antigenu je výhodnější pro imunizaci pacientů, v současné době je ale odklon od používání nepurifikovaných nádorových lyzátů, protože nelze exaktně definovat jaké antigeny a v jakém množství obsahuje. Seznam známých nádorových antigenů obsahuje velmi mnoho proteinů a neustále roste [Novellino et al., 2005]. Nepochybně bude možné v blízké budoucnosti detailně určit u velké většiny lidských nádorů, které nádorové antigeny jsou exprimovány.

Známe-li nádorový antigen, můžeme přistoupit k imunizaci mnoha způsoby. Mezi nejčatěji používané a nejúčinější metody patří imunizace pomocí rekombinantních virů, které exprimují tento nádorový antigen. Dále lze s úspěchem použít imunizaci pomocí syngenních či allogeních buněk a imnunizaci pomocí DNA vakcín. K dalším velmi používaným způsobům patří imunizace pomocí antigenních peptidů buď podaných přímo s adjuvans nebo ve formě

pulzovaných autologních APC, jako jsou třeba DC. Imunizace pomocí DC je velmi moderní metoda nádorové imunoterapie a zároveň je to metoda velmi univerzální neboť lze použít jakoukoliv formu antigenu. Autologní DC mohou být transdukovány virovým vektorem, nebo inkubovány v přítomnosti nádorového lyzátu, proteinu či přímo s antigenními peptidy. Následně jsou DC podány pacientům s cílem navodit imunitní odpověď. Přes toto široké množství způsobů vakcinace, není žádný z nich dostatečně účinný a nevede k indukci efektivní protinádorové imunity.

Virové vektory jsou nejefektivnější protinádorové vakcíny. Mezi nejpoužívanější virové vektory patří hlavně virus vakcínie (VV) a z něho odvozené modifikované vektory [Moss, 1996]. VV má mnoho výhod, je velmi bezpečný a byl prověřen mnoha milióny jedinců, kteří byli imunizováni proti pravým neštovicím. Následnými modifikacemi VV byly vyvinuty ještě bezpečnější virové vektory, které se nereplikují v lidském organismu a zároveň jsou dostatečně imunogenní . Tento fakt zásadním způsobem zvyšuje bezpečnost vektoru, protože ho lze aplikovat i imunodeficientním pacientům, u kterých by replikující se vektor mohl způsobit závažné komplikace. Mezi tyto vektory patří hlavně MVA, ALVAC, NYVAC [Cox et al., 1993; Carroll et al., 1997; de Bruyn et al., 2004]. Viry vakcínie jsou výhodné pro konstrukci rekombinantních vakcín nejen pro dobrou imunogenitu a vysokou bezpečnost, ale i pro relativní jednoduchost vkládání cizích genů do genomu VV a pro dostatečnou kapacitu VV (cca 25 kB) pojmout vložené geny.

Onkogeneze epiteliálních buněk děložního čípku je způsobena produkty genů HPV, tyto časné proteiny zejména E5, E6, E7 jsou exprimovány keratinocyty infikovanými HPV, což má za následek maligní transformaci, ale zároveň tyto virové produkty jsou cílem pro buněčnou imunitní odpověď. Vakcinace proti HPV, která indukuje CD8-specifickou imunitní odpověď specifickou na onkoproteiny E6 a E7 je principem terapeutických vakcín proti HPV-asociovaným nádorům . HPV exprimuje kromě časných nestrukturálních proteinů i proteiny podzní, které tvoří kapsidu viru. Tyto proteiny L1 a L2 mohou navodit tvorbu neutralizačních protilátek po infekci HPV, čehož lze využít při preventivním způsobu vakcinace. L1 a L2 asociují in vitro do pseudovirionů, které nejsou infekční, protože neobsahují virovou DNA, ale navodí tvorbu neutralizačních protilátek proti infekčním HPV. Vakcinace pomocí VLP může

zabránit infekci HPV a tak nepřímo zabránit tvorbě nádorů, v současné době je k dispozici komerční vakcína na bázi VLP.

Terapeutické vakcíny proti HPV-asociovaným nádorům musí ale vyvolat CD8 buněčnu imunitní odpověď proti časným proteinům E6 a E7, které způsobují maligní transfomaci infikovaných keratinocytů. Jeden z možných přístupů je exprese proteinu E7 ve virovém vektoru, jakým je např. VV. Testování účinnosti experimentálních vakcín proti HPV nádorům je nezbytné provádět v imunologicky definovaném prostředí. E7 protein je imunogení pro myši, C57BL/6 kmen tvoří CD8+T lymfocyty specifické na epitop RAHYNIVTF, který se váže na molekuly H2-Db [Feltkamp et al., 1993]. Tyto E7-specifické CD8+ T lymfocyty inhibují růst implantovaných nádorů exprimujících protein E7 in vivo. T buněčná imunitní odpověď na proteiny E6 a E7 je podle současného názoru nejdůležitějším faktorem účinné protekce, existuje řada vakcinačních strategiií, které si kladou za cíl navodit dostatek efektorových CD8+ T lymfocytů, které mají schopnost dlouhodobě přežít v oganismu a migrovat do nádorového stromatu. Mnohé z těchto vakcinačních postupů jsou zkoušeny v klinických studiích, bohužel až na výše zmíněnou vakcinaci pomocí VLP nebylo dosaženo výrazného úspěchu. Výsledný efekt vakcinace je primárně závislý na typu vakcíny a způsobu imunizace, dále ale závisí na vlastnostech antigenu, t.j. v jaké kvantitě je exprimován a jaká je jeho subcelulární lokalizace. Bylo prokázáno, že cílené směřování antigenu do některých buněčných kompartmentů může mít velmi dramatický efekt na účinnost imunizace. Např. bylo ukázáno, že směřování E7 proteinu do lysozomů, ke kterému dochází je-li E7 protein zfůzován se signální a transmembránovou sekvencí lysozomálního asociovaného proteinu vyvolá velmi silnou odpověď specifickou pro E7 [Ji et al., 1999].

V naší práci jsme si kladli otázku jakým způsobem bude změněná imunogenita E7 proteinu v závislosti na jeho subcelulární lokalizaci a testovali jsme, jestli směřování E7 proteinu na povrch buňky zvýší jeho imunogenitu. Pro expresi na povrchu buňky byl E7 protein vložen do genu A56R viru vakcínie, který kóduje virový hemaglutinin. Další virus VV-SigE7Lamp exprimuje fůzní gen mezi E7 a Lamp, který směřuje E7 do lysozomů [Ji et al., 1999] a třetí virus VV-E7 exprimuje nemodifikovaný protein E7. Naše výsledky ukázaly, že směřování proteinu E7 má vliv na charakter indukované odpovědi. Exprese E7 na povrchu buňky preferenčně indukovala Th2 imunitní odpověď zatímco viry VV-SigE7Lamp a VV-E7 naopak indukovaly Th1 imunitní odpověď. Imunizace virem VV-E7-HA nebyla účinná

v inhibici růstu E7-exprimujících nádorových buněk TC-1, což lze právě vysvětlit preferenční indukcí protilátkové a Th2 buněčné odpovědi. Polarizace imunitní odpovědi je tedy kritický parametr pro antivirovou protekci a protinádorovou imunitu. Zvolený experimentální přístup testuje rejekci transplantovaných nádorových buněk, která nastane v důsledku předchozí imunizace, toto uspořádání je tedy ekvivalentní preventívní vakcinaci. Pro léčbu pacientů s nádory je ale nezbytné, aby vakcíny navodily terapeutickou imunitu, z našich experimentů a i z publikovaných údajů je však zřejmé, že navození terapeutické imunity je mnohem obtížnější než dosažení účinné protinádorové imunity v preventivním uspořádání. Připravené virové rekombinanty mohou mít význam pro další studium Th2-polarizované imunitní odpovědi na protektivní imunitu na HPV-asociované nádory a pro vývoj účinějších protinádorových vakcín.

3. Souhrnná diskuze

3.1 Shrnutí výsledků

Disertační práce „Imunoterapie nádorů vyvolaných viry HPV16 a SV40“ studuje aspekty nádorové imunologie využitím dvou modelových systémů. První model je založený na nádorovém antigenu viru SV40 a analyzuje komplexní imunitní odpověď pro několik CD8-specifických epitopů jednak při indukci protinádorové imunity a pak také při navození nádorové suprese. Druhý model je založený na nádorovém onkogenu E7 viru HPV typ 16 a předmětem studia v tomto systému byl vývoj a testování experimentálních protinádorových vakcín na bázi rekombinantních virů vakcínie. Oba experimentální přístupy využívají velmi dobře charakterizované onkogeny malých DNA virů HPV a SV40 což spolu s dostupnostní experimentáních nástrojů umožnilo zodpovědět řadu otázek v oblasti základního výzkumu.

Prezentovaná práce přinesla řadu originálních zjištění, které jsou formulované v přiložených publikacích. Při studiu hierarchické imunitní odpovědi na T Ag jsme zjistili, že imunorecesivita epitopu Db/V je pravděpodobně způsobena kombinací dvou faktorů: neúčinné krosprezentace epitopu Db/V a kompetice dominantních a recesívních CD8 T lymfocytů. Jako první jsme popsali, že neúčinná krosprezentace může být příčinnou imunorecesivity některých epitopů a toto sdělení bylo recenzenty velmi pozitivně přijato (viz přiložený dopis). V druhé práci (odeslané do tisku) jsme popsali, že existuje i podobná hierarchie při rozvoji periferní tolerance na T Ag u myší transgenní linie Rip1-Tag4. Pozorovali jsme vyrazný rozdíl mezi dominantními epitopy Db/I a Kb/IV. Odpověď na epitop Db/I byla velmi silně suprimována zatímco odpověď na epitop Kb/IV byla přítomná. Nicméně ani přítomnost významného počtu CD8+ T lymfocytů specifických pro epitop Kb/IV nezabránila progresi nádorů, pokud byly aktivovány imunizací po nástupu exprese T Ag v periferii Rip1-Tag4 myší. Toto zjištění naznačuje, že vyvíjející se nádory se mohly stát rezistentními na Tag-specifickou imunitu. V poslední práci jsme studovali možnosti indukce protinádorové imunity pomocí vakcinace rekombinantními viry vakcínie exprimujícími protein E7 viru HPV 16. Naše výsledky ukázaly, že Th1/Th2 polarizace E7-specifické imunitní odpovědi závisí na buněčné lokalizaci exprimovaného proteinu E7. Směřování E7 proteinu na buněčnou membránu indukovalo preferenčně Th2 odpověď která nevedla k inhibici růstu E7-transformovaných buněk, zatímco

směřování E7 proteinu do lysozomů nebo do cytoplazmy bylo asociováno s rozvojem protinádorové imunity. V souhrnu naše data nepochybně přinesla zajímavé poznatky a přispěli jsme k formulování moderního pohledu na nádorovou imunoterapii.

3.2 Budoucí směr vývoje nádorové imunoterapie.

Jak se bude vyvíjet nádorová imunoterapie v budoucnosti? Pochopení, že jednoduché vakcinační postupy jsou neúčinné pro navození regrese nádorů vyprovokovalo vývoj alternativních posupů vesměs označovaných jako „komplexní“. Tyto postupy kombinují různé imunoterapeutické strategie, které mají za cíl vyvolat jednak silnou protinádorovou imunitu a dále mají za úkol aktivně zabránit rozvoji nádorové tolerance. Navození protinádorové imunity může být dosaženo vakcinačními metodikami, pro které z principu plyne, že expanze protinádorových T lymfocytů závisí na podmínkách v organismu. Limitace tohoto postupu spočívá na vlastnostech buněk v organismu účastnících se imunitní odpovědi a můžeme si tyto omezující faktory představit jako dvě nezávislé proměnné: účinnost samotné vakcinace a schopnost aktivovaných T lymfocytů zabít nádor. Mnohokrát bylo ukázáno, že přítomnost protinádorových T lymfocytů nevede k regresi nádorů a podobně často bylo ukázáno, že ani samotná vakcinace nemusí aktivovat dostatek T lymfocytů. Bohužel, výsledná pravděpodobnost úspěchu této imunoterapie je součin a nikoliv součet pravděpodobností zmíněných dvou nezávislých faktorů. Jedním z možných způsobů, jak zvýšit šanci na úspěch je eliminovat variabilitu dannou vakcinací: Rosenberg se spolupracovníky již v 80tých letech [Rosenberg et al., 1986] ukázal, že lze izolovat protinádorové T lymfocyty z nádorové tkáně, expandovat je in vitro a následně podat zpět pacientům (tzv. adoptivní imunoterapie). Současné postupy umožňují izolovat i velmi malé procento T lymfocytů specifických na nádorové antigeny a expandovat je in vitro na množství až cca 10^{10} buněk, které mohou být opakovaně podávány pacientům zpět [Dudley et al., 2002]. Žádná známá vakcinační metoda neumožnuje dosáhnout takto srovnatelně velké populace protinádorových T lymfocytů. Výsledky řady skupin ale prokázaly, že ani opakované infůze takto velkého počtu T lymfocytů specifických na nádorové antigeny nevede spolehlivě k regresi nádorů.

Proto je snaha v současnosti adoptivní imunoterapii kombinovat s dalšími postupy zamezujícími rozvoji imunologické tolerance. Mezi nadějné strategie může patřit použití geneticky modifikovaných T lymfocytů. Schumacher se spolupracovníky vyvinul metodu retrovirové transdukce genů kódujících T-buněčný receptor specifický pro definovaný komplex peptid/MHC I což umožňuje připravit dostatečný počet autologních protinádorových T lymfocytů i za situace, kdy se je nepodaří izolovat z pacientů [Kessels et al., 2001]. Informace získané na myších modelech dále naznačují, že inaktivace některých genů v T lymfocytech (například přerušení signalizační kaskády TGFβ, nebo inaktivace genu pro protein Cbl-b) [Gorelik and Flavell, 2001; Jeon et al., 2004] zamezí rozvoji nádorové tolerance, lze očekávat, že nepochybně bude definováno více genů s podobnou funkcí. Další možností jak zamezit rozvoji nádorové tolerance je aplikace látek s imunostimulačními účinky, které aktivují tolerogenní APC přítomné v organismu pacientů s nádorovým onemocněním. Řada experimentů na myších, ale i některé klinické studie naznačují, že aktivace receptoru CD40 prostřednictvím aktivační protilátky nebo přirozeným ligandem (CD40L) zabrání rozvoji tolerance [Diehl et al., 1999]. Mezi látky s podobným účinkem patří nemetylované CpG oligonukleotidy aktivující TLR 9 nebo ligandy TLR 7/8 jako jsou syntetické látky imiquimod a resiquimod [Hemmi et al., 2002; Garbi et al., 2004]. T lymfocyty specifické na nádorové antigeny jsou suprimovány také působením T-regulačních lymfocytů (T_{reg}), proto se současná imunoterapie soustřeďuje i na tuto složku nádorové tolerance. Inhibiční efekt T_{reg} lze nejjednodušeji eliminovat jejich deplecí, ke které dojde po podání protilátky proti receptoru CD25, bohužel tato protilátka odstraní i všechny aktivované T lymfocyty a z tohoto důvodu se hledají jiné vhodnější postupy. Ukazuje se, že existují jiné důležité receptory na povrchu T_{reg}, které mohou být také cíleny podáním terapeutických protilátek. Takovýto receptor je GITR a experimentální data prokázala, že jeho aktivace buď protilátkou nebo přirozeným ligandem (GITR-L) zabrání T_{reg} suprimovat ostatní T lymfocyty [Ko et al., 2005]. Není obtížné si představit, že moderní nádorová imunoterapie bude založena na kombinaci výše zmíněných postupů. Tato kombinovaná nádorová imunoterapie bude pravděpodobně spoléhat na masívní infůze T lymfocytů modifikovaných „na zakázku“ podle potřeb individuálních pacientů současně s aplikací adjuvantní imunomodulační léčby. Tyto nové přístupy si nepochybně vyžádají intenzívní klinické testování a nelze očekávat, že se stanou součástí standartní léčby nádorových onemocnění v blízké budoucnosti.

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5. PUBLIKAČNÍ AKTIVITA AUTORA

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1. Otahal, P., S.C. Hutchinson, L.M. Mylin, M.J. Tevethia, S.S. Tevethia, and T.D. Schell, *Inefficient cross-presentation limits the CD8+ T cell response to a subdominant tumor antigen epitope.* J Immunol, 2005. 175(2): p. 700-12.

2. Otahal, P., et al., *Early Immunization Induces Persistent Tumor-Infiltrating CD8+ T Cells against an Immunodominant Epitope and Promotes Life-long Control of Pancreatic Tumor Progression in SV40 T Antigen Transgenic Mice*. Odesláno do tisku.

3. Mackova, J., L. Kutinova, P. Hainz, J. Krystofova, V. Sroller, P. Otahal, P. Gabriel, and S. Nemeckova, *Adjuvant effect of dendritic cells transduced with recombinant vaccinia virus expressing HPV16-E7 is inhibited by co-expression of IL12.* Int J Oncol, 2004. 24(6): p. 1581-8.

4. Nemeckova, S., R. Stranska, J. Subrtova, L. Kutinova, P. Otahal, P. Hainz, L. Maresova, V. Sroller, E. Hamsikova, and V. Vonka, *Immune response to E7 protein of human papillomavirus type 16 anchored on the cell surface.* Cancer Immunol Immunother, 2002. 51(2): p. 111-9.

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6. Nemeckova, S., P. Hainz, P. Otahal, P. Gabriel, V. Sroller, and L. Kutinova, *Early gene expression of vaccinia virus strains replicating (Praha) and non-replicating (modified vaccinia virus strain Ankara, MVA) in mammalian cells.* Acta Virol, 2001. 45(4): p. 243-7.

7. Bohbot, V., P. Otahal, Z. Liu, L. Nadel, and J. Bures, *Electroconvulsive shock and lidocaine reveal rapid consolidation of spatial working memory in the water maze.* Proc Natl Acad Sci U S A, 1996. 93(9): p. 4016-9.

Přednášky

1. 31st Annual Judy S. Finkelstein Memorial Symposium. May 7, 2005, Hershey, USA. CD8+ T cell based Immunotherapy of Pancreatic Insulinomas in SV40 T Antigen Transgenic Mice.

2. 3rd International Conference on Tumor Microenvironment: Progression, Therapy and Prevention. October 12-16, 2004, Prague. Overcoming Barriers to CD8+ T cell based immunotherapy of Pancreatic Insulinomas in SV40 T Antigen Transgenic Mice.

3. Studentská vědecká konference 2. LF UK. 2002, Praha. Host-range protein C7L viru vakcinie.

4. 2nd International Mini-symposium on Gene Therapy. 16.-18.5.2001 Brno. Antigen transfer into dendritic cells via inactivated vaccinia virus.

Postery

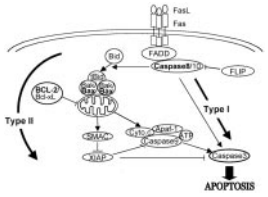
1. DNA Tumor Virus Meeting. July 15-20, 2003, Trieste, Italy. Immunization with Wild Type SV40 T Ag Activates but Fails to Expand CD8 T Cells Specific for Subdominant Epitope.

2. 56th Annual Symposium on Fundamental Cancer Research „Cancer Immunity: Challenges for the Next Decade“. October 14-17, 2003, Houston, USA. Immunization with Wild Type SV40 T Ag Activates but Fails to Expand $CD8^+$ T Cells Specific for Subdominant Epitope.

THE JOURNAL OF IMMUNOLOGY

IN THIS ISSUE

Modeling apoptosis

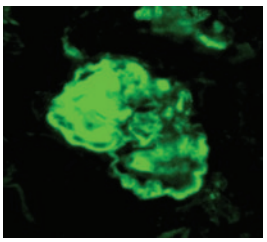


The ability to regulate apoptosis can be useful in treating cancer and autoimmune diseases. Although Fas-induced apoptosis has been extensively studied, complete understanding of the complex interactions of molecules involved in the type I and type II pathways is lacking. Hua et al. (p. 985) developed a computational model based on experimental data obtained from studies on human Jurkat cells. The model uses biochemical reactions incorporating 136 rate constants (with 35 independent values) and 15 initial conditions to describe molecular interactions of type I and type II pathway components. Bcl-2 overexpression was predicted to be most effective in blockade of the mitochondrial pathway (type II) under conditions of its binding to two pathway components rather than to either of three single components. Maximum inhibition of caspase-3 activation was seen at a 10-fold increase of Bcl-2 expression in the model and at a 6-fold increase experimentally. Sensitivity analysis, or using the model to predict effects of increasing or decreasing a single component, analyzed the impact of each type I and type II component on caspase-3 activation. A 10- or 100-fold higher concentration of some molecules inhibited caspase-3 activation, whereas lower concentrations of similar magnitude had no effect. Only decreased concentrations of other molecules inhibited capase-3 activation, while both increased and decreased concentrations of a third group of molecules blocked caspase-3 activation. The model, which traces apoptosis from FasL binding through caspase-3 activation, identifies molecular interactions in the type I and type II pathways sensitive to intervention.

Subdominant tumor Ags

The response of $CD8^+$ T cells to foreign Ags varies with the epitope. Understanding factors influencing immunodominance of epitopes could influence vaccine design. Otahal et al. (p. 700) transferred naive T cells expressing a TCR specific for the subdominant epitope V of SV40 T Ag (TCR-V) into C57BL/6 mice. Transferred cells were activated and proliferated in mice immunized i.p. with cells transformed with epitope V SV40 T Ag (V-only T Ag); immunization with cells transformed with wild-type SV40 T Ag (wt T Ag) resulted in modest TCR-V T cell proliferation and expansion. TCR-V T cell expansion occurred in mice immunized with a mixture of the two transformed cells but not with cells cotransformed with wt and V-only T Ags. $TAP1^{-/-}$ cells expressing wt or V-only T Ag were not lysed by CTL clones specific for epitope I or epitope V, respectively, unless infected with recombinant vaccinia virus expressing TAP1 and TAP2. In vivo, adoptively transferred naive cells transgenic for epitope I TCR were induced to expand by injected $TAP1^{-/-}$ cells expressing wt T Ag; limited expansion of adoptively transferred TCR-V cells was induced by $TAP1^{-/-}$ cells expressing epitope V and only a small fraction of the cells proliferated. $TAP1^{-/-}$ hosts did not support expansion of TCR-V cells after immunization with cells expressing V-only T Ag. B6 mice immunized with V-only T Ag-expressing cells developed few epitope V-specific $CD8^+$ T cells; however, boosting with wild-type, but not $TAP1^{-/-}$, cells transformed with V-only or wt T Ag increased epitope V-specific $CD8^+$ T cells 30- and 10-fold, respectively. The authors conclude that subdominance of T Ag epitope V in mice immunized with wt T Ag-transformed cells results from limited cross-presentation of epitope V by APC and competition from immunodominant T Ag epitope-specific $CD8^+$ T cells for T Ag-expressing cells.

Genetic dissection of lupus susceptibility and suppression



Systemic lupus erythematosus (SLE) immune pathology involves abnormal T and B cell development and phenotype, autoantibody production, splenomegaly, and fatal glomerulonephritis. Interactions among several genes determine the likelihood of developing SLE and the severity of the disease. Critical susceptibility regions in mice are defined as *Sle1* on chromosome 1, *Sle2* on chromosome 4, and *Sle3/5* on chromosome 7; the *Sles1* locus on mouse chromosome 17 suppresses SLE. These major genomic loci were identified by linkage analysis or through the use of congenic mouse strains carrying NZB or NZW loci on a C57BL/6 (B6) background. However, interactions among these regions in SLE pathogenesis are only partially delineated. In the first of three papers addressing this issue, Wakui et al. (p. 1337) transferred congenic bone marrow into sublethally irradiated B6.$Rag^{-/-}$ mice to look at epistatic interactions of regions *Sle1* and *Sle3/5*. Mice receiving cells from B6 mice bicongenic for those two regions had marked splenomegaly exhibiting elevated CD4:CD8 T cell ratios, fewer B cells, greater accumulation of mature B cells at the transition between T1 and T2 cells, and increased numbers of splenic lymphoid dendritic cells along with higher levels of most autoantibodies compared with normal B6 mice. Chimeras of cells monocongenic for either region had values closer to those of normal controls, whereas mice receiving cells from both monocongenic strains had values intermediate to the two groups but closer to those of normal mice. A genetic dissection of the influence of the *Sle2* locus on B cell development by Xu et al. (p. 936) was accomplished using B6 mice congenic for one of three subintervals, *Sle2a*, *Sle2b*, or *Sle2c*. Older *Sle2c* congenics had the greatest expansion of B-1a cells in the peritoneal cavity and spleen compared with the other congenic strains and B6 controls; later B1-a cell expansion was seen in *Sle2a* congenic mice.

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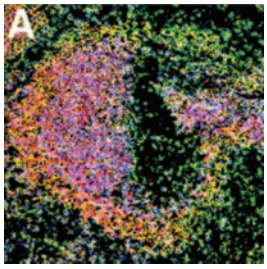
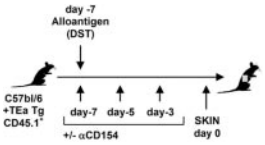
Reduced surface expression of IgM on B-1a cells of splenic origin mapped to *Sle2a*. Mice tricongenic for *Sle1*, *Sle3*, and *Sle2a* or *Sle2b* had more prominent parameters of lupus pathogenesis including extensive remodeling of the splenic architecture with influx of $CD11b^+$ cells in T and B cell zones than animals tricongenic for *Sle1*, *Sle3*, and *Sle2c*. Subramanian et al. (p. 1062) used congenic recombinant fine-mapping to introduce six intervals of the NZW *Sles1* locus into B6 mice congenic for *Sle1*. Progeny homozygous, but not heterozygous, for some *Sles1* subintervals suppressed SLE splenomegaly, anti-chromatin Ab production, and T and B cell activation. *Sles1* gene activity mapped to a 956-kb interval within the *Sles* locus. The authors discovered a complementary *Sles1* modifier allele in 129 mice by creating a 129 × B6 congenic strain homozygous for a specific 129 autoimmunity-promoting region but heterozygous for the suppressive NZW *Sles1* allele. Together, these three manuscripts point out the importance of epistatic interactions among SLE genes and the utility of congenic recombinant mouse strains in understanding contributions from specific cell types in this complex genetic disease.

Tolerizing self-reactive B cells



Central tolerance mechanisms for self-reactive B cells involve anergy, deletion, and receptor editing. However, the contribution of each mechanism to limiting self-reactive B cells is unknown. Hippen et al. (p. 909) bred mice transgenic for membrane hen egg lysozyme (mHEL) with mice carrying double transgenes (anti-HEL H chain plus anti-HEL L chain knock-in at the endogenous locus). Triple transgenic progeny splenic B cell numbers were equivalent to those in transgenic mice carrying randomly integrated anti-HEL H chain plus anti-HEL L chain, but the cells were unable to bind HEL. In contrast, pre-B cells were clonally deleted in transgenic mHEL mice carrying randomly integrated anti-HEL H chain plus anti-HEL L chain. In lethally irradiated mHEL mice injected with bone marrow from anti-HEL H chain plus anti-HEL L chain knock-in transgenic mice, nearly half of the generated B cells did not bind HEL but expressed a human Cκ chain knock-in transgene marker. Only background levels of B cells positive for the human marker were seen in wild-type recipients. Mice triple transgenic for mHEL, anti-HEL H chain plus anti-HEL L chain knock-in had low levels of serum anti-HEL IgM^a Abs compared with controls, and their spleen cells proliferated in response to LPS but not to HEL. Mice triple transgenic for mHEL, anti-HEL H chain plus anti-HEL L chain knock-in had the highest percentage of pre-B cells and a B cell developmental delay of 6 h as demonstrated by BrdU pulse labeling. Mice triple transgenic for soluble HEL, anti-HEL H chain plus anti-HEL L chain knock-in had two populations of splenic B cells. An anergic cell population had low levels of IgM^a, no HEL binding, and localized to splenic follicles; a second non-HEL binding population had high levels of IgM^a and localized to the marginal zone. The data suggest that receptor editing tolerizes B cells reactive to membrane-bound self Ag, whereas both receptor editing and anergy tolerize B cells reactive to soluble self Ag.

Allogeneic skin graft acceptance



Donor-specific transfusion (DST) of allogeneic cells combined with anti-CD154 Ab induces long-term acceptance of allogeneic skin grafts. Yet the underlying cellular and molecular mechanisms of tolerance in this system are unknown. Quezada et al. (p. 771) transfused C57BL/6 mice with TCR transgenic (Tg) $CD4^+$ T cells (TEa-Tg) that recognized a major alloantigen on F_1 skin grafted 7 days later. Mice given DST plus anti-CD154 Ab at the time of cell transfer accepted skin grafts long-term; grafts were not retained by treated recipients depleted of $CD4^+CD25^+$ regulatory T cells (T^{reg}) with anti-CD25 mAb, costimulated with anti-GITR (glucocorticoid-induced TNFR-related gene) Ab, or untreated. TEa-Tg cell expansion in mice given DST plus control hamster Ig at the time of transfer was greater than in mice given DST plus anti-CD154 Ab. Synthesis of IL-2 and IFN-γ was blocked in transfused CFSE-TEa-Tg cells 5 days after DST plus anti-CD154 Ab treatment. Mice tolerized with DST plus anti-CD154 Ab rejected 45-day-old skin grafts after anti-GITR treatment. Naive TEa-Tg cells adoptively transferred into mice tolerized by DST plus anti-CD154 Ab treatment 3 wk earlier had reduced capacity to expand, differentiate, produce cytokines, and reject F_1 skin grafted at the time of transfer compared with TEa-Tg cells given to nontolerized recipients. Anti-GITR or anti-CD40 Ab partially reversed the tolerant state. The data suggest that both effector T cell suppression by T^{reg} cells and anergy induced by costimulation blockade contribute to tolerance of allogeneic skin grafts.

Understanding oral tolerance

Oral tolerance, a form of peripheral tolerance to nonself Ags, is induced by oral administration of soluble Ags and is mediated by hyporesponsive T cells. However, the ability of tolerized T cells to form conjugates and immunological synapses with APCs had not been investigated. Ise et al. (p. 829) developed in vivo tolerized T cells by giving OVA in drinking water to OVA-TCR transgenic mice. Splenic $CD4^+$ T cells from OVA-fed mice had reduced OVA-induced proliferation and IL-2 production, and the mice had lower anti-OVA IgG serum levels than untreated controls. Splenic $CD4^+$ T cells from OVA fed mice formed LFA-1/ICAM-1 integrin-dependent conjugates with OVA-pulsed T cell-depleted splenocytes at comparable levels with splenic T cells from untreated mice. However, Ag-stimulated orally-tolerized T cells had impaired formation of immunological synapses with APCs, i.e., less TCR and protein kinase C-θ were associated with lipid raft fractions. Lower levels of the phosphorylated GTPases, Rac1 and cdc42, and the guanine nucleotide exchange factor, Vav, were found in orally-tolerized T cells than in untreated T cells after OVA stimulation. By contrast, OVA-immunized T cells had proliferation, IL-2 production, conjugate formation and Ag-induced activation of Vav, Rac1, and cdc42 equivalent to untreated T cells. The authors conclude that orally-tolerized T cells can form stable conjugates with APCs but, unlike OVA-immunized T cells, have impaired immunological synapse formation.

Summaries written by Dorothy L. Buchhagen, Ph.D.

Inefficient Cross-Presentation Limits the CD8$^+$ T Cell Response to a Subdominant Tumor Antigen Epitope[1]

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CD8$^+$ T lymphocytes (T_{CD8}) responding to subdominant epitopes provide alternate targets for the immunotherapy of cancer, particularly when self-tolerance limits the response to immunodominant epitopes. However, the mechanisms that promote T_{CD8} subdominance to tumor Ags remain obscure. We investigated the basis for the lack of priming against a subdominant tumor epitope following immunization of C57BL/6 (B6) mice with SV40 large tumor Ag (T Ag)-transformed cells. Immunization of B6 mice with wild-type T Ag-transformed cells primes T_{CD8} specific for three immunodominant T Ag epitopes (epitopes I, II/III, and IV) but fails to induce T_{CD8} specific for the subdominant T Ag epitope V. Using adoptively transferred T_{CD8} from epitope V-specific TCR transgenic mice and immunization with T Ag-transformed cells, we demonstrate that the subdominant epitope V is weakly cross-presented relative to immunodominant epitopes derived from the same protein Ag. Priming of naive epitope V-specific TCR transgenic T_{CD8} in B6 mice required cross-presentation by host APC. However, robust expansion of these T_{CD8} required additional direct presentation of the subdominant epitope by T Ag-transformed cells and was only significant following immunization with T Ag-expressing cells lacking the immunodominant epitopes. These results indicate that limited cross-presentation coupled with competition by immunodominant epitope-specific T_{CD8} contributes to the subdominant nature of a tumor-specific epitope. This finding has implications for vaccination strategies targeting T_{CD8} responses to cancer. ***The Journal of Immunology,*** **2005, 175: 700–712.**

The response of CD8$^+$ T lymphocytes (T_{CD8})[5] to microorganisms, tumors, and tissue grafts is typically focused toward multiple epitopes with one or a few epitopes predominant (1, 2). These epitopes have been grouped into two broad categories, dominant and subdominant, based on the relative frequency of T_{CD8} that respond following Ag exposure. Defining the basis for this hierarchical response will provide insight for monitoring natural T_{CD8} immunity and developing vaccines to cancer and infectious diseases. Multiple mechanisms can contribute to immunodominance following immunization with complex Ags. These include the efficiency of processes involved in Ag presentation such as peptide liberation from protein substrates (3–5), TAP-dependent peptide transport into the endoplasmic reticulum (6), transport of peptide-MHC complexes to the cell surface (7, 8), as well as the stability of peptide-MHC class I complexes (9, 10). In addition, T cell precursor frequency (1, 11, 12), TCR avidity (13, 14), the nature of the APC (15), and T_{CD8} competition for the APC (16–18) have been implicated. Thus, the position of an epitope within the immunodominance hierarchy derives from the interplay of multiple factors important for initiation of T_{CD8} responses. Because no general rules have been established to this point, the basis for subdominance of a particular epitope must be defined empirically.

Professional APC, such as dendritic cells, are capable of activating naive T_{CD8} following engagement of the TCR with MHC class I/peptide complexes in addition to the provision of costimulatory signals such as B7/BB1 engagement with CD28 on the T_{CD8} (19). The antigenic peptides presented by MHC class I molecules on professional APC can be either derived from de novo synthesized proteins within the APC (direct presentation) or from cell-associated Ags via the mechanism of cross-presentation (20–22). Cross-presentation is particularly important for priming T_{CD8} responses to tumor Ags because most tumor cells lack the expression of costimulatory molecules (23–25).

The contribution of cross-presentation in establishing T_{CD8} immunodominance to tumor-specific epitopes remains unknown. Cross-presentation can be influenced by the dose of Ag, as some studies have shown that high levels of Ag are more efficiently cross-presented than low levels of the same Ag (26–29). Whether variability in the T_{CD8} response to multiple epitopes within the same protein can be attributed to differences in the efficiency of cross-presentation has not been investigated. In this study, we assessed the mechanisms that contribute to the subdominant nature of the H-2D^b-resticted epitope V (residues 489–497) from SV40 T Ag. The tumor-specific T_{CD8} response to SV40 T Ag in C57BL/6 (B6) mice is targeted against three dominant epitopes (designated epitopes I, II/III, and IV) and one subdominant epitope (designated epitope V)

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[5] Abbreviations used in this paper: T_{CD8}, CD8$^+$ T lymphocyte; T Ag, SV40 large tumor Ag; TAP1$^{-/-}$, TAP 1 knockout; wt, wild type; TCR-V, epitope V-specific TCR transgenic; V-only, epitope V-only; TCR-I, epitope I-specific TCR transgenic.

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(30). The T_{CD8} response to the H-2D^b-restricted epitope V is undetectable following immunization with wild type (wt) T Ag-transformed cells, SV40, or even a recombinant vaccinia virus expressing full-length T Ag (31). This strict immunodomination is relieved by deletion of the three immunodominant epitopes from T Ag (32) or following immunization with a recombinant vaccinia virus expressing epitope V as a minigene (33). Although epitope V-specific T_{CD8} are not induced following immunization with wt T Ag, this subdominant epitope is efficiently presented by T Ag-transformed cells in vitro (32). One potential mechanism contributing to the subdominant nature of epitope V in vivo is the relative instability of the epitope V/Db complexes compared with the dominant T Ag epitopes (30, 33), particularly under conditions where a fixed amount of Ag is cross-presented. A recent study by Chen et al. (34) demonstrated that the subdominant nature of epitope V is maintained under conditions where T Ag is exclusively cross-presented.

In the present study, we investigated the mechanism of epitope V subdominance by measuring the response of epitope V-specific TCR transgenic (TCR-V) T cells to immunization with syngeneic or TAP1 knockout (TAP1$^{-/-}$) T Ag-transformed cells. The results indicate that epitope V is inefficiently cross-presented, resulting in limited T cell priming and expansion. The additive effect of competition by immunodominant epitope-specific T_{CD8} further inhibits the response to epitope V following immunization with wt T Ag.

Materials and Methods

Mice

C57BL/6 (H-2^b), B6.129S2-Tap1^{tm1Arp} (TAP1$^{-/-}$), and B6.SJL-Ptprc^{a}Pep3^b/BoyJ (B6.SJL) mice were purchased from The Jackson Laboratory and used between the ages of 8 and 16 wk. TCR transgenic mice specific for the T Ag epitope I (TCR-I mice) are on a B6 background and were described previously (35). All mice were maintained at the animal facility of the Milton S. Hershey Medical Center. All animal studies were performed in accordance with guidelines established by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee under an approved protocol.

Cloning of epitope V-specific TCR subunits from the CTL clone Y-5

TCR sequences corresponding to both the α- and β-chains expressed by the SV40 T Ag epitope V-specific CTL clone Y-5 (36) were derived as described previously (35). The TCRα-chain combining region was amplified by PCR from clone Y-5-derived cDNA using an antisense 3′-constant region oligonucleotide (5′-CGAGGATCTTTTAACTGGTA-3′) and a 5′-variable region Vα4 chain sense oligo (5′-GCCACCTCCTTCCACTTG CAGAAAG-3′), whereas the TCR Vβ-chain combining region was amplified using the 3′-antisense constant region oligonucleotide (5′-CTT GGGTGGAGTCACATTTCT-3′) and a 5′-sense Vβ7 oligonucleotide (5′-AAGAAGCGGGAGCATTTCTTC-3′) (37–39). The Y-5 TCRα and TCRβ PCR products were subcloned into pUC19, and sequence analysis revealed Jα18 and Jβ1.3 usage, respectively. Sequencing of additional 5′-extended Vα4 cDNA clones obtained by 5′-RACE (Invitrogen Life Technologies) from total Y-5 RNA was conducted to confirm the identity of the variable region as Vα4.1. Accordingly, 3′-antisense genomic primers corresponding to intron sequences downstream of the respective TCRα and TCRβ joining regions (Jα18, 5′-TGCGGCCGCAAATTTTATACT TACTGGGCTTGATAGATAAC-3′; Jβ1.3, 5′-CACTGCAACCGCG GCACCTCAGAGAGAA-3′) (38, 40) were used in combination, respectively, with sense primers corresponding to sequences located upstream of the Vα4.1 (5′-CTTCCCGGGCTCAAAATATTTGTATTCACACACTCCA-3′) or Vβ7 (5′-CACACTTTCCTCGAGACCACCATGAGAGTTAGG-3′ (37, 39)) coding regions to amplify the corresponding genomic sequences from CTL clone Y-5 nuclear DNA and incorporate restriction endonuclease cleavage sites at the ends of each product (α, 5′-*Xma*I, 3′-*Not*I; β, 5′-*Xho*I, and 3′-*Kpn*I/*Sac*II). The nucleotide sequences of the subcloned genomic V(D)J fragments were verified and liberated from the cloning vector by endonuclease digestion and ligated into the appropriately digested TCRα or β expression cassette plasmids (pTαcass and pTβcass, respectively, obtained from Dr. D. Mathis (Harvard Medical School, Boston, MA) (41)). The full-length Y-5α- and β-chain TCR expression cassette fragments were released by endonuclease digestion as previously described (35) and eluted from unstained agarose gel slices directly into microinjection buffer.

Generation of SV40 T Ag TCR-V mice

Purified Y-5 TCRα- and β-chain expression cassettes were combined before injection. Microinjection of fertilized embryos from B6 mice was performed as described previously (42). The presence of the α and β transgenes in weanlings was determined at 4 wk of age by PCR analysis of tail-derived DNA using the following primer pairs: Vα4.1 chain sense, 5′-GAAGCCACCTCCTTCCACTTGCAG-3′; Jα18 chain antisense, 5′-TGCGGCCGCAAATTTTATACTTACTGGGCTTGATAGATAAC-3′; Vβ7 chain sense, 5′-AAGAAGCGGGAGCATTTCTCC-3′; and Jβ1.3 chain antisense, 5′-CACTGCAACCGCGGCACCTCAGAGAGAA-3′. Amplification of the corresponding 160- and 200-bp fragments from genomic DNA was diagnostic for the presence of the respective transgenes. Expression of the transgene products was confirmed by staining lymphocytes from various lymphoid tissues with a TCR Vβ7-specific mAb and a Db/V tetramer (31). The founder line, line 459, was maintained by backcrossing transgene-positive males with B6 females, and progeny were screened for the presence of both α and β TCR transgenes by PCR analysis (35). A preliminary characterization of TCR-V transgenic mice was reported recently (43). Greater than 90% of T_{CD8} among PBL of TCR-V mice are specific for epitope V, as indicated by positive staining with Db/V tetramer (Fig. 1*A*). Importantly, the surface expression of CD44 on Db/V tetramer$^+$ T cells from TCR-V mice is low, which is consistent with a naive phenotype for TCR-V T cells (Fig. 1*A*).

Cell lines and media

Cell lines used in this study are summarized in Table I. B6/T116A1 cells (B6/V-only T Ag) express a T Ag variant in which epitopes I (residues 207–215) and II/III (residues 223–231) are deleted, and epitope IV is inactivated by alanine substitution of residues 406, 408, and 411 but in which epitope V remains intact (31). B6/T5Aa (B6/wt T Ag) (30) and B6/K-0 (44) cells express wt T Ag. The cell line B6/122B1 (ΔI, II/III, IV, V) expresses a T Ag derivative in which all four CTL determinants were inactivated by substitution of critical MHC class I anchor residues (N210A, N227A, F408A, and N493A) (31). The Ag loss variant B6/K-1,4 was derived by sequential in vitro coculture of B6/K-0 cells with T Ag-specific CTL clones, which resulted in the selection of a clone expressing a T Ag variant in which epitopes I and II/III are deleted, and epitope IV contains an inactivating point mutation (45, 46). The cell line B6/K-1,4-SV was derived previously from the B6/K-1,4 cells by supertransfection with a plasmid encoding the wt T Ag (45). TAP1$^{-/-}$ cells expressing either wt T Ag or V-only T Ag were generated by transfection of B6.129S2-Tap1^{tm1Arp} mouse primary kidney cells with plasmid pPVU0 (47) encoding wt T Ag and pSLM361-11 (31) encoding epitope V-only T Ag, respectively. To ensure that the TAP1$^{-/-}$ cells had the expected phenotype, we determined their ability to activate *LacZ*-inducible T cell hybridomas specific for T Ag dominant (I and IV) and subdominant (V) epitopes (Fig. 1*B*). Coincubation of T cell hybridomas with B6-derived T Ag-transformed cells expressing either wt or V-only T Ag resulted in β-galactosidase production by the epitope V-specific T cell hybridoma. In contrast, T cell hybridomas specific for epitopes I or IV were activated only following coincubation with wt T Ag-expressing cells. T Ag-transformed cell lines on the TAP1$^{-/-}$ background expressing either wt or V-only T Ag failed to activate T cell hybridomas. T Ag expression was confirmed by immunofluorescent staining with T Ag-specific mAbs (data not shown). Thus, the T Ag-transformed cell lines used here have the expected phenotypes and support previous findings that epitope V is efficiently presented from wt T Ag in vitro (30, 33). All cell lines were maintained in DMEM and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, 2 mM L-glutamine, 10 mM HEPES, 0.075% (w/v) $NaHCO_3$, and 5–10% FBS.

Viruses and synthetic peptides

The recombinant vaccinia virus expressing human TAP1 and TAP2 proteins (VV-TAP(1 + 2) (48)) was obtained from Drs. J. R. Bennink and J. W. Yewdell (National Institutes of Health, Bethesda, MD). The VV-SC vaccinia virus contains only empty vector. Viruses were propagated and titrated in HuTK$^-$143 cells essentially as described previously (33). Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by Fmoc chemistry using an automated peptide synthesizer (9050 MiliGen PepSynthesizer; Millipore). Peptides were solubilized in DMSO and diluted to the appropriate concentration with RPMI 1640 medium. Peptides used in these experiments correspond to the SV40 T Ag epitope I (SAINNYAQKL; peptide I), epitope V (QGINNLDNL; peptide V), and influenza virus nucleoprotein 366–374 (ASNENMETM; peptide Flu).

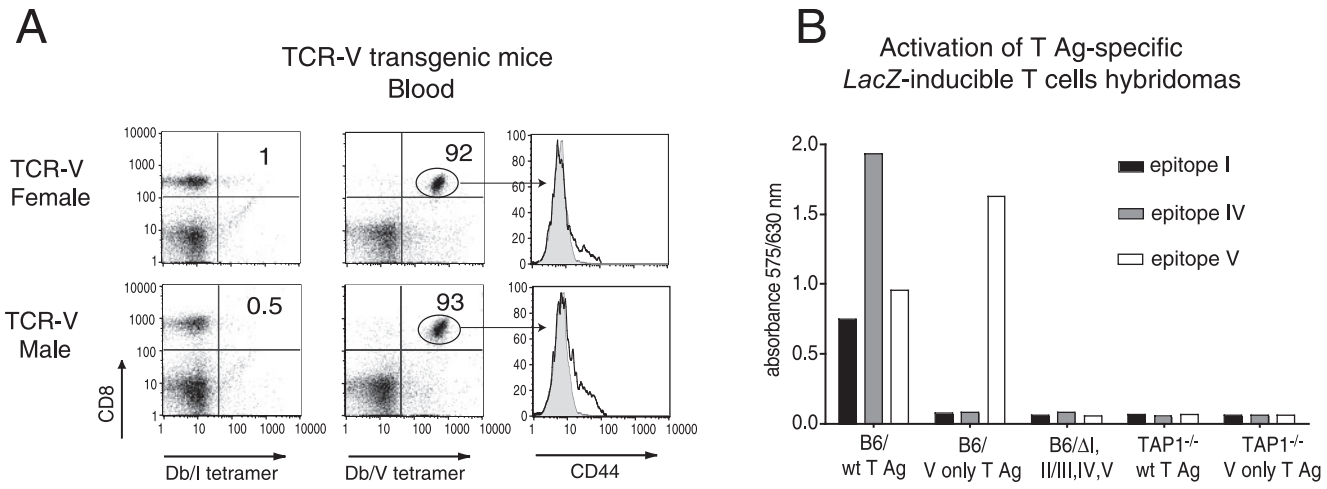

FIGURE 1. Characterization of TCR-V transgenic mice and T Ag-transformed cell lines used for immunization. *A*, Characterization of T Ag epitope V-specific TCR transgenic mice. PBL from TCR-V male and female mice were triple stained with anti-CD8α Ab, the indicated tetramer, and anti-CD44 Ab. Numbers in the *upper right quadrant* indicate the percentage of epitope-specific T_{CD8}. Open histograms indicate the surface expression of CD44 on Db/V tetramer$^+$ T cells. The background fluorescence shown in closed histograms was determined on unstained cells. *B*, Recognition of T Ag-transformed cells by epitope-specific *LacZ* T cell hybridomas. A total of 3×10^4 hybridoma cells specific for T Ag epitope I, IV, or V was incubated overnight with an equal number of B6/wt T Ag, B6/V-only T Ag, B6/Δ I, II/III, IV, and V cells lacking all H-2^b CTL epitopes, TAP1$^{-/-}$ wt T Ag, and TAP1$^{-/-}$ V-only T Ag cells. Accumulation of *LacZ* in activated cells was detected by incubating culture lysates with the *LacZ* substrate, chlorophenol red-β-D-galactopyranoside, and measuring the absorbance of the product at 575 nm using 630 nm as a reference wavelength.

Flow cytometric analysis

Ex vivo staining of T_{CD8} lymphocytes with MHC tetramers and primary-conjugated Abs was performed on single-cell suspension prepared from spleens as described previously (31). Cells were then fixed with 2% paraformaldehyde in PBS and analyzed using a FACScan flow cytometer (BD Biosciences). Routinely, at least 50,000 events were recorded. Data were analyzed and prepared using FlowJo software (Tree Star). Production and characterization of the MHC class I tetramers specific for the H-2D^b/T Ag epitope I (Db/I), H-2D^b/T Ag epitope V (Db/V), H-2K^b/T Ag epitope IV (Kb/IV), and H-2D^b/influenza virus nucleoprotein epitope 366–374 (Db/Flu) was described previously (31). The following Abs were purchased from BD Pharmingen: PE- and cychrome-labeled anti-mouse CD8a (clone 53-6-7), FITC-labeled anti-mouse TCR Vβ7 (clone TR310), FITC-labeled anti-mouse CD44 (clone IM7), FITC-labeled anti-mouse CD62L (clone MEL-14), and FITC-labeled anti-mouse CD122 (clone TM-β1). PE-labeled anti-mouse CD45.1 (clone A20). The percentage of T_{CD8} cells that stained positive for T Ag-specific tetramer was determined by subtracting the percentage of cells that stained positive for Db/Flu tetramer within the same population.

In vivo proliferation assay

RBC-depleted lymphocytes derived from spleens and lymph nodes of TCR-V transgenic mice were resuspended at 1×10^7/ml in PBS/0.2% BSA and labeled with 5 μM 5- and 6-CFSE (Molecular Probes) for 10 min at 37°C. Cells were than washed three times with PBS, resuspended in HBSS, and injected i.v. at a dose of 5×10^6 clonotypic TCR-V T cells/B6 mouse. The mice were then immunized i.p. the following day with T Ag-expressing cells. After 4 days, the dilution of the CFSE label was determined by tetramer staining of splenic lymphocytes.

SV40-specific CTL clones and T Ag-specific LacZ-inducible T cell hybridomas

SV40 epitope V specific CTL line 96 (T. D. Schell, unpublished results) was derived from line SV11 mice by rVV-ES-V immunization followed by booster with B6/WT-19 cells (49). Epitope I-specific CTL were obtained by in vitro activation of spleen cells from line TCR-I mice using gamma-irradiated T Ag-transformed stimulator cells. *LacZ*-inducible T cell hybridomas specific for the T Ag epitopes I, IV, and V were generated by fusing T Ag-specific CTL clones K-11 (epitope I (50)), Y-4 (epitope IV (45)), and H-1 (epitope V (30)) with BWZ.36.1/CD8 cells (kindly provided by Dr. N. Shastri, University of California, Berkeley, CA), using an approach detailed elsewhere (51). After in vitro selection in the presence of hypoxanthine/aminopterin/thymidine and hygromycin, the hybridoma clones responding to peptides specific for epitopes I, IV, and V were identified and further cloned by limiting dilution.

In vitro and in vivo cytotoxicity assays

In vitro cytotoxicity assays were performed as described previously (30). T Ag-transformed cell lines were treated with γ-IFN (40 U/ml) for 48 h followed by labeling overnight with 1 mCi of ^{51}Cr per T-75 flask. The cells were then trypsinized, washed once with PBS/0.1% BSA, resuspended at 5×10^6 cells/ml, and infected with the indicated vaccinia viruses at multiplicity of infection of 10 for 1 h at 37°C with occasional agitation. Cells were then diluted with 10 ml of complete RPMI 1640 medium-10% FBS and rocked at 37°C for an additional 4 h. After centrifugation, target cells were resuspended in complete RPMI 1640 medium-10% FBS and added in 0.1-ml aliquots to 96-well V-bottom plates to yield 1×10^4 cells/well. Effector cells were added to targets in 0.1-ml aliquots to give the E:T ratio of 15 for CTL clone 96 and 10 for in vitro-activated TCR-I cells. Plates were incubated for 4 h at 37°C in 5% CO_2, and cells were then pelleted by centrifugation ($200 \times g$ for 2 min). A total of 0.1 ml of supernatant was transferred to glass tubes, and the radioactivity was counted in a Packard Cobra model 5005 gamma counter. Percent-specific lysis was calculated as follows: percent-specific lysis = ((experimental − spontaneous)/(maximum − spontaneous)) × 100, where spontaneous is the counts per minute released from target cells incubated with medium alone, while maximum is the counts per minute released from target cells incubated in the presence of 2.5% SDS. All data represent the means of triplicate samples.

For in vivo cytotoxicity assays, targets were prepared from sex-matched B6.SJL (CD45.1$^+$) spleen cells incubated in the presence of the indicated peptides (1 μM) in RPMI 1640 medium/10% FBS at 37°C for 90 min and washed three times. Targets were then labeled with different concentrations of CFSE (5 μM/peptide IV; 0.5 μM/peptide I; 0.025 μM/peptide Flu) for 10 min at 37°C in PBS/0.1% BSA, washed twice, and 2×10^6 cells/target (6×10^6 total cells) were injected i.v. into the tail vein in 0.2 ml of HBSS. The elimination of CFSE-labeled targets was assessed the next day by staining splenic cells with PE-labeled anti-CD45.1 mAb. The following formula was used to determine the percentage of specific killing: percentage = (1 − (ratio unprimed/ratio primed) × 100), where ratio = (percentage of CFSE ^{low}/percentage of CFSE$^{high\ or\ medium}$).

^{35}S-metabolic labeling and pulse-chase immunoprecipitation of SV40 T Ag

T Ag-expressing cells grown in T-75 flasks were starved for 1 h at 37°C in methionine-free DMEM supplemented with 2% dialyzed FBS and then pulsed for 1 h at 37°C with 400 μCi of [^{35}S]methionine in 1 ml of medium. After washing the cells three times with cold PBS, the label was chased for

Table I. *SV40 T Ag-transformed cell lines used in this study*

Cell Designation	Cell Type	Transforming Agent	T Ag Construct	H-2^b CTL Epitopes Present	References
B6/T5Aa	C57BL/6 embryo fibroblasts	pLM234	WT	I, II/III, IV, V	30
B6/T116A1	C57BL/6 embryo fibroblasts	pSLM361–11	Δ207–215, Δ223–231, Y406A, F408A, C411A (V-only T Ag)	V	31
B6/K-0	C57BL/6 kidney	pPVU0	WT	I, II/III, IV, V	45
B6/K-1,4[a]	C57BL/6 kidney	Derived from K-0	Δ134–263, V405L	V	45
B6/K-1,4-SV[b]	C57BL/6 kidney	Derived from K-1,4 + pSV2neo-SV40	Δ134–263, V405L T Ag + WT	I, II/III, IV, V	45
B6/122B1 (B6 ΔI, II/III, IV, V T Ag)	C57BL/6 embryo fibroblasts	PLMTS364–1	N210A, N227A, F408A, N493A (no CTL epitopes)	None	31
TAP1$^{-/-}$ wt	B6.129S2-Tap1^{tm1Arp} kidney	pPVU0	WT	I, II/III, IV, V	This study
TAP1$^{-/-}$ 361–11	B6.129S2-Tap1^{tm1Arp} kidney	pSLM361–11	Δ207–215, Δ223–231, Y406A, F408A, C411A (V-only T Ag)	V	This study

[a] K-0 epitope loss variant selected by coculture with T Ag-specific CTL clones in vitro.
[b] K-1,4 cells transfected with pSV2neo-SV40 encoding WT T Ag.

the indicated time in the presence of 100-fold molar excess of cold methionine. Subsequently, cells were washed three times with cold PBS, scraped into tubes, and centrifuged. The cell pellet was lysed in 1 ml of lysis buffer (50 mM Tris-HCl (pH 8.5), 120 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors 1:100 (Sigma-Aldrich)) for 20 min on ice followed by centrifuged at 12,000 × *g* for 5 min. Each cell lysate was precleared with protein A-Sepharose beads conjugated with 2 mg/ml BSA for 2 h at 4°C. Precleared samples were immunoprecipitated overnight with Pab 901 (47) directed to the C-terminal of T Ag and a control Ab to herpes simplex virus glycoprotein D (52). The immune complexes were collected on protein A-Sepharose beads, washed three times with radioimmunoprecipitation assay buffer (10 mM Tris-HCl (pH 8.0), 140 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS), once with SNNTE (50 mM Tris, 5 mM EDTA, 0.5 M NaCl, 5% sucrose, and 1% Nonidet P-40) buffer, and denatured for 5 min at 95°C in 30 μl of 2× sample buffer (100 mM Tris-HCl, 4% SDS, 20% glycerol, 2% 2-ME, and 0.01% bromphenol blue). Proteins were separated on 7.5% SDS polyacrylamide gel under reducing conditions. Gels were fixed in methanol, acetic acid, and water and were treated with Amplify (Amersham Biosciences) and dried. Autoradiography was performed at −80°C using Kodak X-omat LS film.

Results

Naive TCR-V T cells recognize wt T Ag in vivo but only a subset of cells proliferate

One explanation for the subdominant phenotype of epitope V is that the precursor frequency in naive B6 mice might be limiting (1, 11, 12). To determine whether an increase in the precursor frequency of epitope V-specific T_{CD8} could overcome subdominance following immunization with wt T Ag-expressing cells, we developed transgenic mice that express a TCR specific for epitope V to provide a source of naive epitope V-specific T_{CD8}. Line TCR-V mice express the TCRα- and β-chains from the epitope V-specific CTL clone Y-5 on the B6 background (see *Materials and Methods*). Lymphocytes from TCR-V mice were transferred into B6 mice to determine their responsiveness to immunization with T Ag-transformed B6 cells expressing either wt or V-only T Ag. Groups of naive B6 mice received two different doses of TCR-V T cells (1×10^6 or 1×10^7) followed by i.p. immunization with wt or V-only T Ag-transformed cells the next day. Seven days postimmunization, CD8$^+$,Db/V tetramer$^+$ spleen cells were quantitated. TCR-V T cells expanded dramatically in mice immunized with B6/V-only T Ag-transformed cells (Fig. 2*A*). In mice that received 1×10^6 donor cells, TCR-V T cells expanded to 24% of T_{CD8} following immunization with B6/V-only T Ag-transformed cells. Limited expansion of TCR-V T cells, representing 3.4% of T_{CD8}, was observed in mice immunized with B6/wt T Ag-transformed cells (Fig. 2*A*, *left middle panel*). A 10-fold increase in the initial TCR-V donor population failed to result in further increases in the percentage of T_{CD8} specific for epitope V following immunization (Fig. 2*A*, *right panels*). Likewise, a 5-fold increase in the immunizing dose of B6/wt T Ag cells failed to result in increased expansion of TCR-V T cells (data not shown). The absolute number of TCR-V T cells per spleen also was calculated to ensure that the magnitude of the response was not biased by varying numbers of total spleen cells among individual mice. The results are consistent with the data presented as percentages of T_{CD8} (Fig. 2*A*). Thus, only limited accumulation of TCR-V T cells was observed following immunization with wt T Ag, despite the presence of large numbers of naive TCR-V T cells.

This result might be explained by a quantitatively small proportion of naive TCR-V T cells recognizing epitope V in vivo, despite the large Ag dose used (1×10^7 B6/wt T Ag cells). Examination of the cell surface phenotype of TCR-V T cells following immunization revealed that CD44 surface expression was up-regulated on the majority of cells following immunization with either V-only T Ag or wt T Ag-transformed cells (Fig. 2*B*). In contrast, only a subset of TCR-V T cells down-regulated the CD62L lymph node homing receptor or up-regulated the CD122 IL-2β-chain receptor following immunization with wt T Ag. Taken together, these results suggest that only a fraction of the TCR-V T cells are fully activated following exposure to wt T Ag, consistent with limited T cell expansion.

Because suboptimal T cell activation might fail to result in cell division (53), we monitored the proliferation of CFSE-labeled TCR-V T cells 4 days after transfer into B6 mice immunized with either wt or V-only T Ag-transformed cells. Immunization with V-only T Ag induced robust proliferation of TCR-V T cells, representing 71–81% of splenic CD8$^+$ Db/V tetramer$^+$ cells (Fig. 2*C*). In contrast, immunization with wt T Ag-transformed cells induced proliferation of only a small proportion of TCR-V T cells, representing 17–19% of recovered TCR-V T cells. No proliferation was detected after immunization with cells expressing a T Ag variant that lacks all defined T_{CD8} epitopes (data not shown).

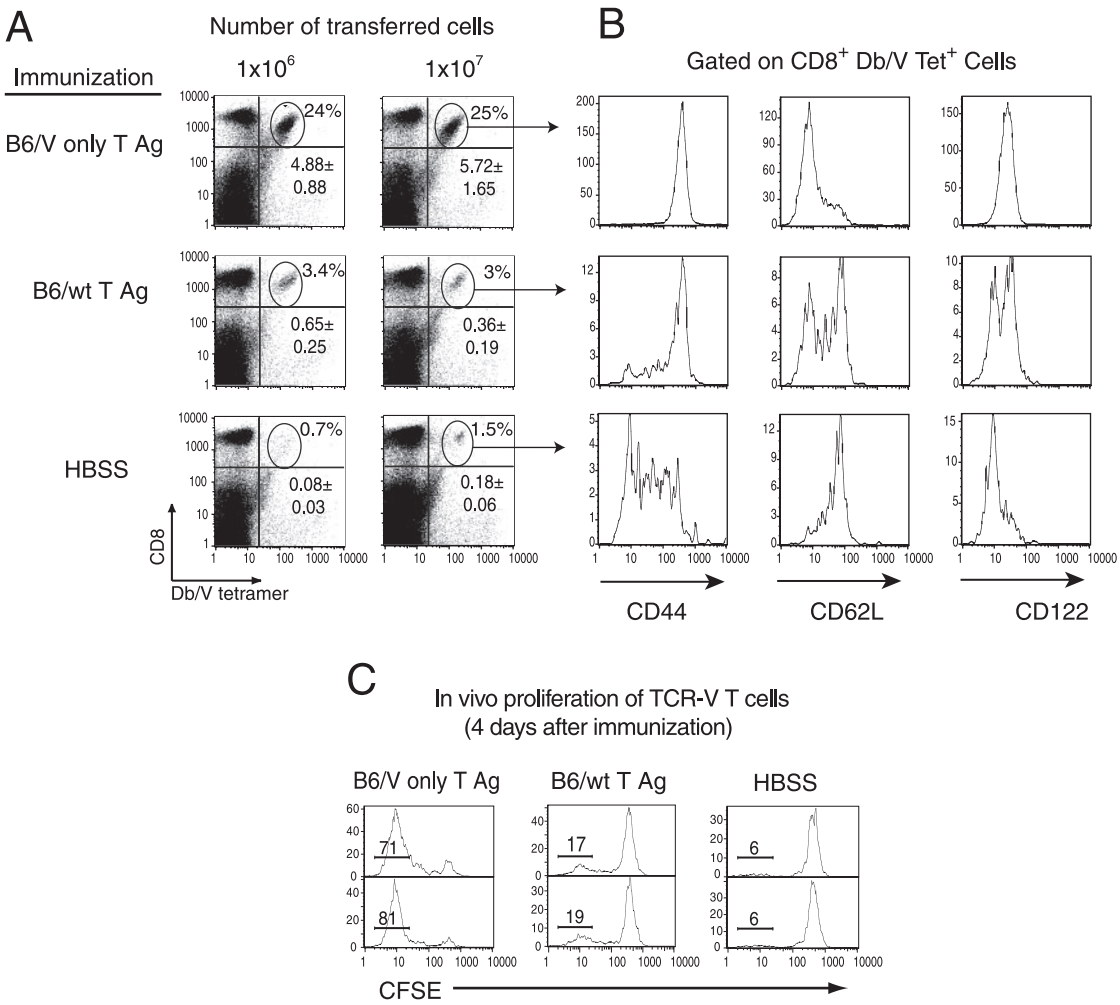

FIGURE 2. Activation and proliferation of TCR-V T cells in response to T Ag immunization. *A*, TCR-V expansion following immunization with T Ag-transformed cells. B6 mice were adoptively transferred with either of two doses of TCR-V T cells (1×10^6 or 1×10^7) followed by immunization the next day with 1×10^7 B6/V-only T Ag or B6/wt T Ag cells or remained unimmunized. Seven days postimmunization, spleen cells were triple stained for CD8α, Db/V tetramer, and cell surface markers CD44, CD62L, or CD122. The *top value* in each dot plot indicates the percentage of splenic T_{CD8} cells positive for Db/V tetramer in one individual mouse, and the bottom value indicates the total number of TCR-V T cells per spleen $\times 10^6 \pm$ SD ($n = 2$ mice/group). *B*, Flow cytometric analysis of T cell activation markers. Histograms show the level of expression of the indicated cell surface markers on the gated population of Db/V tetramer$^+$,T_{CD8} cells shown in *A*. *C*, Immunization with wt T Ag-expressing cells induces extensive proliferation in a small proportion of naive TCR-V T cells. A total of 5×10^6 CFSE-labeled TCR-V T cells was adoptively transferred into naive B6 mice; the next day, recipients were immunized with 1×10^7 B6/V-only T Ag or B6/wt T Ag cells or remained unimmunized. Four days postimmunization, spleen cells were stained for CD8α and Db/V tetramer to reveal the intensity of CFSE fluorescence on TCR-V T cells. Two individual mice per group are shown.

These experiments indicate that under conditions where T cell precursor frequency is not limiting, the subdominant phenotype of epitope V is maintained.

Coexpression of wt T Ag inhibits the immunogenicity of the epitope V-only T Ag variant

The finding that TCR-V T cells expanded dramatically following immunization with V-only T Ag but not wt T Ag indicates that T_{CD8} responding to the dominant epitopes inhibit the T_{CD8} response to the subdominant epitope V. To exclude the possibility that the V-only T Ag is inherently more immunogenic than the wt T Ag due to factors other than the lack of the dominant T Ag epitopes, we determined whether wt T Ag would affect the immunogenicity of V-only T Ag when coexpressed in the same cell. To perform this set of experiments, we used a panel of cell lines derived from the wt T Ag-transformed cell line B6/K-0. In a previous study (44), sequential in vitro selection of B6/K-0 cells with CTL clones specific for epitopes I and IV resulted in the isolation of cells expressing a T Ag variant in which residues 134–263 are deleted, which removes epitopes I (206–215) and II/III (223–231), and an additional mutation at residue 405 (V→L) inactivates epitope IV (404–411) (30, 46). This cell line, called B6/K-1,4, was subsequently supertransfected with a plasmid encoding wt T Ag (32) such that both wt and V-only T Ag constructs are expressed in the same cell. Coexpression of the two T Ags in B6/K-1,4-SV cells was demonstrated previously by immunoprecipitation of the two different-sized T Ag proteins (45). The expression of wt T Ag in B6/K-1,4-SV cells restores presentation of all T Ag epitopes in vitro, as shown by reactivity with epitope-specific CTL clones (36, 45).

TCR-V T cells were transferred into B6 mice followed 1 day later by immunization with 1) B6/K-0 (wt T Ag) cells, 2) B6/K-1,4 (V-only T Ag) cells, or 3) B6/K-1,4-SV (V-only + wt T Ags) cells. Seven days after immunization, mice were sacrificed, and the T_{CD8} response was evaluated by MHC tetramer staining (Fig. 3*A*). Consistent with the data in Fig. 2, immunization with B6/K-0 (wt

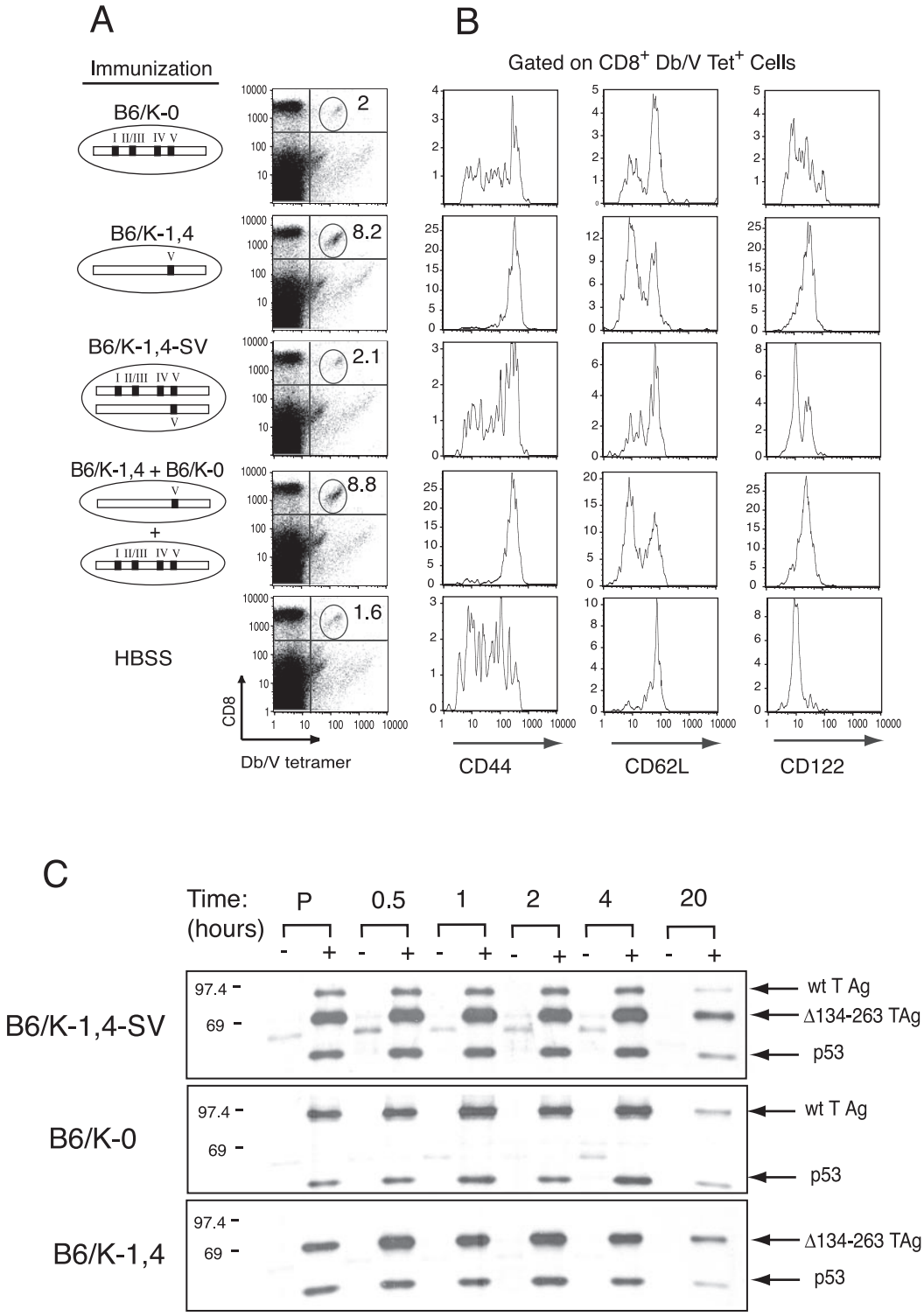

FIGURE 3. Coexpression of wt T Ag and V-only T Ag inhibits TCR-V T cell expansion. *A*, Quantitation of TCR-V T cells following immunization with cells coexpressing wt and V-only T Ags. B6 mice were adoptively transferred with 5×10^6 TCR-V T cells and immunized the next day with 5×10^7 B6/K-0 (wt T Ag), B6/K-1,4 (V-only T Ag), or B6/K-1,4-SV (expresses simultaneously wt T Ag and V-only T Ag) cells. Some mice received 5×10^7 B6/K-0 cells mixed with 5×10^7 B6/K-1,4 cells or remained unimmunized. Seven days later, splenic T_{CD8} specific for epitope V were quantitated by flow cytometry following staining with Db/V tetramer and anti-CD8 Ab. The values shown in the *upper right quadrant* indicate the percentage of splenic T_{CD8} cells positive for Db/V tetramer. *B*, Flow cytometric analysis of activation markers on TCR-V T cells. Histograms show the level of expression of the indicated cell surface marker on the population of Db/V tetramer$^+$ T_{CD8} cells shown within the gate in *A*. *C*, Pulse-chase immunoprecipitation of T Ag from T Ag-transformed cell lines. Cell lines, as indicated on the *left*, were metabolically labeled with [^{35}S]methionine followed by chase in medium containing unlabeled methionine for the indicated times (P; pulse, without chase). After preclearing with BSA-conjugated Sepharose 4B beads, lysates were immunoprecipitated using Sepharose 4B beads conjugated with PAb901 Ab (+) or with negative control Ab anti-HSV gD (−). The beads were then washed, denatured in sample buffer, and proteins were separated by SDS/7.5% PAGE. Size markers in kDa are shown on the *left*. The location of immunoprecipitated proteins is indicated on the *right*.

T Ag) cells led to only a weak expansion of naive TCR-V T cells and the activation of a subset of cells as measured by modulation of CD44, CD62L, and CD122 on the cell surface (Fig. 3*B*). In contrast, immunization with B6/K-1,4 (V-only) cells led to a 5-fold expansion of TCR-V T cells (8.2% of T_{CD8} vs 1.6% of T_{CD8} in the HBSS group) and the induction of an activated phenotype on the majority of cells. Importantly, immunization with B6/K-1,4-SV cells (coexpressing wt and V-only T Ags) mimicked the results obtained with cells expressing wt T Ag alone. In this group, only partial activation and minimal expansion of TCR-V T cells occurred (2.1% of T_{CD8}). Thus, the potent immunogenicity of cells expressing the V-only T Ag is inhibited by coexpression of wt T Ag in the same cells.

To determine whether this inhibitory effect required that wt and V-only T Ags be expressed in the same cells, a fourth group of mice was immunized with B6/K-0 (wt T Ag) cells mixed with an equal number of B6/K-1,4 (V-only T Ag) cells. TCR-V T cells isolated from mice immunized with this cell mixture were fully activated and expanded to the same level as in mice immunized with B6/K-1,4 cells alone (Fig. 3, *A* and *B*). These results indicate that the epitope V-specific T_{CD8} response is inhibited when T_{CD8} specific for the immunodominant epitopes can recognize the same but not different cells. Whether this competition affects the T_{CD8} response to epitope V at the level of APC that are cross-presenting T Ag or by preventing access to the tumor cells themselves remains to be determined.

Coexpression of wt T Ag does not alter the stability of epitope V-only T Ag

Recent studies have suggested that cross-presented Ag is derived from long-lived proteins found in the donor cell (54–56). Thus, a change in V-only T Ag stability might alter its immunogenicity. To determine whether coexpression of wt T Ag alters the stability of V-only T Ag, we performed pulse-chase immunoprecipitation of the T Ags in B6/K-0, B6/K-1,4, and B6/K-1,4-SV cells (Fig. 3*C*). The T Ags were detected as two prominent bands: 94 kDa corresponding to wt T Ag and 75 kDa corresponding to the epitope V-only variant (Δ134-263 T Ag; Fig. 3*C*). The stability of V-only T Ag was similar in both B6/K-1,4-SV cells and the parental B6/K-1,4 cells. This finding demonstrates that wt T Ag does not adversely affect the stability of the V-only T Ag variant when coexpressed in the same cell, although it dramatically reduces the epitope V immunogenicity of these cells. Therefore, the inability of K-1,4-SV cells to induce significant expansion of naive TCR-V T cells does not correlate with differences in protein stability within the transformed cells.

Cross-presentation of T Ag leads to inefficient expansion of TCR-V cells

The initiation of T_{CD8} responses to cell-associated Ags has been shown to depend on cross-presentation by professional APC in several experimental systems (57–60). Thus, one possible explanation for the weak activation of TCR-V T cells following immunization with wt T Ag-transformed cells is that epitope V might be poorly cross-presented compared with the immunodominant epitopes. To examine the role of cross-presentation in the activation and the expansion of TCR-V T cells, we used $TAP1^{-/-}$ cells transformed with either wt or V-only T Ag for immunization. $TAP1^{-/-}$ cells are defective in the transport of cytosolic antigenic peptides into the endoplasmic reticulum due to the absence of the TAP1 component of the peptide transporter (61). Thus, these cells are defective in the presentation of most endogenous Ags by MHC class I molecules but are capable of donating Ag for cross-priming in vivo (62).

To ensure that T Ag epitopes are not directly presented by T Ag-transformed $TAP1^{-/-}$ cells and that no other defects in these cells could inhibit T cell recognition, we reconstituted the TAP1 protein by infecting each $TAP1^{-/-}$ cell line with a recombinant vaccinia virus expressing the TAP1 and TAP2 proteins ((VV-TAP1 + 2); Ref. 48). $TAP1^{-/-}$ wt T Ag cells were efficiently recognized by in vitro-activated T cells derived from TCR-I mice after infection with VV-TAP(1 + 2) but not when infected with vaccinia recombinant VV-SC expressing an empty vector (Fig. 4*A*). Likewise, $TAP1^{-/-}$ V-only T Ag cells were lysed by a CTL clone specific for epitope V after infection with VV-TAP(1 + 2) but not after infection with empty vector VV-SC. Thus, the expected T Ag epitopes are presented by $TAP1^{-/-}$ cells following restoration of functional TAP. No CTL lysis was detected following infection of T Ag epitope null cells (B6/122B1) with VV-TAP(1 + 2) or with VV-SC. In addition, T Ag epitope-specific *LacZ* T cell hybridomas failed to recognize the T Ag-transformed $TAP1^{-/-}$ cells (see *Materials and Methods*). These results demonstrate that $TAP1^{-/-}$ cells expressing wt or V-only T Ag do not directly present T Ag epitopes for T cell recognition unless functional TAP is restored. In the absence of direct presentation of epitopes on the surface of T Ag-transformed cells, the T_{CD8} response induced by immunization with $TAP1^{-/-}$ cells can be attributed solely to the cross-presentation of T Ag by host APC.

To determine the efficiency of epitope V cross-presentation in vivo, we measured the activation, proliferation, and accumulation of TCR-V T cells in B6 mice immunized with $TAP1^{-/-}$ cells. These results were compared with cross-presentation of a dominant T Ag epitope, using TCR transgenic T cells specific for the immunodominant T Ag epitope I (TCR-I cells; Ref. 35). Mice were adoptively transferred with naive TCR-I (Fig. 4*B*) or TCR-V (Fig. 4*C*) T cells. The following day, mice that received TCR-I T cells were immunized with B6/wt T Ag or $TAP1^{-/-}$ wt T Ag-transformed cells or remained unimmunized. Similarly, TCR-V T cell recipients were immunized with B6/V-only T Ag or $TAP1^{-/-}$V-only T Ag-transformed cells or remained unimmunized. After 7 days, spleen cells were analyzed. Naive TCR-I T cells expanded to 49 and 12% of splenic T_{CD8} cells, respectively, following priming with the B6- or $TAP1^{-/-}$-derived wt T Ag-expressing cells, (Fig. 4*B*). TCR-I T cells recovered from both sets of mice expressed high levels of CD44. Likewise, TCR-V T cells expanded to 32% of splenic T_{CD8} cells and up-regulated CD44 after priming with B6/V-only T Ag-expressing cells (Fig. 4*C*) but failed to expand significantly after priming with $TAP1^{-/-}$ V-only T Ag-expressing cells. In addition, only a small population of TCR-V T cells showed up-regulation of CD44. This result suggests that only a small fraction of naive TCR-V T cells detected the presence of cross-presented epitope V following immunization with $TAP1^{-/-}$ V-only T Ag-expressing cells and failed to expand significantly.

Because no apparent increase in TCR-V frequency was observed 7 days postimmunization with $TAP1^{-/-}$ V-only T Ag-expressing cells, we asked whether any TCR-V cells were induced to proliferate early after immunization. Thus, the experiment shown in Fig. 4, *B* and *C*, was repeated using CFSE-labeled TCR-I and TCR-V donor cells. Three days after immunization, spleen cells were analyzed to determine the extent of TCR-I and TCR-V T cell proliferation. The data in Fig. 4*D* show that TCR-I T cells proliferated extensively after immunization with B6/wt T Ag or $TAP1^{-/-}$ wt T Ag cells. The TCR-V T cells similarly proliferated after immunization with B6/V-only T Ag. Importantly, immunization with $TAP1^{-/-}$ V-only T Ag cells triggered proliferation in only a subset of TCR-V T cells (28% CFSE negative). This result demonstrates that cross-presentation of epitope V results in proliferation of only a fraction of naive TCR-V T cells and implies

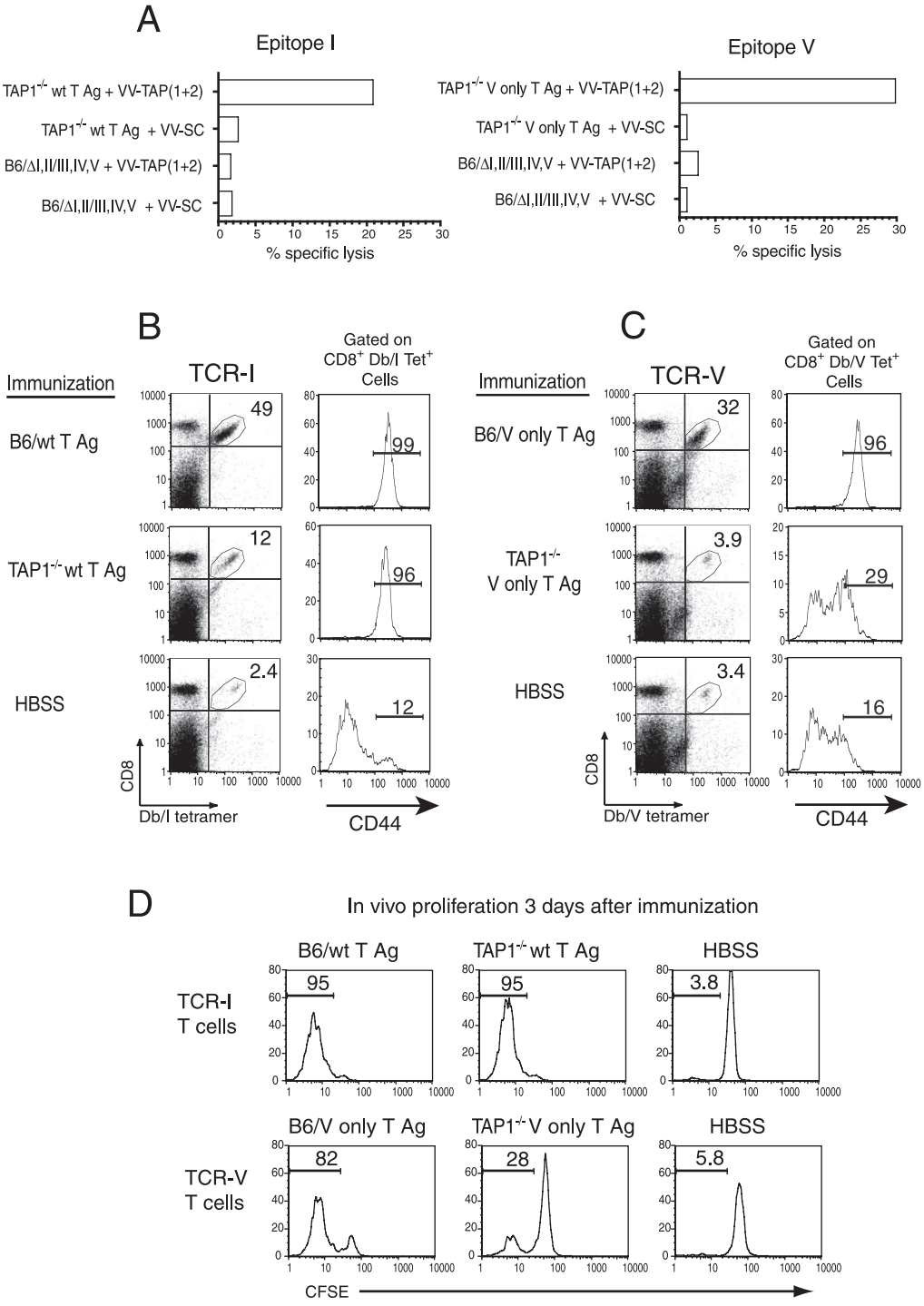

FIGURE 4. Cross-presentation of epitope V leads to proliferation of only a subset of naive TCR-V T cells. *A*, CTL recognition of T Ag-expressing TAP1$^{-/-}$ cells. ^{51}Cr-labeled T Ag-transformed cells were infected at multiplicity of infection = 10 with vaccinia viruses expressing TAP 1 + 2 (VV-TAP(1 + 2)) or VV-SC containing no insert. Following 5 h of infection, target cells were combined with a CTL clone specific for T Ag epitope V at an effector-to-target cell ratio (E:T) of 15:1 and with in vitro-activated TCR-I T cells at an E:T of 10:1 for 4 h. B6/T122B1 cells express a T Ag variant lacking all H-2^b CTL epitopes (Δ I, II/III, IV, V). *B*, Flow cytometric analysis of TCR-I T cell response to immunization with TAP1$^{-/-}$ cells. Naive B6 mice were adoptively transferred with 1×10^6 TCR-I T cells followed by immunization with 5×10^7 B6/wt T Ag (TAP$^{+/+}$) cells, TAP1$^{-/-}$ wt T Ag cells, or with vehicle (HBSS). Seven days postimmunization, spleen cells were triple stained with anti-CD8α Ab, the indicated tetramer and anti-CD44 Ab. The values in *upper right quadrants* indicate the percentage of splenic T_{CD8} that are positive for either Db/I tetramer or Db/V tetramer. Histograms show the level of surface expression of CD44 on the gated population of tetramer$^+$,T_{CD8}. *C*, Flow cytometric analysis of TCR-V T cell response to immunization with TAP1$^{-/-}$ cells. Naive B6 mice were adoptively transferred with 1×10^6 TCR-V T cells followed by immunization with 5×10^7 B6/V-only T Ag (TAP$^{+/+}$) cells, TAP1$^{-/-}$V-only T Ag cells, or with vehicle (HBSS). Cells were analyzed as in *B*. *D*, In vivo proliferation of CFSE-labeled TCR transgenic T cells. Mice were adoptively transferred with CFSE-labeled TCR-I (*top*) or TCR-V (*bottom*) T cells and immunized the next day with the indicated B6 or TAP1$^{-/-}$ cells or left unimmunized (HBSS). Three days after immunization, spleen cells were stained for CD8α and Db/I or Db/V tetramer, and the intensity of CFSE fluorescence on TCR-I and TCR-V T cells was determined by flow cytometry.

that additional direct presentation of epitope V by the B6/V-only T Ag-transformed cells also is required for maximal expansion of these activated T_{CD8}. By comparison, direct presentation of epitope I by wt T Ag-transformed cells was shown to be dispensable for inducing proliferation of TCR-I T cells by day 3 postimmunization. However, a role for direct presentation of epitope I by wt T Ag-transformed cells in obtaining maximal TCR-I T cell expansion is suggested because TCR-I T cells accumulated to higher levels by day 7 postimmunization with B6 vs TAP1$^{-/-}$ wt T Ag cells (Fig. 4*B*).

Direct presentation alone by T Ag-transformed cells is not sufficient to prime naive TCR-V T cells

To address the possibility that T Ag-transformed cells can prime naive TCR-V T cells directly and initiate an immune response in the absence of costimulatory signals provided by professional APC cross-presenting T Ag, we compared the response of adoptively transferred TCR-V T cells to immunization in B6 vs TAP1$^{-/-}$ hosts. To ensure that only naive TCR-V T cells were transferred, T_{CD8} expressing low amounts of cell surface CD44 were sorted by flow cytometry before adoptive transfer (Fig. 5*B*). B6 and TAP1$^{-/-}$ mice were adoptively transferred with TCR-V T cells and immunized on the same day with B6/V-only T Ag-expressing cells. Seven days later, mice were sacrificed, and TCR-V T cell expansion was evaluated.

Consistent with our previous results, naive TCR-V T cells expanded dramatically in B6 mice immunized with B6/V-only T Ag (Fig. 5*A*). In contrast, immunization of TAP1$^{-/-}$ mice with B6/V-only T Ag-expressing cells did not lead to detectable expansion of TCR-V T cells. To ensure that TCR-V T cells could respond to specific immunization in TAP1$^{-/-}$ hosts, one group of TAP1$^{-/-}$ mice was immunized with a vaccinia virus recombinant expressing epitope V as a minigene preceded with an endoplasmic reticulum-targeting sequence (rVV-ES-V) in addition to B6/V-only T Ag cells. The use of rVV-ES-V bypasses any requirements for TAP in the presentation of epitope V (33). TCR-V T cells expanded to ~18% of T_{CD8} in these mice, confirming the ability of the sorted TCR-V T cells to expand in TAP1$^{-/-}$ mice. Thus, direct presentation of epitope V by B6/V-only T Ag-transformed cells is not sufficient to induce the extensive accumulation of TCR-V T cells observed in B6 mice. This finding is consistent with the results of others who demonstrated that cross-presentation of Ag by host APC is required for activation of naive T_{CD8} (60, 63). Taken together, the results in Figs. 4 and 5 suggest that limited cross-presentation of epitope V is needed to activate a few naive TCR-V T cells in B6 mice, but direct presentation of Db/V complexes by T Ag-transformed cells drives the more extensive proliferation observed following immunization with V-only T Ag-transformed cells.

Cross-presentation of epitope V inefficiently boosts memory T_{CD8}

We next determined the extent to which Ag experienced T_{CD8} specific for epitope V were reactivated following cross-presentation of epitope V. For this experiment, we used the endogenous epitope V-specific T_{CD8} established in B6 mice following primary immunization with B6/V-only T Ag-transformed cells. At days 14 and 21 postimmunization, primed mice were boosted with either B6 or TAP1$^{-/-}$ cells expressing wt or V-only T Ag. Seven days later, the immune response to T Ag was analyzed using both MHC tetramer staining and the in vivo cytotoxicity assay (Fig. 6). Mice that received only primary immunization 28 days earlier with B6/V-only T Ag cells had low levels of epitope V-specific T_{CD8} (HBSS; 0.2%) and failed to show any significant elimination of peptide V-coated targets in the in vivo cytotoxicity assay. Epitope V-specific in vivo cytotoxicity was observed at earlier times after immunization (data not shown). Epitope V-specific T cells increased 30-fold following boosters with B6/V-only cells, representing 6% of splenic $T_{CD8,}$ and this resulted in the elimination of 79% of peptide V-pulsed target cells. As expected, there was no elimination of peptide I-pulsed target cells in these mice. Boosting with B6/wt T Ag cells led to a 10-fold increase in the percentage of T_{CD8} specific for epitope V (B6/wt; 2%) compared with unboosted mice and resulted in a detectable population of epitope I- and IV-specific T cells (2 and 12%, respectively). Accordingly, the in vivo cytotoxicity assay revealed killing of both peptide I- and V-coated targets (82 and 63% elimination, respectively) following boosting with B6/wt T Ag cells.

In contrast to boosting with B6-derived T Ag-transformed cells, TAP1$^{-/-}$ cells expressing either epitope V-only T Ag or wt T Ag failed to induce significant expansion of the epitope V-specific memory T cells. However, some reactivation of epitope V-specific memory T cells was indicated by increased levels of killing against peptide V-pulsed target cells following boosting with TAP1$^{-/-}$ V-only T Ag cells (20% elimination) and TAP1$^{-/-}$ wt T Ag cells (8% elimination). In contrast, immunization with TAP1$^{-/-}$ wt T Ag cells induced detectable responses against epitopes I and IV (1 and 2% of

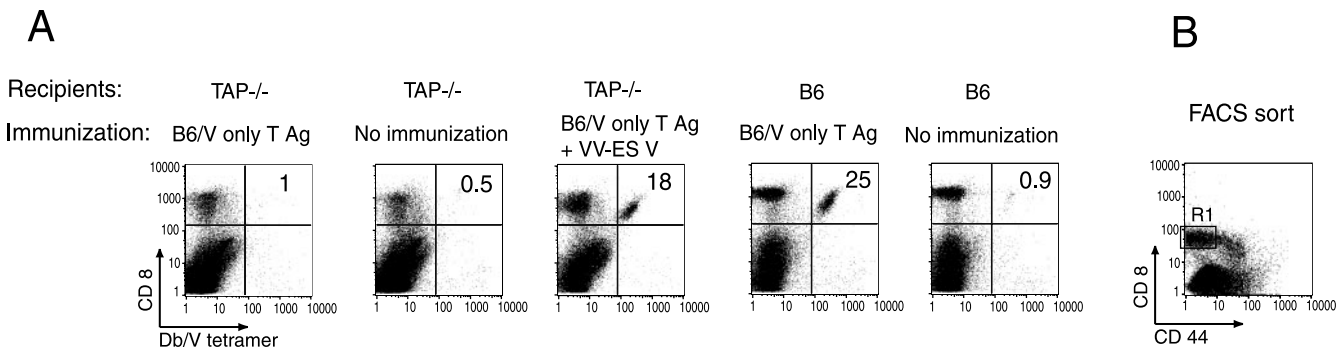

FIGURE 5. Direct priming by T Ag-transformed cells in the absence of cross-presentation is not sufficient to expand TCR-V T cells. *A*, Analysis of TCR-V T cell expansion in TAP1$^{-/-}$ mice. FACS-sorted naive TCR-V T cells (1×10^6) were adoptively transferred into gamma-irradiated (400 rad) TAP1$^{-/-}$ mice or nonirradiated B6 control mice. Recipient mice were immunized with 5×10^7 B6/V-only T Ag (TAP$^{+/+}$) on the same day or received HBSS as a control. One group of TAP1$^{-/-}$ mice was immunized with 5×10^7 B6/V-only T Ag cells plus i.p. immunization with 1×10^7 PFU of VV-ES-V. The values show the percentage of Db/V tetramer$^+$ T cells of splenic T_{CD8}. This experiment was repeated twice with similar results. *B*, Phenotype of donor TCR-V T cells. CD44^{low}-expressing CD8$^+$ T cells from naive TCR-V mice were sorted by flow cytometry before transfer into TAP1$^{-/-}$ mice.

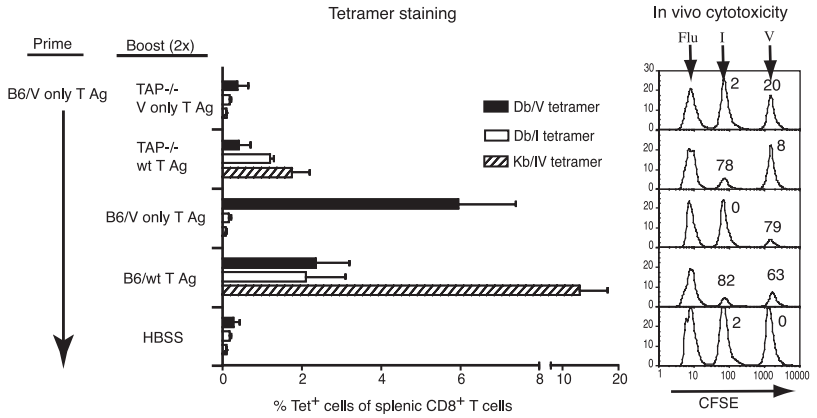

FIGURE 6. Epitope V memory T_{CD8} preferentially expand in response to direct presentation by T Ag-transformed cells. Groups of two or three B6 mice were immunized with 5×10^7 B6/V-only T Ag cells. Two and 3 wk later, mice were boosted with the same dose of B6/V-only T Ag, B6/wt T Ag, TAP1$^{-/-}$ V-only T Ag, or TAP1$^{-/-}$ wt T Ag cells or received no boost (HBSS). One week later, spleen cells from individual mice were analyzed for the presence of T_{CD8} specific for epitopes I, IV, and V by costaining with MHC class I tetramers. The data are presented as a percentage of splenic T_{CD8} that stained specifically with the indicated tetramer, and the background staining on naive B6 mice was subtracted and did not exceed 1% of T_{CD8}. Mice were simultaneously analyzed for the presence of epitope I- and V-specific effector T cells by in vivo cytotoxicity assay. B6.SJL spleen cell targets were incubated with peptides V, I, or Flu control peptide. Peptide-pulsed cells were then differentially labeled with CFSE (5, 0.5, and 0.025 μM, respectively), and 2×10^6 of each target population were injected 18 h before analysis of CD45.1$^+$ cells reisolated from spleens by flow cytometry. Histograms for representative mice are shown. The values indicate the percentage of specific elimination of CD45.1$^+$ targets (see *Materials and Methods*).

splenic T_{CD8} cells, respectively) and efficient killing of peptide I-coated targets (78% elimination). These results suggest that cross-presentation of epitope V alone is unable to expand epitope V-specific memory T_{CD8}. Efficient expansion required direct presentation of epitope V/Db complexes by B6-derived V-only or wt T Ag-transformed cells. By comparison, cross-presentation of wt T Ag led to expansion of the endogenous epitope I- and IV-specific T_{CD8}, although the frequencies achieved were reduced compared with immunization with B6/wt T Ag cells.

Discussion

Transfer of Ag from tumor cells to professional APC provides a viable mechanism to present tumor Ag epitopes for activation of naive T_{CD8} in cases where tumor cells lack the necessary costimulatory molecules. In support of this mechanism, cross-presentation of tumor Ags has been documented in multiple tumor systems (28, 58, 64–67). In this article, we demonstrate that the efficiency of cross-presentation can vary for epitopes within the same tumor Ag. Our results indicate that the subdominant epitope V is only weakly cross-presented in vivo from T Ag-transformed cells. Inefficient cross-presentation led to priming of only a subset of naive TCR-V T cells and also failed to significantly expand epitope V-specific memory T_{CD8}. However, under cross-priming conditions, all naive immunodominant TCR-I T cells were induced to proliferate and expanded to substantial levels. In addition, immunization of B6 mice with TAP1$^{-/-}$ wt T Ag cells primed endogenous T_{CD8} specific for epitopes I and IV. These data indicate that the subdominant epitope V is poorly cross-presented, whereas the immunodominant T Ag epitopes are more efficiently cross-presented under the same conditions. Thus, limited cross-presentation in vivo contributes to the subdominant nature of T Ag epitope V following immunization with T Ag-transformed cells.

Previous investigations of epitope V revealed that this antigenic peptide forms relatively short-lived complexes with H-2D^b compared with the dominant epitopes of T Ag (30, 33). Thus, in a system where only a limited amount of epitope is available, such as might occur during cross-presentation of cell-associated Ags, epitopes that are generated less efficiently or form more labile complexes with MHC class I molecules would be at a disadvantage regarding T cell priming by the APC (68). Cross-presentation involves the transfer of cell-associated Ag from a donor cell to a host professional APC (60). Although the nature of the cross-presented substrate has not been clearly identified, recent reports (54–56) suggest that the substrate for cross-presented Ags are native proteins or larger protein fragments. Our data indicate that cross-presentation of the epitope V is severely compromised even though cross-presentation of immunodominant T Ag epitopes within the same protein is maintained. Thus, one possible explanation is that the relatively short half-life of epitope V/D^b complexes generated in the APC from a fixed amount of transferred T Ag might provide limited opportunity for cross-priming to occur before epitope V/Db complexes fall below detectable levels. Such a mechanism has been proposed to explain immunodominance to some minor histocompatibility Ags (69). Furthermore, the instability of peptide/MHC complexes has been shown to limit the immunogenicity of an epitope derived from the gp100 tumor-associated Ag (70) and may explain the subdominant nature of a *Listeria monocytogenes*-derived epitope from the p60 protein (71). Recent studies investigating T cell activation in vivo have suggested that naive T cells require only a brief encounter with the Ag (4–8 h) to result in the modulation of cell surface receptors such as CD44 and CD69 but require more extended Ag exposure to enter productive proliferation and acquire effector function (72, 73). It should be noted that these studies were performed using immunodominant epitope-specific T cells and have yet to be confirmed using T cells specific for a subdominant epitope. Given the need for such time periods and the lability of epitope V/Db complexes, the opportunity for extended Ag engagement with APC cross-presenting epitope V in vivo might be limited, resulting in proliferation of only a small number of epitope V-specific T cells. Pamer and colleagues (74) previously demonstrated that premature termination of *L. monocytogenes* infection at 12 h by antibiotic treatment resulted in a dramatic decrease in the number of subdominant epitope-specific TCR transgenic T_{CD8} that proliferate, whereas the expansion of dominant epitope-specific TCR transgenic T_{CD8} was reduced only 2-fold. As in the model used here, this difference is consistent with the relative low stability of the subdominant epitope/

MHC complexes. Thus, the rapid loss of peptide/MHC complexes correlates with triggering of relatively fewer naive T_{CD8}.

Alternatively, epitope V may be inefficiently processed and presented by professional APC compared with the immunodominant epitopes as has been reported for some subdominant epitopes (75). We have shown previously that epitope V-specific CTL efficiently recognize IFN-γ-treated wt T Ag-transformed fibroblast cells, suggesting that induction of immunoproteasomes in general does not preclude presentation of epitope V in vitro (33). However, whether epitope V may be generated less efficiently by APC in vivo is unknown.

The ability of tumor cells to directly prime T_{CD8} responses in vivo has been implicated under conditions in which the tumor cells are able to migrate to the draining lymph nodes (25). The finding that cross-presentation of epitope V alone induces inefficient expansion of naive TCR-V T cells prompted us to investigate whether direct presentation by the B6/V-only T Ag cells was sufficient to result in priming and expansion of TCR-V T cells. However, lack of TCR-V T cell expansion in TAP1$^{-/-}$ hosts following immunization with B6/V-only T Ag cells indicated that direct priming alone was not sufficient to activate and expand naive TCR-V T cells. Why then do TCR-V T cells expand so dramatically in B6 mice following immunization with B6/V-only T Ag cells? We suggest that TCR-V T cell activation requires initial cross-presentation by host APC, but more extensive expansion occurs only after direct presentation by the T Ag-transformed cells themselves. This scenario is supported by the finding that a small population of TCR-V T cells are initially induced to proliferate following immunization with TAP1$^{-/-}$ V-only T Ag cells but fail to expand significantly. B6/V-only T Ag immunization could provide substrate for both cross-presentation to activate a few naive TCR-V T cells and direct presentation by the tumor cells themselves to drive further expansion. In this manner, constitutive direct presentation by the tumor cells could supplement the limited number of labile complexes found on the APC. Whether this interaction might happen simultaneously or serially remains to be determined but could be explained by the formation of three cell complexes in the lymphoid organs such that host APC cross-presenting epitope V, tumor cells directly presenting epitope V and TCR-V T cells interact simultaneously.

The participation of direct presentation by the tumor cells in driving T_{CD8} expansion in this model is also implied for the immunodominant epitopes. In this case, we found that efficient priming of naive TCR-I T cells was similar 3 days following immunization with TAP1$^{-/-}$ wt T Ag cells or B6/wt T Ag cells. However, total accumulation by day 7 was significantly higher following immunization with B6/wt T Ag cells, suggesting that additional direct presentation by the tumor cells led to enhanced expansion of T_{CD8} cells. This scenario is also supported by the finding that immunization of B6 mice with TAP1$^{-/-}$ wt T Ag cells induced fewer endogenous epitope I and IV-specific T_{CD8} than the B6-derived cells. Thus, the combination of cross-presentation of T Ag epitopes for activation of naive T_{CD8} plus additional direct presentation of T Ag epitopes by the tumor cells might lead to more efficient expansion of activated T_{CD8}. However, this effect might be more dramatic for weaker T_{CD8} responses.

In addition to inefficient cross-presentation of epitope V, our results indicate that the presence of T_{CD8} responding to the dominant T Ag epitopes contribute to the subdominant nature of epitope V. This is most evident in experiments in which immunization with B6/V-only T Ag cells led to extensive expansion of naive TCR-V T cells, while B6/wt T Ag cells led to only minimal expansion. This was demonstrated using two different epitope V-only T Ag constructs, indicating that this effect is due to the absence of immunodominant T Ag epitopes and not other intrinsic factors of the particular cell line. The frequency of epitope-specific T cell precursors has been shown to alter the immunodominance hierarchy in several Ag systems (1, 11, 12). Our results demonstrate that epitope V remains subdominant, even in the presence of supraphysiological numbers of naive TCR-V T cells and pre-existing endogenous memory T cells specific for epitope V. The inability of TCR-V T cells to expand significantly following immunization with B6/wt T Ag-expressing cells is likely due to the combination of inefficient cross-presentation of epitope V coupled with competition by the immunodominant T Ag epitope-specific T_{CD8}.

Previous studies on T cell competition revealed that presentation of dominant and subdominant epitopes by the same APC is required for maintenance of the immunologic hierarchy (11, 16–18, 76, 77). Kedl et al. (17) suggested that T cell interaction with specific peptide-MHC complexes on the surface of the APC induced the loss of that particular complex, thereby preventing activation of lower-affinity T_{CD8} specific for the same epitope. However, this mechanism does not explain domination of one epitope over another. Experiments using minor histocompatibility Ags demonstrated that T_{CD8} cells can compete for different epitopes if presented by the same APC (18, 78, 79). The nature of this competition was proposed to be of either a steric nature, competition for cytokines in the local environment, inactivation of the APC via cell killing or another unknown mechanism. Although this phenomenon, called cross-competition (76), was reported to be far less efficient than the competition with T_{CD8} cells of the same specificity, we reasoned that it might play a significant role under certain conditions, particularly if Ag is limiting as is suggested for epitope V.

Although our results indicate that T_{CD8} responding to the immunodominant T Ag epitopes inhibit expansion of epitope V-specific T_{CD8}, the mechanism remains unknown. We suggest that competition may occur at the level of the tumor cells themselves. The finding that epitope V memory T_{CD8} are less efficiently expanded by B6/wt T Ag cells than B6/V-only T Ag cells suggests that competition for the tumor cells following priming of endogenous T_{CD8} specific for the immunodominant epitopes limits the expansion of epitope V-specific memory T_{CD8}. Conversely, there was minimal expansion of epitope V-specific memory T_{CD8} following immunization with TAP1$^{-/-}$ wt or V-only T Ag-expressing cells, suggesting that the endogenous epitope V-specific memory T_{CD8} are responding preferentially to direct presentation by the tumor cells. The additional finding that coimmunization with a mixture of wt and V-only T Ag-transformed B6 cells led to efficient expansion of naive TCR-V T cells suggests that immunodomination requires copresentation of the dominant and subdominant epitopes by the same cell. Our results do not rule out the possibility that T_{CD8} responding to the dominant epitopes also can act at the level of the cross-presenting APC. In fact, this mechanism might be more important for inhibiting priming of the endogenous epitope V-specific T_{CD8} response because fewer precursor T_{CD8} are available in the normal repertoire of B6 mice than in the experiments using adoptively transferred TCR-V T cells.

The results presented in this article suggest that when multiple T_{CD8} epitopes derive from the same antigenic protein, epitope-specific factors that affect cross-presentation can limit T_{CD8} immunity. Thus, even if an epitope is directly presented on tumor cells that constitutively express the antigenic protein, transfer of a fixed amount of tumor Ag to the APC might result in subthreshold levels of peptide/MHC complexes to activate a significant number of T_{CD8}. One potential benefit of this effect for the tumor-specific T_{CD8} repertoire is that T_{CD8} specific for poorly cross-presented

self-tumor epitopes might be less susceptible to tolerance induction due to limited presentation of epitopes in the steady state. We have shown previously that epitope V-specific T_{CD8} are less susceptible to both central and peripheral tolerance mechanisms than the immunodominant T Ag epitopes in T Ag transgenic mice developing spontaneous tumors (43, 49, 80). Thus, T cells specific for epitopes that are poorly cross-presented might represent good vaccine candidates for cancer as they may be less susceptible to tolerance yet capable of responding to specific immunization approaches. Our results, and those of others (11, 16), also suggest that immunization with individual epitopes, as opposed to multisubunit vaccines, would reduce the development of immunological hierarchies for epitopes that are limited by cross-presentation, allowing efficient priming of both dominant and subdominant epitope-specific T_{CD8}. Thus, future vaccination approaches that require cross-presentation should consider the epitope-specific factors that affect the efficiency of T_{CD8} responses.

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Disclosures

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Early Immunization Induces Persistent Tumor-Infiltrating CD8$^+$ T Cells against an Immunodominant Epitope and Promotes Life-long Control of Pancreatic Tumor Progression in SV40 T Antigen Transgenic Mice[1]

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Summary

The ability to recruit the host's $CD8^+$ T lymphocytes (T_{CD8}) against cancer is often limited by the development of peripheral tolerance toward the dominant tumor-associated antigens. Since multiple epitopes derived from a given tumor antigen can be targeted by T_{CD8}, vaccine approaches should consider whether peripheral tolerance differentially compromises T_{CD8} specific for individual epitopes. In this study, we investigated the effect of peripheral tolerance on the T_{CD8} response to two immunodominant epitopes, designated epitopes I and IV, from the SV40 large T antigen (T Ag) in line RIP1-Tag4 transgenic mice which express T Ag from the rat insulin promoter (RIP), resulting in the progressive growth of pancreatic insulinomas. We demonstrate that immunization of 5 week old RIP1-Tag4 mice elicits T_{CD8} against both epitope I and IV, but that only epitope IV-specific T_{CD8} are maintained long-term. Immunization of RIP1-Tag4 mice at 5 weeks of age against epitope IV resulted in complete protection from tumor progression over a 2 year period in which T Ag expression was maintained in the pancreas. This extensive control of tumor progression was associated with the persistence of functional epitope IV-specific, but not epitope I-specific, T_{CD8} within the pancreas for the lifetime of the mice. These results demonstrate that T_{CD8} specific for two distinct epitopes from the same tumor antigen are differentially affected by peripheral tolerance and that activation of T_{CD8} against epitope IV induces lifelong surveillance against spontaneous tumor progression without disrupting organ function.

Introduction

A variety of experimental models have been utilized to examine the role of $CD8^+$ T lymphocytes (T_{CD8})[5] in the immunotherapy of tumors. These models include transplantable tumors as well as spontaneously arising tumors that develop in transgenic mice expressing cell- or virus-derived oncogenes. Such studies have revealed a multitude of factors that may inhibit an effective T_{CD8}-mediated response against the tumor [reviewed in (1)], including but not limited to the deletion of tumor specific T_{CD8} by central tolerance (2-5) or their inactivation by peripheral tolerance (6-8). Mechanisms which allow the tumor to resist even an active T_{CD8} response also contribute to failed immunotherapy of cancer, such as the inability of tumor-specific T_{CD8} to access the tumor (9). Some experimental models have utilized surrogate tumor antigens expressed in transplantable and transgene induced tumors to monitor the role of endogenous T_{CD8} in the control of tumor growth (5, 10-12) or alternatively have assessed the response of T_{CD8} derived from T cell receptor (TCR) transgenic mice toward transplantable or oncogene-induced spontaneous tumors (13-17). Few studies, however, have assessed the role of the endogenous natural T_{CD8} for the control of spontaneous tumors expressing a known tumor antigen (18-21). The identification of successful immunotherapeutic approaches in mice that develop spontaneous tumors may provide key information for developing translational approaches toward the immunotherapy of human cancers.

T_{CD8} recognize peptides bound to MHC class I molecules on the tumor cell surface. These peptides are derived from the processing of intracellular proteins, with each MHC class I allele binding different combinations of the available peptides for presentation at the cell surface (22). Thus, a wide variety of peptide/MHC complexes is presented at the tumor cell surface and the tumor-specific T_{CD8} response can be targeted against multiple epitopes simultaneously, although with varying efficiencies

(23). This phenomenon, known as immunodominance is controlled by multiple factors affecting antigen processing and presentation as well as the T cell repertoire of the host (24). Since multiple tumor epitopes could be potentially targeted, assessing the nature of the immune response toward epitopes that lead to the most effective control of tumor progression in experimental models will provide clues toward better approaches to immunotherapy.

Our previous studies using two different transgenic mice expressing SV40 tumor antigen (T Ag) indicate that immunodominance among T Ag epitopes contributes toward the efficiency of T_{CD8}-mediated control of progressively growing tumors due to differential inactivation of T_{CD8} specific for four distinct T Ag epitopes (3, 6). SV40 T Ag encodes four H-2^b-restricted epitopes (25). Epitopes I (residues 206-215), II/III (residues 223-231) and V (residues 489-497) are H-$2D^b$-restricted whereas epitope IV (residues 404-411) is H-$2K^b$-restricted. These four T Ag-derived T_{CD8}-recognized epitopes fall on a hierarchical scale; epitope IV being the most immunodominant followed by epitopes I and II/III (26, 27). Epitope V is subdominant and is not immunogenic unless the dominant epitopes are inactivated in T Ag (28, 29). The ability of T_{CD8}-specific for T Ag to control tumor progression was established by showing that transfer of T_{CD8} specific for the immunodominant T Ag epitopes into SV11 T Ag transgenic mice induced regression of advanced-stage spontaneous tumors (30).

To better understand the role of the endogenous T_{CD8} repertoire specific for the T Ag epitopes in the control of spontaneous tumor progression, we took advantage of the well-characterized tumor model of RIP1-Tag4 transgenic mice on the C57BL/6 (B6) background (31). In RIP1-Tag4 mice, T Ag is expressed as a transgene from the rat insulin II promoter in pancreatic β cells, which leads to highly predictable formation of β cell tumors. The transformation of β cells by T Ag is a gradual process,

initially inducing development of islet hyperplasia by three months of age and followed by appearance of macroscopic insulinomas between five and six months of age (18, 32). The average life span of RIP1-Tag4 mice is around 200 days with 100% of mice developing tumors. Transformed β cells secrete high amounts of insulin leading to the development of severe hypoglycemic shock and death of tumor-bearing mice. Previously, Knowles and colleagues (18) demonstrated that immunization of RIP1-Tag4 mice with SV40 prior to or during T Ag-induced tumorigenesis resulted in the development of T_{CD8} capable of lysing syngeneic T Ag transformed cells in vitro. However, only immunization prior to the expression of T Ag in the β cells (at 3 weeks of age), led to long-term protection against tumor development. Whether the repertoire of T Ag-specific T_{CD8} was altered by expression of the endogenous T Ag was not addressed. Although this previous study demonstrated that the presence of T Ag-specific T_{CD8} correlates with long-term control of tumor progression in RIP1-Tag4 mice, it remained to be determined whether peripheral tolerance differentially affects T_{CD8} responding to individual T Ag epitopes over the course of tumor progression and whether long-term protection from tumor progression is associated with a particular T Ag epitope.

Our results show that activation of endogenous T_{CD8} specific for the dominant SV40 T Ag epitope IV prior to T Ag expression in the pancreatic β cells leads to life-long surveillance against T Ag-induced tumors. Importantly, functional epitope IV-specific, but not epitope I-specific T_{CD8} persist in the host without being deleted or inducing tissue destruction or diabetes in RIP1-Tag4 mice.

Materials and Methods

Mice

C57Bl/6 (H-2^b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME), B6.SJL mice (CD45.1^+) were purchased form Taconic Farms (Germantown, NY). Line RIP1-Tag4 mice are on a C57Bl/6 background and expresses the SV40 early region as a transgene under control of the rat insulin promoter and were described previously (31). All mice were maintained at the animal facility of the Milton S. Hershey Medical Center. Animal studies were performed in accordance with guidelines established by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee under an approved protocol.

Reagents and immunizations

The B6/WT-19 cell line was derived previously by transformation of C57Bl/6 mouse embryo fibroblasts with wild type SV40 strain VA45-54 (33). B6/122B1 cells express a T Ag variant containing wild type epitope IV but in which epitopes I, II/III, and V have been inactivated by site-directed mutagenesis (26). Cell lines were maintained in DMEM, supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 100 μg/ml of kanamycin, 2 μM L-glutamine, 10 mM HEPES, 0.075% (wt/vol) $NaHCO_3$ and 5-10 % fetal bovine serum (FBS). Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by Fmoc chemistry using an automated peptide synthesizer (9050 MiliGen PepSynthesizer ; Milipore, Bedford, MA). Peptides used in these experiments correspond to the SV40 T Ag epitope I (SAINNYAQKL; 206-215), epitope IV (VVYDFLKL; 404-411), and an epitope from herpes simplex virus (HSV) glycoprotein B (SSIEFARL; gB 498-505). Immunization of mice with T Ag-expressing cell lines was carried out by i.p. injection of 3-5 x 10^7 cells in 0.5 ml HBSS at the indicated ages.

Flow cytometric analysis and in vitro stimulation of bulk CTL cultures

Ex vivo staining of T_{CD8} with MHC tetramers and primary conjugated antibodies was performed on single-cell suspensions prepared from spleens or collagenase D and DNAse digested tumors as described previously (26). For dissociation of tumors, tissue was mined with a razor blade and incubated in HBSS containing 1% FBS, 1 mg/ml collagenase D (Roche) and 50 U/ml DNAse I (Roche) for one hour at 37º C. Any remaining clumps were dissociated by gentle pipeting and cells were washed prior to tetramer staining. In some cases, spleen cells were cultured in vitro for 6 days in the presence of gamma-irradiated B6/WT-19 stimulator cells as described previously (3). After staining, cells were fixed with 2% paraformaldehyde in PBS and analyzed using a FACScan or FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, San Carlos, CA). Production and characterization of the H-2D^b/T Ag epitope I (Db/I), H-2K^b/T Ag epitope IV (Kb/IV), H-2D^b/influenza virus NP epitope 366-374 (Db/Flu) and H-2K^b/HSV gB epitope 498-505 (Kb/gB) tetramers was previously described (26). The following monoclonal antibodies were purchased from BD Pharmingen: PE-, and Cychrome-labeled rat anti-CD8α (clone 53-6-7), FITC-labeled hamster anti-mouse CD69 (clone H1.2F3), FITC-labeled rat anti-mouse IFN-γ (clone XMG1.2). The percentage of CD8$^+$ cells that stained positive for T Ag specific tetramer was determined by subtracting the percentage of cells that stained positive with the control Db/Flu or Kb/gB tetramers within the same population. Determination of TCRß chain (TCRß) usage by epitope IV-specific T_{CD8} was performed by direct ex vivo staining of splenocytes with a panel of Vß-specific antibodies (BD-Pharmingen), Kb/IV tetramer and anti-CD8α Ab.

Intracellular cytokine assay and tetramer dissociation assay

For staining of intracellular IFN-γ, RBC-depleted lymphocyte suspensions were prepared as described above and incubated at $5x10^6$/ml with 1 μM of the indicated synthetic peptides, representing T Ag or control epitopes, and 1 μg/ml brefeldin A in complete RPMI 1640 containing 10% FBS for 4 h at 37°C in 5% CO_2. The Cytofix/Cytoperm Kit (BD PharMingen) was used to stain for IFN-γ production according to the manufacturer's instructions and as described previously (26). For MHC tetramer stability assays, in vitro-restimulated splenocytes derived from immunized mice were incubated with a 1:20 dilution of the Kb/IV tetramer and anti-CD8α Ab for 1 h on ice. Cells were then washed three times with PBS/2% FBS. Cells were resuspended in PBS/2% FBS and placed at 37˚C. Aliquots of cells were removed at the indicated time points, fixed immediately with 4 % paraformaldehyde and kept at 4°C prior to flow cytometric analysis.

In vivo cytotoxicity assay

Splenocytes from B6.SJL mice ($CD45.1^+$) were incubated in the presence of the indicated peptides (1μM) in RPMI/10% FBS at 37˚C for 90 min and washed three times to remove excess peptide. Peptide-coated targets were then labeled with varying concentrations (5mM; $CFSE^{high}$, 0.5mM;$CFSE^{medium}$, 0.025mM;$CFSE^{low}$) of 5- and 6-CFSE (Molecular Probes, Eugene, OR) for 10 min at 37˚C in PBS/0.1% BSA, washed 2 times, mixed together at 1:1:1 ratio and then $6x10^6$ cells were injected i.v. into the tail vein of sex-matched mice in 0.2 ml HBSS. The elimination of CFSE-labeled targets was assessed after 16 hours by analyzing spleen cells for the presence of $CD45.1^+$, $CFSE^+$ cells. The following formula was used: percentage of specific killing = [1 – (ratio of control mice/ratio of immunized mice) x 100], where ratio = (% of$CFSE^{low}$/$CFSE^{high\ or\ meidium}$). $CFSE^{low}$ targets were pulsed with control peptide gB and used as an internal control for nonspecific lysis.

Tumor histology and immunohistochemistry, islet size measurement

For T Ag immunohistochemistry, pancreata were fixed with 10% buffered formalin prior to embedding in paraffin blocks. Eight-micrometer-thick sections were cut on a microtome and collected onto positively charged slides. Sections were deparaffinized in xylene and rehydrated in ethanol. After two washes in PBS, slides were treated with antigen unmasking solution (Vector Laboratories) for 30 min in a steamer. Cooled slides were washed in PBS, incubated with 10% normal goat serum in PBS containing 0.1% Tween 20 for 30 min, washed and incubated with primary anti-T Ag antibody for 1 h at room temperature. Primary antibody consisted of an equal mixture of monoclonal antibody Pab419 (34) and Pab901 (35) culture supernatants which bind to epitopes in the amino- and carboxyl-terminal portions of SV40 T Ag, respectively. Sections were then incubated for 1 h with biotinylated anti-mouse antibody and visualized with peroxidase/DAB detection [Chemmate detection kit; Ventana, Tuscon, AZ)] for 2 to 10 min, as needed. Parallel sections were stained with H&E. Sections were examined using a Nikon Microphot-FXA microscope and representative images captured using a Sony DKC-ST5 digital camera. To measure the size of individual islets, H&E stained pancreatic section were examined under the microscope using a micrometer. The radius of all islets per section was measured and their individual sizes were plotted.

Lifespan analysis and measurement of blood glucose levels

Mice in lifespan analyses were monitored for symptoms of hypoglycemia, indicative of advanced stage tumors, and sacrificed when blood glucose levels dropped below 50 mg/dl. Blood glucose levels were measured using an Elite® glucometer (Bayer Corporation, Elkhart, IN). Kaplan-Mier survival plots were constructed using Prism software (Graphpad, San Diego, CA).

Results

RIP1-Tag4 mice with progressively growing tumors remain responsive to T Ag epitope IV, but not to T Ag epitope I.

To determine if T_{CD8} specific for the immunodominant T Ag epitopes remain sensitive to immunization in aged RIP1-Tag4 mice, groups of three RIP1-Tag4 mice aged 35 days, 90 days and 180 days and 90-day old B6 mice were immunized intraperitoneally with T Ag-transformed B6/WT-19 cells that express wild type T Ag. Nine days later, splenic T_{CD8} lymphocytes were stained with anti-CD8 Ab and MHC class I tetramers capable of detecting T cells specific for epitope I (Db/I) or epitope IV (Kb/IV). A tetramer composed of the influenza nucleoprotein (NP) epitope $NP_{366-374}$ (Db/Flu) was used as a control tetramer. The data in Figure 1A show that epitope IV-specific T cells expanded in 35-day old RIP1-Tag4 mice to levels similar for control B6 mice. The number of epitope IV-specific T_{CD8} recruited by immunization gradually declined in older RIP1-Tag4 mice, but remained significant even at 180 days of age. In contrast, the T_{CD8} response against epitope I already was significantly decreased in 35-day old RIP1-Tag4 mice compared to B6 mice and was undetectable by tetramer staining in 180-day old RIP1-Tag4 mice.

Since the immune response in the lymphoid organs may not necessarily reflect the extent of the immune response at the tumor site, we determined if T Ag-specific T_{CD8} recruited by immunization with B6/WT-19 cells localized to the tumors in RIP1-Tag4 mice. Groups of 180-day old RIP1-Tag4 mice were immunized with B6/WT-19 cells. Nine days later tumors were enzymatically digested and epitope-specific T_{CD8} were directly enumerated by staining with MHC tetramers. The data in Figure 1B show that epitope IV-specific T_{CD8} (29 % of total T_{CD8}) infiltrated the pancreatic tumors. In parallel experiments we found that approximately half the number of Kb/IV tetramer+ T_{CD8} detected in the tumor

at this time point were capable of producing IFN-γ in response to short term incubation with epitope IV peptide as determined by intracellular cytokine staining (data not shown). Staining of tumor-derived T_{CD8} with Db/I tetramer did not show positive cells above the background staining obtained with control Db/Flu tetramer. These results show that epitope I and IV-specific T_{CD8} are differentially affected by peripheral tolerance in RIP1-Tag4 mice with only epitope IV-specific T_{CD8} remaining responsive to immunization and capable of infiltrating tumors in 180-day old RIP1-Tag4 mice.

Early immunization of RIP1-Tag4 mice against epitope IV prevents the development of tumors without inducing diabetes.

An initial report by Ye *et* al (18) showed that the development of tumors in RIP1-Tag4 mice can be most significantly delayed (average lifespan of 622 days) by immunization with live SV40 only if carried out at three weeks of age. Immunization of 6 week old mice was less effective in controlling tumor progression (average lifespan of 487 days versus 263 days for control mice). To determine if specific immunization against the immunodominant epitope IV alone could increase the survival of RIP1-Tag4 mice, 35-day old RIP1-Tag4 mice were immunized i.p. with B6/15Bb cells expressing a T Ag variant (epitope IV-only) containing epitope IV but lacking the other H-2^b-restricted epitopes I, II/III, and V (26). Immunization with B6/15Bb cells results in a quantitatively similar T_{CD8} response to epitope IV as following immunization with B6/WT-19 expressing the wild type T Ag (data not shown). Another group of RIP1-Tag4 mice was similarly immunized at 180 days of age. The data in Figure 2A demonstrate, that early immunization at 35 days of age with epitope IV-only T Ag expressing cells completely prevented the development of tumors since all mice in the group (n=8) lived for two years, at which time these mice were euthanized for further analysis (see below). In contrast, immunization of RIP1-Tag4 mice with epitope IV-only T Ag expressing cells at 150 days of age was not effective at

controlling tumor progression (n=8, median 204 days) and their life span was similar to untreated RIP1-Tag4 mice (n=10, median 194 days). Thus, immunization of RIP1-Tag4 mice at 35 days of age against epitope IV is associated with a dramatic increase in survival while immunization of tumor-bearing mice had a minimal effect on survival.

To monitor the effect of immunization on tumor growth, we measured the size of individual islets at various ages in RIP1-Tag4 mice that remained unimmunized (Fig. 2C) or were immunized with IV-only T Ag-expressing cells at 35 days of age (Fig. 2D). Increased islet size in unimmunized RIP1-Tag4 mice can be detected as early as 84 days of age, which is consistent with the reported development of initial islet hyperplasia (36). Islet size further increased by 175 days of age which corresponded with the appearance of macroscopic tumors. Histological analysis, on days 180 and 720, of pancreata from immunized RIP1-Tag4 mice (Fig. 2D) revealed that islet size was consistent with modest hyperplasia, although small tumors could be detected in some mice (1-1.5 mm in diameter). Thus, the protective effect of early immunization against epitope IV correlates with inhibition of tumor development in RIP1-Tag4 mice.

Immunization against the T Ag expressed in the pancreatic β cells might be expected to induce an autoimmune phenotype and subsequent diabetes if β cells are specifically destroyed by responding immune cells. However, we found mice immunized with B6/15Bb cells at 5 weeks of age which survived long-term maintained normal levels of blood glucose (Fig. 2B), indicative of continued β cell function. In contrast, naïve RIP1-Tag4 mice became hypoglycemic by 27 weeks of age, consistent with the progression of β cell tumors producing high levels of insulin (31). Thus, immunization against T Ag epitope IV results in continuous and long term protection from tumor progression without disrupting

pancreatic function.

Immunized RIP1-Tag4 mice survive long-term despite continued expression of T Ag

Long-term survival of RIP1-Tag4 mice immunized with T Ag transformed cells at 35 days of age could be explained by the loss of β cells expressing the T Ag transgene. To assess this possibility, we determined if long-term surviving RIP1-Tag4 mice which were immunized at 35 days of age maintain T Ag expression in the pancreatic islets as detected by immunohistochemistry (Fig. 3). Islet cells from unimmunized B6 mice lacked T Ag expression (Fig. 3C), whereas the majority of cells in the islets of 6 month old naïve RIP1-Tag4 mice were positive for T Ag expression (Fig. 3D). We then analyzed pancreatic tissue obtained from long-term surviving RIP1-Tag4 mice which were immunized at 35 days of age. The histological analysis of pancreatic sections revealed that T Ag positive cells were detected in the islets of one-year (Fig. 3G and H) and two-year old (Fig. 3K and L) RIP1-Tag4 mice. H&E staining of pancreatic tissue from these T Ag-immune mice showed that most islets were smaller than the islets found in naive 6-month-old RIP1-Tag4 mice, although some hyperplastic islets were slightly larger (Fig. 3F). It should be noted that the number of islet cells positive for T Ag from immunized mice was reduced (Fig. 3K and L) compared to the percentage of T Ag positive cells in unimmunized RIP1-Tag4 mice (Fig. 3D). Importantly, despite continuous expression of T Ag in the islets, large tumors did not develop in immunized mice although islets of varying sizes were detected. These results demonstrate that immunization at 35 days with T Ag epitope IV-only expressing cells prevented the development of tumors in RIP1-Tag4 mice. These data also suggest that islet cells expressing high levels of T Ag may be susceptible to T_{CD8} mediated elimination, but that some T Ag-expressing β cells persist to produce insulin over the lifespan of the mice.

Life-long persistence of T Ag epitope IV-specific T_{CD8} in immunized RIP1-Tag4 mice.

In some models, T cells responding to self antigens have been shown to be deleted shortly after the initial immune response or persist in an anergic state (37-39). Thus, an important question is whether epitope IV-specific T_{CD8} induced by immunization at 35 days of age persist and remain functional in two-year old RIP1-Tag4 survivor mice. Representative survivors were sacrificed to quantitate the epitope IV-specific T_{CD8} present in the spleens and pancreas by tetramer staining, and to examine T cell function by in vivo cytotoxicity assay (Fig. 4A). This analysis revealed that epitope IV-specific T_{CD8} were present at detectable levels in the spleens (8.1% of total T_{CD8}) of long-term survivors and expanded dramatically (53% of total T_{CD8}) following in vitro culture (Fig. 4A, group 5). Analysis of epitope IV-specific in vivo cytotoxicity in the same animal showed that epitope IV-pulsed, but not epitope I-pulsed targets were eliminated (99% vs 0%). To determine if these persisting epitope IV-specific T_{CD8} could expand following antigenic challenge in vivo, representative mice were immunized with B6/WT-19 cells. After nine days, epitope IV-specific T_{CD8} had expanded to 15% of T_{CD8} in the spleen (Fig. 4A, group 6). Epitope IV-pulsed target cells were also completely eliminated in these mice, although no epitope I-specific lysis was detected indicating that these RIP1-Tag4 mice remained fully tolerant to epitope I.

One possible explanation for the inability of immunization against T Ag to induce control of advanced stage tumors is that the responding T_{CD8} in older mice are rendered nonfunctional by toleragenic mechanisms. Thus, we determined whether RIP1-Tag4 mice immunized at 6 months of age could eliminate target cells pulsed with epitope IV peptide. Epitope IV-specific T_{CD8} were recruited to 2.2% of T_{CD8} following immunization of 171 day old RIP1-Tag4 mice with wild type T Ag-expressing B6/WT-19 cells (Fig. 4A, group 4). In addition, epitope IV-pulsed targets were completely eliminated

in the in vivo killing assay while no lysis of epitope I-pulsed target cells was observed. In contrast, both epitope I- and IV-specific killing was detected in control B6 mice immunized with B6/WT-19 cells (Fig. 4A, group 2). We considered that since epitope IV-specific T_{CD8} persisted in 6 month old naïve RIP1-Tag4 mice, that some priming against the T Ag in tumor-bearing RIP1-Tag4 mice might occur. Spleen cells from unimmunized 6 month old RIP1-Tag4 mice were analyzed directly by tetramer staining. The results in Fig. 4A (group 3) show that although epitope IV-specific T_{CD8} were not detected directly ex vivo by MHC tetramer analysis, a modest level of epitope IV specific killing was detected (39%). Moreover, epitope IV-specific T_{CD8} were expanded after in vitro culture (6.2% of T_{CD8}), indicating that RIP1-Tag4 mice spontaneously develop low numbers of epitope IV-specific T_{CD8}, a finding consistent with those reported by Ye et al (18). No epitope IV-specific T_{CD8} were detected following in vitro culture of splenocytes from naïve B6 mice (Fig. 4A, group 1).

For continuous inhibition of tumor growth to occur, epitope IV-specific T_{CD8} induced by immunization should be present in the pancreas of long-term surviving RIP1-Tag4 mice. Thus, we analyzed by tetramer staining enzymatically-digested pancreas from one year old RIP1-Tag4 mice immunized with epitope IV-only T Ag expressing B6/15Bb cells at 35 days of age. The data in Fig. 4B demonstrate that 7.4 % of T_{CD8} in the pancreas stained with epitope IV-specific Kb/IV tetramer at this late time point. Similarly, a slightly lower number (6.2%) of Kb/IV tetramer positive T_{CD8} were detected in the spleen. Taken together, our data clearly show that epitope IV-specific T_{CD8} persist in the spleens and pancreas of RIP1-Tag4 mice immunized at 35 days of age and remain functional throughout the lifetime of the mice without being significantly tolerized or deleted.

The repertoires of epitope IV-specific T_{CD8} are similar in RIP1-Tag4 and B6 mice

The ability of at least a portion of epitope IV-specific T_{CD8} to escape peripheral tolerance in RIP1-Tag4 mice might be the result of purging the pool of epitope IV-specific T_{CD8} of high-avidity cells. Thus, the repertoire of epitope IV-specific T_{CD8} found in older RIP1-Tag4 mice might consist only of low-avidity T_{CD8} which survive in the toleragenic peripheral environment, due to their lower sensitivity to antigenic stimulation (7, 40). To address this issue, we determined the TCR variable region β chain (TCRβ) usage among $CD8^+$, Kb/IV $tetramer^+$ spleen cells by direct ex vivo staining with anti-TCRβ-specific monoclonal antibodies. The data in Fig. 5A show that in control B6 mice, the epitope IV-specific primary response consisted of a multiclonal population of T_{CD8}. The most predominant subunit utilized was TCRβ8. Other frequently used subunits were TCRβ3, β5, β12 and β17. Analysis of epitope IV-specific T_{CD8} in 2 year-old RIP1-Tag4 mice that were immunized against epitope IV at 35 days of age revealed that the multiclonality of TCRβ usage was retained. The most predominant TCRβ subunit utilized was TCRβ8, but T cells expressing B5, β12 and β17 were also present. Thus, the TCRβ repertoire of epitope IV-specific T_{CD8} derived from immunized B6 and long-term survivor RIP1-Tag4 mice does not vary, suggesting that peripheral tolerance within a particular clonotype does not occur. Since the presence of progressive tumor growth might be expected to influence the T cell repertoire, the TCRβ repertoire of epitope IV-specific T_{CD8} in tumor-bearing RIP1-Tag4 mice also was analyzed. The results again were similar to that observed in immunized B6 mice (data not shown), indicating that the overall diversity of epitope IV-specific T_{CD8} is unaffected by tumor progression.

Although little difference was detected in the TCRβ repertoires of B6 and RIP1-Tag4 mice, these T cell populations might vary in their avidity for epitope IV/Kb complexes. We determined the relative avidity of epitope IV-specific T_{CD8} for epitope IV/Kb complexes by measuring the stability of

Kb/epitope IV tetramer binding to *in vitro* restimulated spleen-derived cultures from long-term surviving two year old immunized RIP1-Tag4 mice and B6 mice immunized with epitope IV-only T Ag cells (Fig. 5B). The results from two individual mice are shown. The loss of fluorescence intensity among tetramer-stained T_{CD8} proceeded at similar rates for T_{CD8} derived from B6 immunized mice and long-term surviving RIP1-Tag4 mice immunized at 35 days of age. These result indicate that the avidity of epitope-IV specific T_{CD8} from long-term survivor RIP1-Tag4 mice is comparable to the avidity of epitope IV-specific T_{CD8} in generated during the primary response in B6 mice. Similar results were obtained using epitope IV-specific T_{CD8} induced in tumor-bearing RIP1-Tag4 mice (data not shown). These results indicate that higher avidity epitope IV-specific T_{CD8} persist in RIP1-Tag4 mice over their lifespan.

Discussion

Differential susceptibility to peripheral tolerance

The overall goal of this study was to determine whether tolerance differentially limits the T_{CD8} response against two immunodominant epitopes derived from a single oncogenic protein, subverting control of spontaneous tumor growth. Our results demonstrate that tumor progression in RIP1-Tag4 mice can be controlled for the natural lifespan by targeting T_{CD8} specific for the single immunodominant K^b-restricted epitope IV only if mice are immunized at the time of initial oncogene expression. Control of tumor development is associated with the persistence of functional epitope IV-specific T_{CD8} in the peripheral lymphoid organs as well as in the pancreas. Despite the persistence of epitope IV-specific T_{CD8} in these mice, T_{CD8} to a second immunodominant epitope, the H-$2D^b$-restricted epitope I, are deleted or anergized during the early stages of tumor development as a result of their interaction with the endogenous T Ag. Thus, tolerance onset is epitope-specific, even though both epitopes are derived from the same protein.

The finding that T_{CD8} specific for epitope I are highly sensitive to peripheral tolerance is consistent with a previous study using a different line of SV40 T Ag transgenic mice in which epitope I-specific T_{CD8} were shown to be more rapidly tolerized by the endogenous T Ag than epitope IV-specific T_{CD8}. Line 501 mice express T Ag from the α-amylase promoter resulting in the formation of osteosarcomas around one year of age (41). Similar to RIP1-Tag4 mice, T_{CD8} specific for the T Ag epitopes are initially detected following immunization of young 501 mice (6), but these mice become tolerant to epitope I by 6 months of age when high levels of T Ag expression are detected. Epitope IV-specific T_{CD8} were efficiently recruited in 501 mice at 6 months of age, but unlike the present study, these cells were eventually tolerized coinciding with tumor appearance. Taken together, these studies

indicate that T_{CD8} specific for epitope I are more susceptible to peripheral tolerance than T cells specific for epitope IV. One possible explanation for this finding may lie in the fact that epitope I forms highly stable complexes with H2-D^b (42), perhaps leading to prolonged high level presentation in vivo. Alternatively, the responding T cells may be differentially susceptible to self-antigen presentation in the periphery.

A unique finding in this study is that epitope IV-specific T_{CD8} survive the effects of peripheral tolerance in RIP1-Tag4 mice. This contrasts with other models of T Ag-induced spontaneous cancer in which epitope IV-specific T_{CD8} are eventually tolerized in mice with progressively growing tumors (6, 21). Epitope IV-specific T_{CD8} are apparently deleted from the repertoire of line 501 mice developing osteosarcomas as they are undetectable following immunization of tumor-bearing animals, but respond to immunization in age-matched littermates without tumors (6). This difference might be due to contrasting properties of osteosarcomas developing in 501 mice and insulinomas in RIP1-Tag4 mice**.** Tumors in 501 mice appear at a later age, grow invasively into surrounding tissues and are metastatic (41). In contrast, tumors in RIP1-Tag4 mice do not infiltrate surrounding tissues and do not form metastases. In addition, T Ag is expressed in multiple tissues in 501 mice while expression is localized to the pancreatic β cells in RIP1-Tag4 mice.

A different scenario is observed when a silent T Ag transgene, expressed from the β-actin/β-globin promoter, is activated and leads to sporadic tumor formation in various tissues (21). In this case, epitope IV-specific T_{CD8} appear to be rendered non-cytolytic by the tumor environment, perhaps due to the presence of immunosuppressive cytokines such as TGF-β. This finding contrasts with our observation in which epitope IV-specific T_{CD8} remained cytotoxic even in mice with advanced-stage

tumors. These differences are a reminder that unique tumor environments can differentially affect T_{CD8} responding to the same tumor-specific epitope and that the results cannot be generally applied to all tumor models.

Tumor protection is life long

Our results provide support for an earlier observation (18) that RIP1-Tag4 mice immunized with SV40 prior to endogenous T Ag expression led to long-term control of tumor progression (average lifespan of 622 days in the previous study). However, immunization with SV40 was ineffective after T Ag was expressed from the host's own β cells despite the persistence of SV40-specific CTL. While the vehicle used for immunization (SV40 versus T Ag transformed cells) differs from the previous study, our findings here suggest that control of tumor progression following SV40 infection of young RIP1-Tag4 mice was likely mediated by epitope IV-specific T_{CD8}.

We found no evidence for elimination or inactivation of endogenous epitope IV-specific T_{CD8} precursors in RIP1-Tag4 mice, although the number of responding cells is somewhat reduced in tumor-bearing mice. Indeed, epitope IV-specific T_{CD8} generated in tumor-bearing mice acquired in vivo killing activity, the ability to produce IFN-γ and had a similar avidity for Kb/IV tetramers as those from normal B6 mice. Yet these T_{CD8} failed to significantly control tumor growth. Our finding contrasts with those from other tumor systems (43) in which tumor-infiltrating T_{CD8} were impaired in their ability to effectively destroy progressing tumors due to production of immunosuppressive cytokines (44, 45), inhibition by regulatory T cells (46-48) or induction of apoptosis (49). Whether T_{CD8} within the tumor stroma of RIP1-Tag4 mice are inhibited in situ remains to be determined.

Since epitope IV-specific T_{CD8} remain responsive and functional in RIP1-Tag4 mice with progressing tumors, escape from immune mediated control of tumor progression may be related to changes in the tumor cells themselves. One such mechanism is the downregulation of MHC class I expression, enabling tumor escape from immunosurveillance (50-52). Extensive analysis of MHC class I expression on islet cells in RIP1-Tag4 mice by Ye et al. (53) showed that normal islets in B6 and RIP1-Tag4 mice express MHC class I while insulinomas from unimmunized RIP1-Tag4 mice contained a heterogeneous population of cells that were either negative or weakly positive for MHC class I expression. This observation suggests that heterogeneity of MHC class I expression on tumor cells could limit the efficiency of immunosurviellance in six-month old RIP1-Tag4 mice by preventing the recognition of T Ag-expressing tumor cells.

The basis for complete protection in RIP1-Tag4 mice immunized prior to, but not after, tumor formation may lie in the steps that lead to T Ag transformation and tumorigenesis. T Ag-expressing cells undergo immortalization with the capacity to proliferate followed by acquisition of a tumorigenic phenotype (54). A brief interaction with activated functional T Ag-specific T_{CD8} can interfere with this process. Our previous studies (55) have shown that T Ag transfected primary mouse embryo fibroblasts abort the transformation process upon a brief interaction with a T Ag-specific CTL clone. Here we show that a portion of pancreatic islets of RIP1-Tag4 mice that were immunized at 35 days of age still contain cells positive for T Ag expression at two years of age and do not appear to be hyperplastic. The mechanism by which epitope IV-specific T_{CD8} suppress tumor progression in vivo could be mediated by the production of cytokines such as IFN-γ or TNF-α (56, 57). Alternatively, those cells expressing the highest levels of T Ag might be eliminated prior to transitioning to tumor cells. This mechanism requires sufficient levels of MHC class I expression to present T Ag epitopes to functional T_{CD8}, whereas

escape variants arising because of low MHC class I expression would be expected to quickly advance to progressively growing tumors.

Tumor control without organ destruction

We found that long-term control of tumor development by epitope IV-specific T_{CD8} does not result in diabetes in RIP1-Tag4 mice since sufficient numbers of T Ag-expressing β cells survive. This is despite the persistence of high avidity epitope IV-specific T_{CD8}. Thus, an equilibrium is reached in which tumor progression is controlled but organ function is maintained; an ideal scenario for tumor immunotherapy. These results contrast with those from mice expressing either the glycoprotein or NP from lymphocytic choriomenengitis virus (LCMV) in β cells. In this case, virus infection resulted in T cell infiltration followed by diabetes due to destruction of the β cells (58, 59). Indeed, we detected T Ag expression in a low percentage of islet cells in the pancreata of long-term survivors immunohistochemically. Whether β-islet cell survival in these mice is dependent on the level of T Ag expression is unknown.

A similar scenario has been observed in some transplantable tumor models in which immunization against a self-tumor antigen induces tumor regression without leading to autoimmunity (5, 60, 61). Thus, the number of insulin-secreting T Ag-positive β cells that remain produce enough insulin to prevent the development of diabetes. It should be pointed out that all β cells in RIP1-Tag4 transgenic mice are expected to express T Ag, although the expression levels may vary (31). In the RIP-HA transgenic model, immunization with influenza virus also induces the onset of diabetes due to the activation of endogenous self-reactive T_{CD8} (39). In this model, however, RIP-HA mice must be immunized prior to 1 week of age to induce diabetes, since high avidity HA-specific T_{CD8} are eliminated

by peripheral tolerance at later time points (7). This contrasts with our data in which high avidity T Ag epitope IV-specific T_{CD8} persist throughout the lifespan of RIP1-Tag4 mice. It is possible that the LCMV and Flu HA proteins are expressed at higher levels in the pancreas than T Ag, such that β cells in these mice are more readily destroyed.

A recently published study argued against a role for immunosurveillance of spontaneous cancer (21). That suggestion may not be generalized to all immunogenic spontaneous models of cancer (62). While the role of spontaneously primed T_{CD8} to control autochthonous tumors will continue to be debated, data presented here and by others (18, 21) argue that long-term immunosurveillance against spontaneous tumors can be effective if T cells are activated prior to the appearance of tumors or if the tumor-bearing host is preconditioned prior to immunotherapy (14, 30, 63). A key to effective T cell-mediated therapy is to target epitopes less likely to lead to peripheral tolerance following prolonged exposure to the endogenous antigen. In addition, understanding the mechanisms by which tumor-specific T cell responses can mediate control of tumor progression without disrupting organ function will be a major advance toward developing immunotherapeutic strategies.

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[5]Abbreviations used in this paper: T_{CD8}, $CD8^+$ T lymphocytes; T Ag, T antigen; B6, C57BL/6; RIP, rat insulin promoter; HSV, herpes simplex virus; glycoprotein B; NP, nucleoprotein; LCMV, lymphocytic choriomeningitis virus; Db/I, epitope I; Kb/IV, epitope IV; Flu, influenza virus; HA, hemaglutinin.

Figure Legends

Figure 1. RIP1-Tag4 mice develop tolerance to T Ag epitope I but not epitope IV. A. Groups of three RIP1-Tag4 mice aged 35 days, 90 days, 180 days and control 90 day old B6 mice were immunized i.p. with wild type T Ag-transformed WT-19 cells. Nine days later the frequency of epitope I- and epitope IV-specific T_{CD8} was determined by tetramer staining of spleen cells. The values obtained with a control Db/Flu tetramer specific for influenza $NP_{366-374}$ were subtracted to obtain the percentage of epitope-specific T cells per total T_{CD8}. *B,* Epitope IV-specific but not epitope I-specific T_{CD8} are detected in pancreatic tumors following immunization. RIP1-Tag4 mice aged 180 days were immunized i.p. with WT-19 cells and nine days later the frequency of epitope I- and epitope IV-specific T_{CD8} was determined by tetramer staining of the resulting cell suspension prepared from pancreatic tumors by enzymatic digestion. Control Db/Flu tetramer was used to determine the specificity of staining. Values in the upper right quadrant indicate percentage of total T_{CD8}.

Figure 2 Immunization against epitope IV induces lifelong protection from tumor progression in RIP1-Tag4 mice. A. Groups of RIP1-Tag4 mice were immunized with T Ag transformed B6/15Bb cells, expressing a T Ag variant containing only epitope IV but not epitopes I, II/III and V, at 35 (median survival >2 years) or 150 days of age (median survival=204 days). A third group remained unimmunized (median survival=194 days). B. Blood glucose levels were measured at the indicated times in naïve mice or in RIP1-

Tag4 mice that had been immunized with B6/15Bb cells at 5 weeks of age. C and D. The pancreatic islet size was measured using H&E stained pancreatic sections prepared from untreated RIP1-Tag4 mice (C) or from RIP1-Tag4 mice immunized at 35 days with B6/15Bb cells (D). Data in each column show the size of individual islets in each mouse. Islet sizes from untreated B6 mice are shown for comparison.

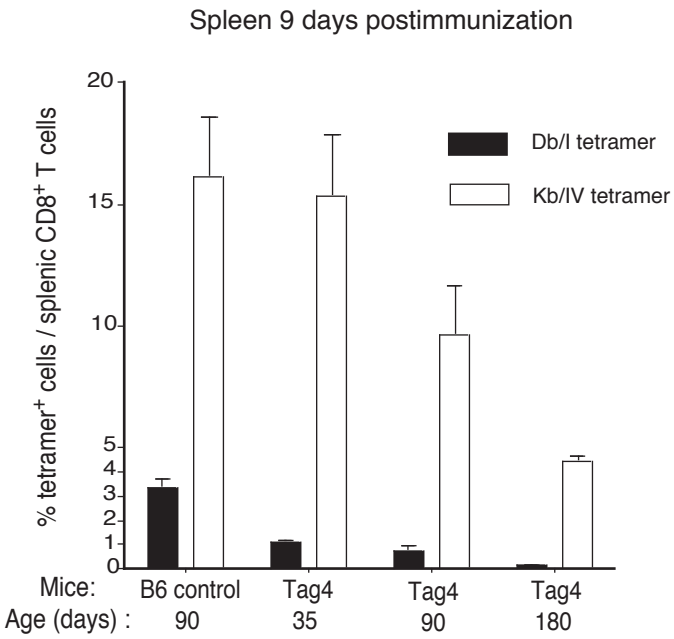
Figure 3 Immunized, long-term surviving RIP1-Tag4 mice maintain T Ag expression in the pancreas. Paraffin-embedded sections were prepared from pancreas of representative B6 mice (panels A and C), unimmunized six-month old RIP1-Tag4 mice (panels B and D), one-year old RIP1-Tag4 mice immunized at 35 days of age with B6/15Bb cells (panels E-H, two individual mice are shown) and two-year old RIP1-Tag4 mice immunized at 35 days of age (panels I-L, two individual mice are shown). Parallel sections were stained by H&E (panels A,B,E,F,I and J) and immunohistochemistry for T Ag (panels C,D,G,H,K and L). Black arrows indicate T Ag-positive cells within the islets. Original magnification x200. Staining of nuclear T Ag appears brown.

Figure 4 Epitope IV-specific T_{CD8} remain functional in RIP1-Tag4 mice despite continuous expression of T Ag. A. RIP1-Tag4 mice and control B6 mice were analyzed for the presence of epitope IV-specific T_{CD8} in the spleen by staining with epitope IV-specific Kb/IV tetramer ex vivo (left panels) and after in vitro culture with gamma-irradiated B6/WT-19 cells (middle panels). The values in the upper right quadrant indicate the percentage of epitope IV-specific T cells per total T_{CD8}. The effector functions of T Ag-specific T_{CD8} were determined using an in vivo cytotoxicity assay (right panels) against

B6/SJL target cells incubated with peptide IV ($CFSE^{high}$), peptide I ($CFSE^{medium}$) and control peptide HSV gB ($CFSE^{low}$). Data represent one individual mouse from a group of two to three RIP1-Tag4 mice. Values over each histogram represent the percentage of target cells eliminated in vivo. B. Pancreata from long-term surviving immunized RIP1-Tag4 mice contain epitope IV-specific T_{CD8}. The presence of infiltrating T Ag-specific T_{CD8} in the pancreas was determined by flow cytometry. Pancreatic tissue from untreated RIP1-Tag4 mice and from one-year old RIP1-Tag4 mice immunized at 35 days of age was enzymatically digested and stained with Kb/IV tetramer or with control Kb/gB tetramer. Values indicate the percentage of total T_{CD8} which stained positive with each tetramer.

Figure 5 Conservation of TCRβ usage and avidity among epitope IV-specific T_{CD8} from B6 and RIP1-Tag4 mice. A. Splenic lymphocytes from two year old RIP1-Tag4 mice immunized at 5 weeks of age and from control B6 mice immunized nine days before analysis were stained ex vivo with Kb/IV tetramer, anti-CD8 Ab and TCRβ-specific Ab's. Two individual mice are shown. The percentage of $CD8^+$Kb/IV $tetramer^+$ cells that stain with each TCRβ-specific antibody is indicated. B. Splenocytes from A were restimulated with T Ag-expressing stimulator cells for 5 days. Dissociation of bound Kb/IV tetramers was measured over time at 37˚C. The values represent the percentage of maximal fluorescence intensity at time 0 for two individual mice.

A


Spleen 9 days postimmunization
% tetramer+ cells / splenic CD8+ T cells
20
15
10
5
4
3
2
1
0
Db/I tetramer
Kb/IV tetramer
Mice:
B6 control
Tag4
Tag4
Tag4
Age (days) :
90
35
90
180

B

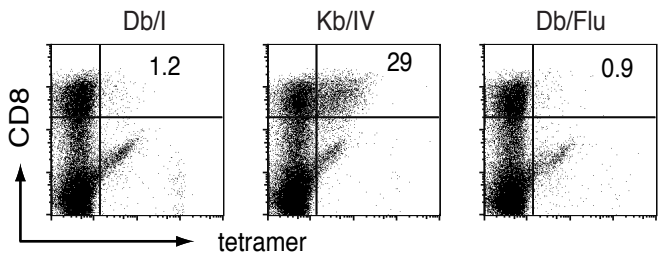

Tumor 9 days postimmunization
Db/I
Kb/IV
Db/Flu
1.2
29
0.9
CD8
tetramer

Figure 1

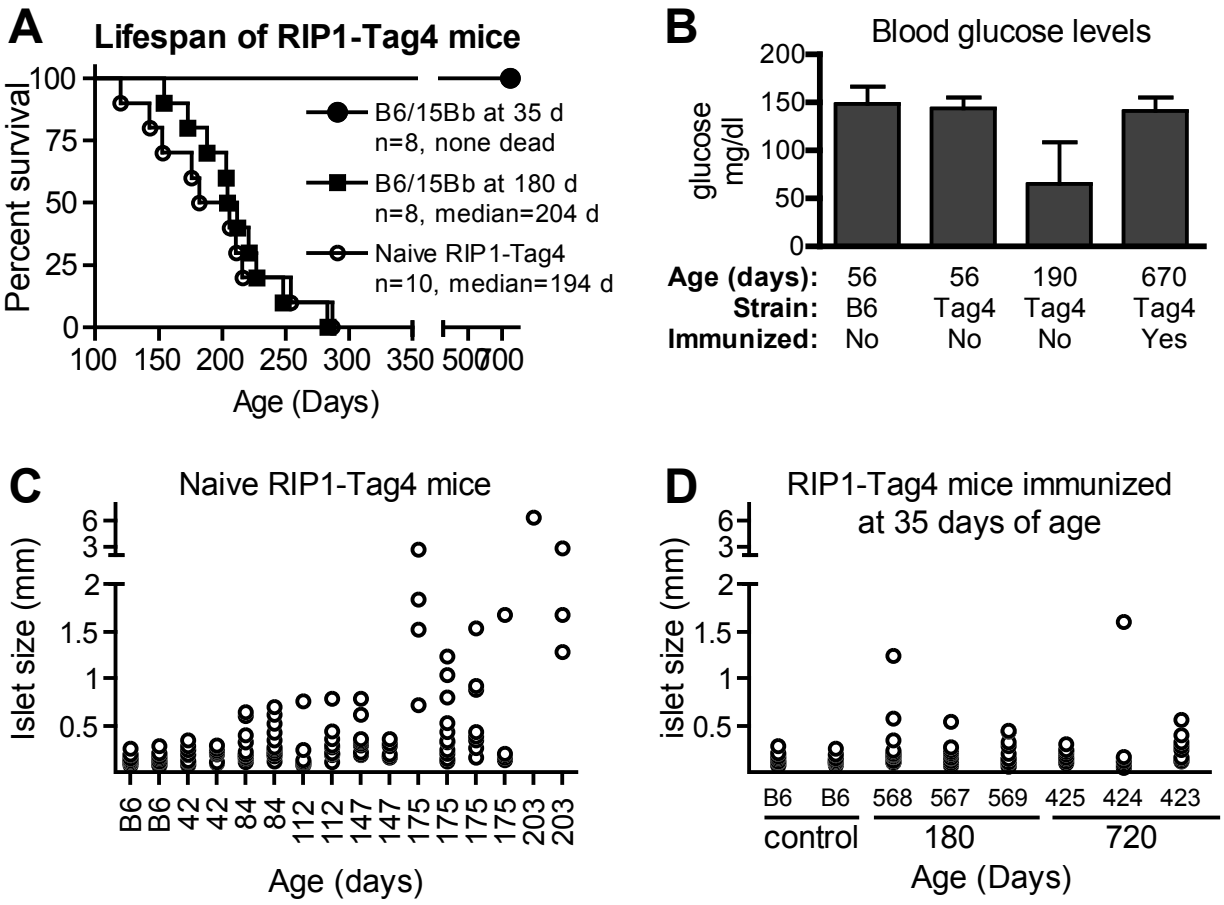

A
Lifespan of RIP1-Tag4 mice
Percent survival
100
75
50
25
0
100 150 200 250 300 350 500 700
Age (Days)
B6/15Bb at 35 d
n=8, none dead
B6/15Bb at 180 d
n=8, median=204 d
Naive RIP1-Tag4
n=10, median=194 d
B
Blood glucose levels
glucose
mg/dl
200
150
100
50
0
Age (days): 56 56 190 670
Strain: B6 Tag4 Tag4 Tag4
Immunized: No No No Yes
C
Naive RIP1-Tag4 mice
Islet size (mm)
6
3
2
1.5
1
0.5
B6 B6 42 42 84 84 112 112 147 147 175 175 175 175 203 203
Age (days)
D
RIP1-Tag4 mice immunized
at 35 days of age
islet size (mm)
6
3
2
1.5
1
0.5
B6 B6 568 567 569 425 424 423
control
180
720
Age (Days)

Figure 2

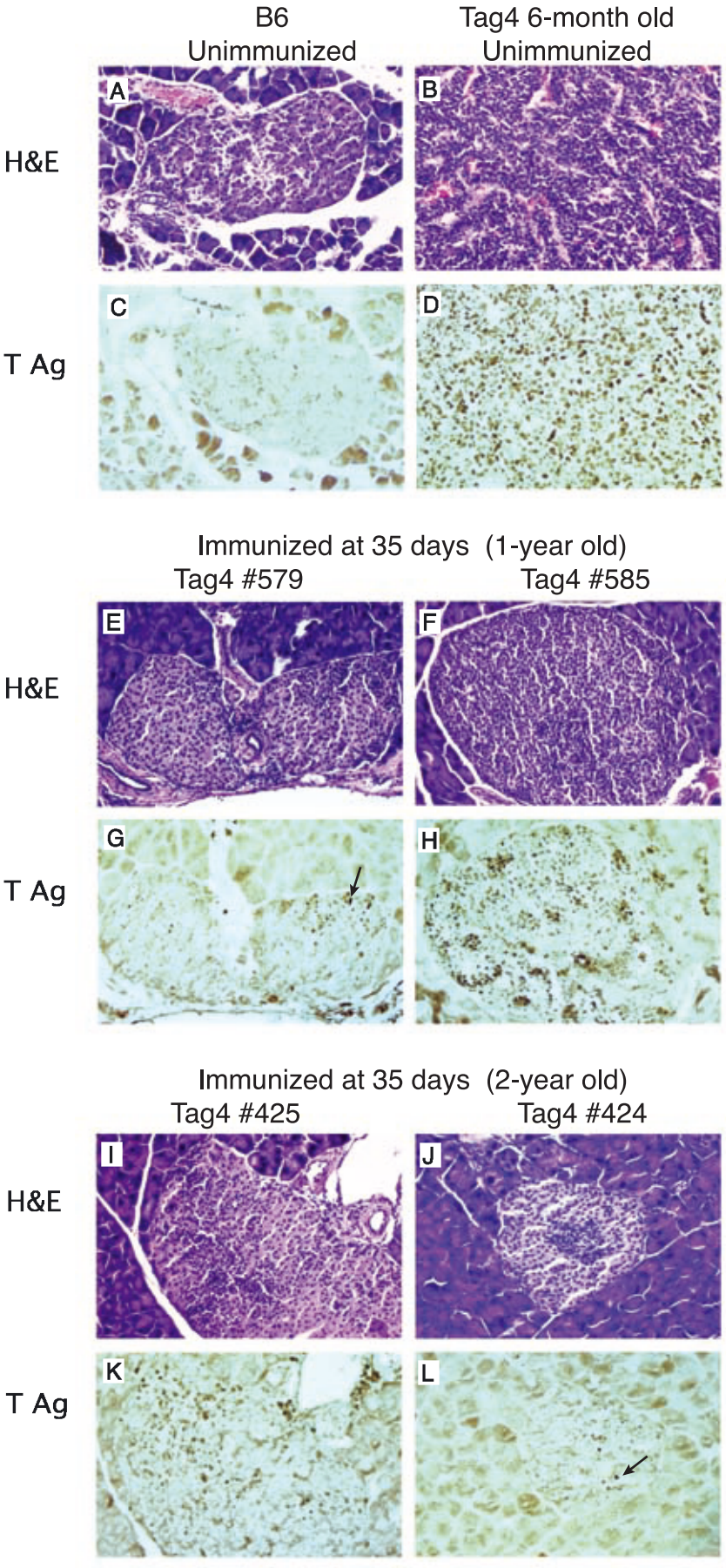
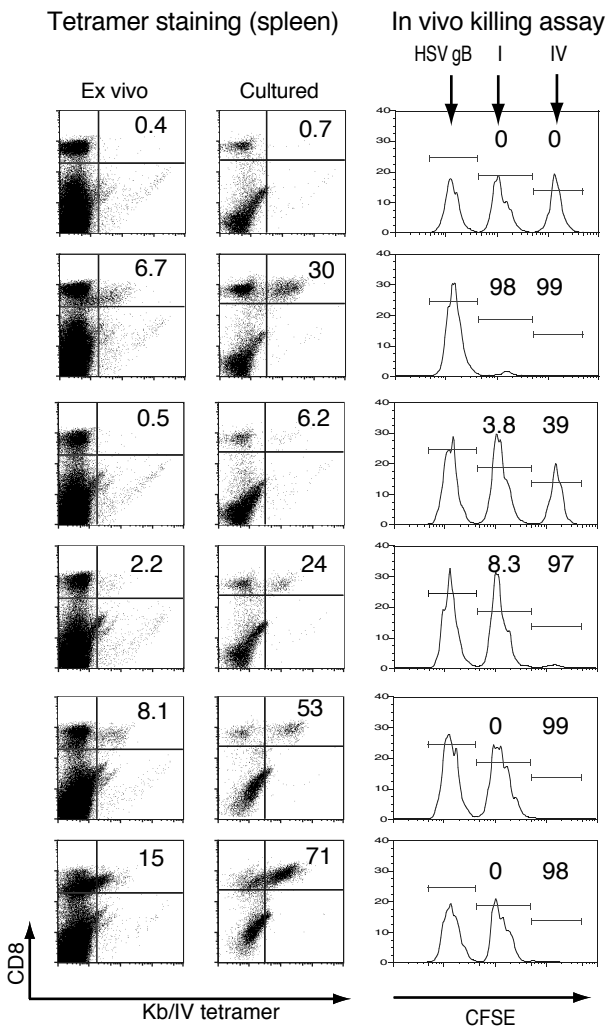

B6
Unimmunized
Tag4 6-month old
Unimmunized
H&E
T Ag
A
B
C
D
Immunized at 35 days (1-year old)
Tag4 #579
Tag4 #585
H&E
T Ag
E
F
G
H
Immunized at 35 days (2-year old)
Tag4 #425
Tag4 #424
H&E
T Ag
I
J
K
L

Figure 3

A

Group #	Mice	Age when immunized (days)	Age when analyzed (days)
1	B6	unimmunized	90
2	B6	81	90
3	Tag4	unimmunized	180
4	Tag4	171	180
5	Tag4	35	720
6	Tag4	35 (prime) 711 (boost)	720



B

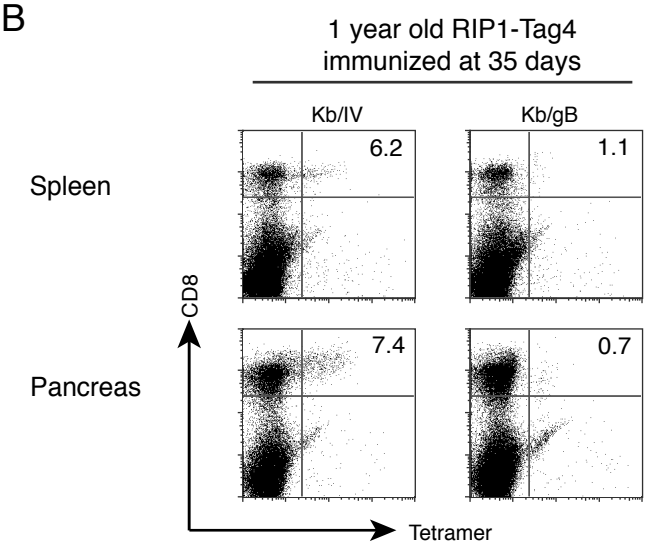


Figure 4

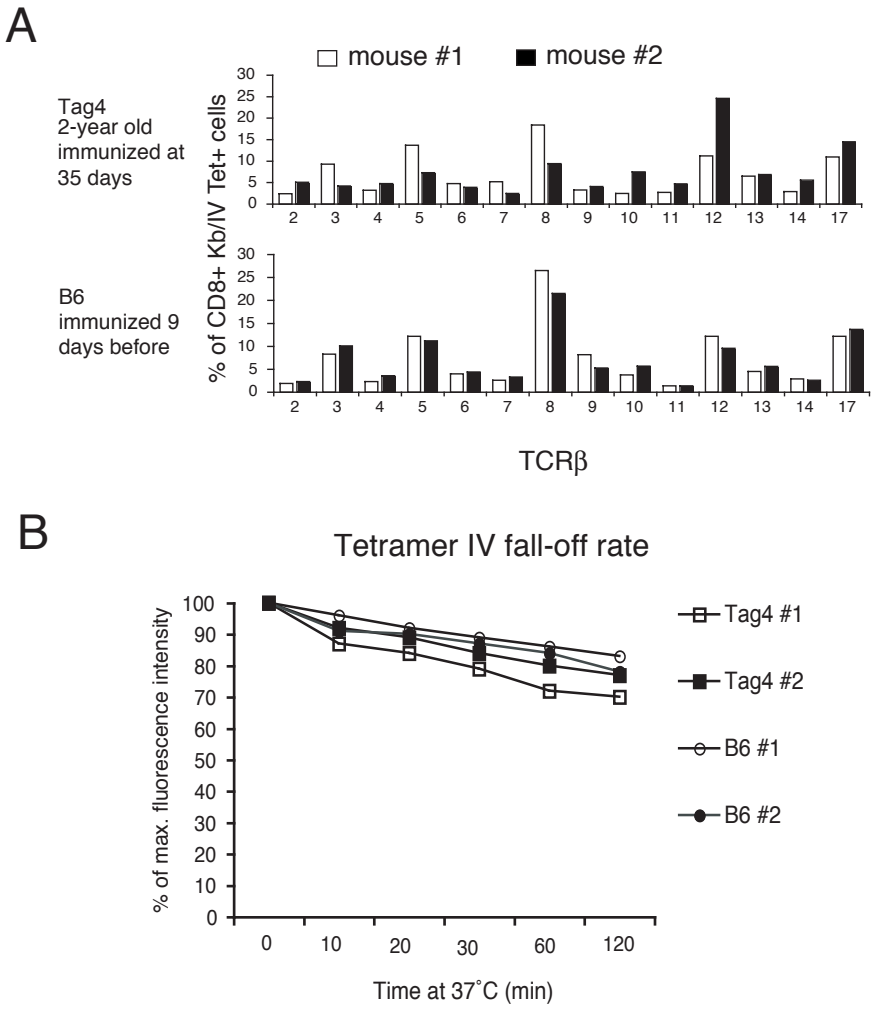

A
mouse #1
mouse #2
Tag4
2-year old
immunized at
35 days
B6
immunized 9
days before
% of CD8+ Kb/IV Tet+ cells
TCRβ
B
Tetramer IV fall-off rate
% of max. fluorescence intensity
Time at 37˚C (min)
Tag4 #1
Tag4 #2
B6 #1
B6 #2

Figure 5

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ORIGINAL ARTICLE

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Immune response to E7 protein of human papillomavirus type 16 anchored on the cell surface

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Abstract To target the E7 protein of human papilloma virus 16 to the cell surface, a fusion gene was constructed. It encodes the signal peptide, part of the immunoglobulin (IgG)-like domain, the transmembrane anchor of vaccinia virus (VV) hemagglutinin (HA), and the complete E7-coding sequence. The fusion gene was expressed under the HA late promoter by a recombinant VV, designated VV-E7-HA. The E7-HA protein was displayed on the surface of cells infected with the recombinant virus and was more stable than unmodified E7. The biological properties of the VV-E7-HA virus were compared with those of a VV-E7 virus that expressed the unmodified E7 and with a VV expressing the Sig-E7-LAMP fusion protein. While the first two of these recombinants were based on VV strain Praha, the third was derived from the WR strain of VV. Infection of mice with the VV-E7-HA virus induced the formation of E7-specific antibodies with the predominance of the IgG2a isotype, whereas the other two viruses did not induce the formation of E7-specific antibodies. Unlike the other two viruses, VV-E7-HA did not induce a response of cytotoxic T lymphocytes or Th1 cells and did not protect mice against the growth of E7-expressing tumors. Thus, VV-E7-HA induced a differently polarized immune response to the E7 protein than the other two viruses.

Keywords Antibody · Cellular immunity · E7 · Hemagglutinin · HPV16 · Protein targeting · Vaccinia virus

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Introduction

Recombinant vaccinia virus (VV) vectors have been used to induce immune responses to many different antigens derived from viruses, bacteria, parasites and mammals (for a review, see [23]). It has been shown that the anchoring of an antigen on the surface of cells infected with a recombinant virus encoding this antigen can increase its immunogenicity. For example, appending the transmembrane domain of membrane immunoglobulin (IgG1) to the carboxy terminus of a secreted *Plasmodium* antigen results in a change in the subcellular location of the S-antigen and increases the immune response to the otherwise weak immunogen [18]. Enhanced immune responses have been observed after fusing the MSA1 signal and anchor sequences with malaria merozoite surface antigen 1 [32]. Similarly, the addition of the membrane anchor to the C terminus of rotavirus VP7 glycoprotein has enhanced the immunogenicity of this protein when expressed by recombinant VV [2].

It has been demonstrated that the hemagglutinin (HA; A56R gene) of VV is not required for infection and replication of this virus [10]. The substitution of the external IgG-like domain of HA (AA 34–103) with a single-chain antibody results in the synthesis of a fusion protein which is exposed on the envelope of extracellular virus (EEV) and on the surface of virus-infected cells. The specificity (anti-ErbB2) of the single-chain antibody is retained, and the surface of EEV can bind the corresponding antigen [7].

An etiological association between human papillomavirus type 16 (HPV16) infection and cervical neoplasia has been firmly established [22]. The viral E7

oncoprotein, which is localized in the nucleus and cytoplasm of HPV16-infected and transformed cells, represents an attractive target for therapeutic vaccines.

In this study we report on our attempts to increase the immunogenicity of the E7 protein by changing its subcellular location via fusing it to the transmembrane sequence of VV HA. The immunogenicity of the recombinant VV expressing this fusion protein has been compared with that of two other VV-E7 recombinants.

Material and methods

Plasmid vectors

Plasmid pRS3 was prepared as follows. An internal portion of the VV HA, between its 64th and 275th amino-acid residues, was replaced by the entire E7 polypeptide. The E7 gene of HPV16 was amplified with the oligonucleotide primers E7–1: 5'-GCTGACGTCATGCATGGAGATACACC-3' and E7–2: 5'-AGACCAAGGTTTCTGAGAACAGATGGG-3' by PCR, with plasmid pEA16E7 (prepared by Ingrid Jochmus, Heidelberg, Germany) as the DNA template. The resulting 300-bp *Aat*II-StyI fragment containing the E7 gene was inserted between the *Aat*II and *Sty*I sites of plasmid pHA. To ensure translation into the complete E7-HA fusion protein in frame, the *Sty*I ends were ligated as blunt ends. The resulting plasmid was denoted pHA-E7. Plasmid pHA was prepared by cleaving the G fragment of a *Sal*I genomic library of VV strain Praha with restriction enzymes *Hin*dIII and *Sal*I, and then cloning the 1,796-bp fragment obtained which contained the HA gene, into pUC18. To obtain a plasmid that would allow "transient dominant selection" of recombinant viruses, plasmid pHA-E7 was cleaved with *Bam*HI and ligated with a *Bam*HI fragment, which contains the β-galactosidase gene of *Escherichia coli* and the 7.5-promoter of VV. The resulting plasmid was denoted pRS3. Plasmid pH5-E7 was prepared by amplifying the E7 gene with primers E7-B: 5'-ATAGGATCCCTGTAATCATGCATG-GAG-3' and E7-E: 5'-GGCGAATTCGATTATGGTTTCTGA-GAACAG-3' by PCR, using plasmid pEAE7 as the DNA template. The amplified 325-bp fragment was cleaved with *Bam*HI and *Eco*RI and ligated with plasmid pSC59-H5 cut with the same enzymes. Plasmid pSC59-H5 had been prepared by insertion of the VV H5 promoter into plasmid pSC59 (obtained through the courtesy of B. Moss, Bethesda, Md.). The 167-bp fragment containing the H5 promoter [8] had been prepared by PCR with primer H5–1: 5'-GCCAGATCTGACACTGTCTTTATTCTATACTTAAAAA-GTGAAAATAAATAC-3' and primer H5–2:5'- GCTGTCGACG-AGCTCCTAGGATCCTATTTACGATACAAACTTAACGG-ATATCG-3', with the H fragment of the *Hin*dIII VV genomic library used as the DNA template. The resulting fragment was cleaved with *Bgl*II and *Sal*I, and was ligated with pSC59 cut with *Bam*HI and *Sal*I.

Viruses and cells

Vaccinia virus clone P13, generated from the Sevac VARIE smallpox vaccine (strain Praha) [16], was used as the parental virus for the construction of recombinants. The recombinant viruses were grown in human-embryo diploid cells (LEP) or monkey-kidney cells (CV-1). Thymidine kinase-deficient (TK^-) RAT 2 rat cells [30] were used for the selection of TK^- VV recombinants. HPV16 E6E7-expressing TC-1 cells, derived from C57BL/6 mice [19], were kindly provided by T.C. Wu (Baltimore, Md.). All cells were cultivated in modified E-MEM (EPL) medium containing bovine serum growth-active proteins, but no complete serum [21]. The VV recombinants used for immunization experiments were grown in chorioallantoic membranes of 11-day-old chicken embryos and were partially purified by the modified method of Joklik [13, 17].

Construction of VV recombinants

Vaccinia virus recombination and selection of TK- recombinants were performed by standard procedures [24]. VV-HA-E7 was prepared using plasmid pRS3. Recombinant viruses produced by a single crossing over were selected by their co-expression of β-galactosidase [4]. "Blue" virus was plaque-purified. After the second plaque purification, "colorless plaque" viruses were isolated and recombinants with double crossing over were identified as carriers of the E7 insert by dot-blot hybridization and PCR. Expression of the E7 fusion protein and absence of HA in selected virus clones were confirmed by immunoblotting using VV-specific antisera. The E7-HA fusion gene was expressed from the late promoter of HA. A schematic view of the protein produced by VV-E7-HA is shown in Fig. 1.

A VV expressing the unmodified E7 protein was prepared by recombination with plasmid pH5-E7. The E7-coding sequence was inserted into the thymidine kinase (TK) gene, and its expression was controlled by the H5 early-late promoter. The third virus used in this study, VV-SigE7LAMP, expresses a fusion molecule consisting of the E7 protein with signal and a transmembrane sequence of the lysosome-associated membrane protein (LAMP1). This recombinant virus, originally prepared with the WR strain, was obtained through the courtesy of T.C. Wu (Baltimore, Md.) [19]. The viruses VV-pS2S (TK^-) and WR-pS2S (TK^-), which express the middle envelope protein (preS2 + S) of hepatitis B virus, had been prepared using plasmid pM3 [15]. The VV-gE (HA^-, TK^+) virus had also been prepared previously [17].

Peptides

The synthesis of peptides $E7–1_{(1–20)}$, $E7–2_{(10–30)}$, $E7–3_{(20–40)}$, $E7–4_{(30–50)}$ and $E7–5_{(40–60)}$ derived from the sequence of HPV16 E7 used in ELISA has been described earlier [14]. The peptide HPV16 $E7_{(49–57)}$ (RAHYNIVTF) [6] was used for the production of MHCI tetramer; the peptide $E7_{(49–57)}$ and E7 peptide-8Q $_{(44–62)}$ (QAEPDRAHYNIVTFCCKCD) [28] were used for restimulation of splenocyte bulk cultures and in ELISPOT.

MS2E7 protein

MS2E7 protein was produced in *Escherichia coli* transformed with plasmid pEX 8mer-HPV16 E7 and purified according to Jochmus-Kudielka et al. [12]. MS2E7 molecule contains E7 protein (encoded by HPV16 nt 585–855) fused to the first 100 amino acids of the bacteriophage MS2 polymerase.

Fig. 1 Schematic diagram showing structure of E7-HA fusion protein. Indicated amino-acid positions refer to the wild-type HA sequence. HA-Sig: HA signal peptide; HA-Ig-domain: part of the IgG-like domain; HA-TM: transmembrane domain; E7: full-length E7 polypeptide (98 AA)

Antibodies

Rabbit antiserum against E7, which was used in immunoblot and immunofluorescence tests, was prepared by immunization with four i.m. doses, each containing 2.5 mg MS2E7 fusion protein [12] in Freund's adjuvant. The first dose was administered in complete adjuvant, the subsequent three doses in incomplete adjuvant. Mouse anti-MS2E7 serum was prepared by the same procedure. Each dose contained 0.5 mg protein.

Preparation of H-2D^b/E7$_{(49–57)}$ tetramers

Major histocompatibility complex (MHC)-I tetramers were prepared as described by Altmann et al. [1]. In brief, heavy and light chains were expressed separately in *E. coli* and used in form of inclusion bodies for folding reaction. The mouse H-2D^b (MHC-I heavy chain), human β2-microglobulin (light chain) and E7$_{(49–57)}$ peptide were folded in vitro to preform MHC-1 monomers. After concentrating the reaction mixtures and buffer exchange, the preformed monomers were subjected to enzymatic biotinylation by BirA biotin synthetase, and then purified with S300 column chromatography and Mono Q ion exchange column chromatography. Tetramers were obtained by mixing the biotinylated protein complex with streptavidin-R–phycoerythrin conjugate (Molecular Probes) at a molar ratio of 4:1.

Immunoblotting

Infected cell cultures were washed twice with PBS and lysed on ice with RIPA buffer (1% NP40, 150 mM NaCl, 50 mM Tris–HCl, pH 8.0) for 30 min. Cell lysates were clarified by centrifugation at 19,000 *g* for 10 min at 4°C. Supernatants were mixed with Laemmli buffer containing 2-mercaptoethanol. Samples were heated for 5 min at 95°C. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 15% gel. The proteins separated were then transferred onto a nitrocellulose membrane using a semi-dry electrophoretic blotting method. Membrane, preincubated with 10% skimmed milk in phosphate-buffered saline (PBS) for 1 h, was incubated with E7 specific rabbit antiserum diluted 1:1,000 in PBS containing 5% milk at 4°C overnight. After being washed (PBS–0.2% Tween, five times for 5 min) the membrane was incubated for 1 h with goat anti-rabbit IgG labeled with horseradish peroxidase (HRP; Sigma) diluted 1:5,000 in PBS–5% milk. Finally, the membrane was washed again as above, developed by ECL (Amersham) and exposed to autoradiography film.

Immunofluorescence

CV1 cells ($4{\cdot}10^3$) were grown in 16-well chamber slides (Nunc). Confluent monolayers were infected with recombinant VV at MOI 0.1. After overnight incubation, infected cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 min, and washed with PBS 3×5 min. Then 0.1% Triton X-100 was added for 2 min and cells were washed with PBS 3×5 min and blocked with 10% skimmed milk in PBS for 1 h. Next, rabbit E7 antiserum diluted 1:50 in 5% milk–PBS was added for 1 h at room temperature. The monolayers were washed as above and incubated with a FITC-conjugated swine anti-rabbit secondary antibody (diluted 1:200) at room temperature for 1 h. Finally the cells were washed with PBS, overlaid with 50% glycerol in PBS, and examined with a Nikon 600 Eclipse microscope.

The expression of E7 on the surface of infected cells was examined by FACS analysis. Briefly, monolayers of CV1 cells in Petri dishes were infected at MOI 0.2. After overnight incubation, when the CPE in all cultures was about 50%, cells were harvested by trypsinization and resuspended in FACS buffer (PBS, 3% bovine serum albumin (BSA), 0.1% NaN_3). The cells were incubated with rabbit E7 antiserum diluted 1:50 in FACS buffer for 1 h at 0°C, then washed three times with the same buffer and incubated with a FITC-conjugated swine anti-rabbit secondary antibody (diluted 1:200 in FACS buffer) for 1 h at 0°C. Samples were analyzed on a Becton Dickinson FACScan instrument using WinMDI 2.8 software.

Subcellular fractionation

Preparation of subcellular fractions was performed as described by Jin et al. [11]. Briefly, CV-1 cells ($8{\cdot}10^6$) were infected with viruses at MOI 2 at 37°C. After 1 h, unadsorbed virus was removed and cells washed with medium were incubated for another 8 h. The cells were scraped into medium, centrifuged, washed once with PBS and resuspended in 1 ml of hypotonic buffer (42 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, pH 7.4) with protease inhibitor cocktail (Sigma) diluted 1:100 and incubated on ice for 15 min. Cells were then passed through a 29-G needle six times; and cell disruption was verified under a microscope. The extract was centrifuged at 200 *g* for 10 min at 4°C, and sediment containing the nuclear fraction was further purified (see below). The supernatant was centrifuged at 10,000 *g* for 10 min at 4°C to separate the heavy membrane fraction and then at 150,000 *g* for 90 min to collect the light membrane fraction. The remaining supernatant was used as the cytosolic fraction. Nucleus purification was performed according to the published protocol [9]. To nuclear fraction resuspended in 4 ml of sucrose buffer I [0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–Cl, pH 8.0, 1 mM DTT, 0.5% (v/v) NP-40], another 4 ml of sucrose buffer II (2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris–Cl, pH 8.0, 1 mM DTT) were added and thoroughly mixed. The extracts were layered over 4.4 ml of sucrose buffer II in Beckman polyallomer SW 40.1 tubes and centrifuged at 30,000 *g* for 45 min at 4°C to prepare sediment containing nuclei. The sediments were resuspended in 100 µl of Laemmli buffer containing 2-mercaptoethanol. 0.8 ml of cytosolic fraction was mixed with 200 µl of 5×Laemmli buffer. All samples were denatured in boiling water for 3 min, separated on 12% SDS–PAGE and analyzed by immunoblot.

Immunization of mice

Six-week-old inbred mice, strain C57BL/6 (H-2^b; Charles River) were injected intraperitoneally with 0.5 ml PBS containing 10^7 PFU of recombinant VVs. All experiments on laboratory animals were conducted maintaining the principles of the Czech law 246/92 Sb. on "Breeding and Utilization of Experimental Animals."

Restimulation of splenocytes in vitro

HPV16E7-specific lymfocyte bulk cultures were generated from splenocytes obtained from immunized mice 12 days after virus inoculation. In brief, mouse spleens were homogenized with cell dissociation (Sigma) in complete RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Gibco), $5{\times}10^{-5}$ M 2-mercaptoethanol, 1.5 mM glutamine, and antibiotics. Lymphocytes were separated on Histopaque-1083 (Sigma) and cultivated at a concentration of $5{\times}10^6$ cells/ml in complete RPMI 1640 with addition of 0.3 µg or 15 µg E7$_{(49–57)}$ peptide or 1 µg (8Q) peptide for 6 days.

Tetramer-staining and FACS analysis

Splenocytes were used for analysis immediately after their isolation or after 6-day in vitro restimulation. Lymphocytes for tetramer-staining were adjusted onto $2{\times}10^7$ cells per ml with FACS buffer (PBS supplemented with 2% FBS and 10 mM sodium azide), incubated with 2 µl of rat anti-mouse CD16/CD32 (Fc-block; Pharmingen; 0.05 mg/ml) for 20 min on ice, washed and an aliquot of $2{\times}10^6$ cells was resuspended in 80 µl of ice-cold FACS buffer. The samples were stained on ice each with 20 µl of a mixture of 2 µl of tetramer–PE and 2 µl of rat anti-mouse CD8a-FITC antibody (Pharmingen; stock concentration 0.5 mg/ml) in FACS buffer for at least 1 h in the dark. The lymphocytes were washed and resuspended

in 200 µl of FACS buffer. The stained cells were analyzed on a FACScan instrument, using CellQuest software (Becton Dickinson).

ELISPOT assay

Ninety-six well plates MAHA 45 (Millipore) were coated with 5 µg/ml of the anti-mouse IFNγ monoclonal antibody R4–6A2 (Pharmingen) or anti-mouse IL4 monoclonal antibody BVD4–1D11 (Pharmingen) in 0.1 M Na–phosphate buffer (pH 9.0) at room temperature overnight. The antibody-coated plates were washed four times with PBS and blocked with RPMI 1640 with 10% FBS for 1 h at room temperature. 100 µl of in vitro restimulated splenocytes in culture medium were added to the wells and incubated for 20 h at 37°C in 5% CO_2 in the presence or absence of peptides. Wells were washed three times with PBS and three times with PBS containing 0.05% Tween 20, followed by overnight incubation at 4°C with 2 µg/ml of the biotinylated anti-mouse IFNγ monoclonal antibody XMG 1.2 (Pharmingen) or the biotinylated anti-mouse IL4 monoclonal antibody BVD6–2462 (Pharmingen) in PBS, respectively. The wells were washed with PBS, 0.05% Tween 20. Avidin–horseradish peroxidase (HRP) conjugate (Pharmingen) diluted 1:1,000 in PBS, 0.05% Tween 20 was added to the wells for 3 h at 37°C. After washing the plates with PBS, the spots were stained with 3-amino-9-ethyl carbazol.

Serological tests for E7 antibodies

Sera of immunized mice were individually tested by ELISA for the presence of specific anti-E7 antibody. Wells of microtiter plates (Maxisorb, Nunc, Denmark) were coated with 2 µg of the oligopeptides E7–1 to E7–5 in 100 µl carbonate buffer, pH 9.6, at 37°C. Unbound antigen was removed and free potential binding sites were blocked with 1% bovine serum albumin (BSA). After threefold washing, the wells were incubated with 100 µl of 1:25 dilution of serum for 1 h. The plates were then repeatedly washed and 100 µl of 1:2,000 dilution of peroxidase-conjugated rabbit anti-mouse IgG (Sigma, USA) was added for 1 h. The plates were washed again, stained with *o*-phenylene-diamine and absorbance at 492 nm was measured. Control sera known to be positive and negative were tested on each plate. Antibody isotypes were determined in plates coated with the E7–1 peptide, using HRP-conjugated rat anti-mouse IgG2a or IgG1 (Pharmingen) diluted 1:500.

Results

The VV-E7-HA recombinant virus expresses the E7 polypeptide fused into VV hemagglutinin

The VV-E7-HA construct was designed to express the E7-HA fusion protein. This protein contained the N-terminal portion of the VV hemagglutinin polypeptide chain including the signal sequence and part of the IgG-like domain (AA 1–62), the entire HPV16 E7 sequence (98 AA) in place of the internal part of the hemagglutinin polypeptide chain (211 AA), and the C-terminal portion of hemagglutinin including the transmembrane anchor (AA 273–315) (Fig. 1).

E7-HA is a glycoprotein targeted to the cell surface

In vitro expression of the E7-HA protein

To determine whether the VV recombinants induced synthesis of the E7 protein in CV1 cells, lysates of infected cells were analyzed by immunoblotting using E7-specific antibody. The lysates analyzed in Fig. 2A were prepared from cells harvested 18 h after infection. Fusion proteins produced by VV-E7-HA virus were found in two main bands of m.w. 33 and 35 kDa (lane 3) and in two minor bands of smaller size that probably represented fragments of the longer polypeptides. All polypeptides must have been glycosylated, because neither of the bands was found if the infected cells were kept in the presence of tunicamycin (5 µg/ml). The m.w. of the unglycosylated E7-HA polypeptide was about 30 kDa (lane 4). The upward shifts of 5 and 3 kDa might indicate the presence of one or two carbohydrate residues. With the use of the PCGENE program, two potential N-linked glycosylation sites were indeed predicted in the E7-HA protein: in the 34th position of HA and in the 29th AA of E7. The VV-E7 virus produced a 19 kDa E7 protein (lane 5), which was of the same size as that of the E7 protein produced in cells transfected with expression plasmid pBK-E7 or in Caski cells (not shown). The size of the E7 protein was not influenced by the addition of tunicamycin; however, the latter apparently reduced the amount of E7 produced (lane 6). A faint E7 band was discernible in lane 6 after longer film exposure (not shown). The

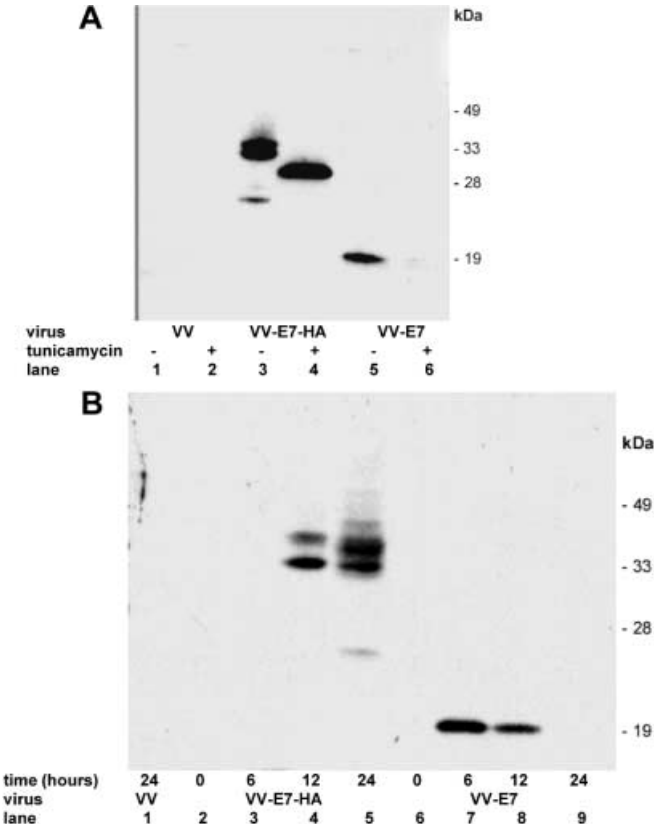

Fig. 2 Immunoblot analysis of E7 proteins expressed by recombinant VV. **A** CV1 cells were infected with parental VV, VV-E7-HA or VV-E7, cultivated in the absence or presence of tunicamycin of 5 µg/ml and harvested after 18 h. **B** Cells were harvested at different times after infection. The samples were analyzed on SDS–15% PAGE and by immunoblot using E7-specific rabbit antiserum

kinetics of production of the E7-HA glycoprotein and the unmodified E7 in cells infected with the respective recombinant viruses appeared to be different (Fig. 2B); E7-HA was detected at 12 h after infection and its amount was higher at 24 h (lanes 4 and 5), whereas the E7 molecule was detected already at 6 h (lane 7) after infection and thereafter its amount kept decreasing; a low amount of E7 present in cells at 24 h after infection (lane 9) was only visible on autoradiograms after a longer exposure time (not shown). Cells infected with non-recombinant VV did not express any E7-specific bands (Fig. 2A and B; lanes 1 and 2 and 1, respectively).

Subcellular location of the E7 antigen

The presence of the E7 antigen inside permeabilized cells was detected by immunofluorescent staining with specific antibodies (Fig. 3A). The cell infection with VVE7-HA was accompanied by formation of large syncytia. Their presence is typical of cultures infected with HA^- mutants [10]. The E7 antigen was found at the nuclear membranes and in cytoplasmic structures of VV-E7-HA-infected cells (a). Unmodified E7 expressed by VV-E7 was mainly present in the nuclei of infected cells (b). Cells infected with parental VV exhibited some fluorescence of very low intensity (c). E7 antigen on the surface of non-permeabilized infected cells was determined by flow cytometry. Fluorescence of high intensity was found on the surface of cells infected with VV-E7-HA only, whereas the FACS profile of VV-E7 infected cells showed no difference from cells infected with parental VV (Fig. 3B). To determine the amounts of E7 proteins in subcellular fractions, CV1 cells, infected with recombinant VVs for 8 h, were lysed in hypotonic solution and fractionated by centrifugation into nuclear,

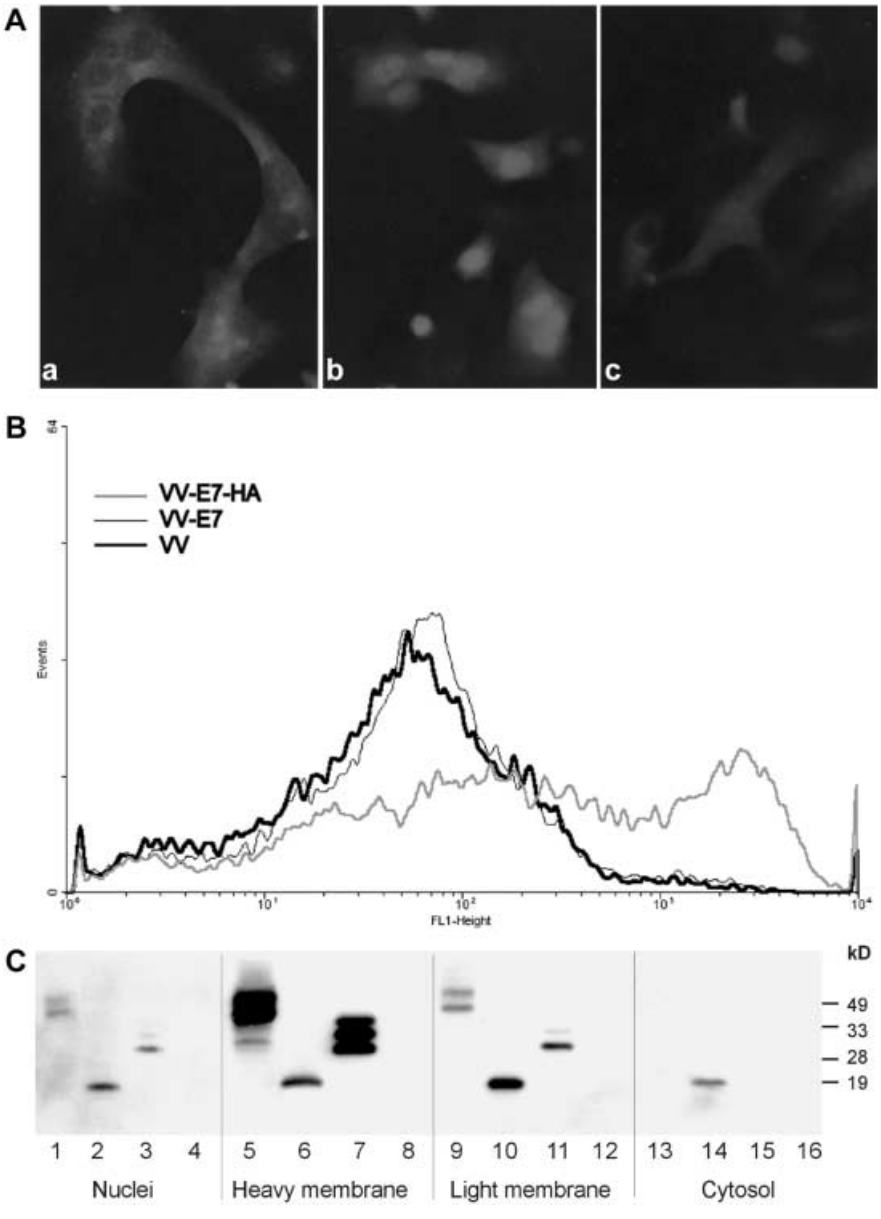

Fig. 3 Location of E7 proteins in CV1 cells after infection with recombinant VVs. **A** Immunofluorescence of E7 antigen in cells infected with: (a) VV-E7-HA; (b) VV-E7; or (c) VV. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and subsequently stained with E7 specific antibodies. **B** Detection of E7 on the surface of cells infected with VV-E7-HA (*grey line*), VV-E7 (*hair line*) or parental VV (*thick line*) by cytofluorometry. **C** Location of recombinant E7 proteins in subcellular fractions by immunoblot. The cells were infected with VV-E7-HA (*lanes 1, 5, 9, 13*), VV-E7 (*lanes 2, 6, 10, 14*), WR-SigE7LAMP (*lanes 3, 7, 11, 15*), parental VV (*lanes 4, 8, 12, 16*) and fractionated. Subcellular fractions were analyzed on SDS–12% PAGE and by immunoblot using E7 specific rabbit antiserum

heavy membrane, light membrane and cytosolic fractions. The amounts of E7 proteins in these fractions were analyzed by immunoblot (Fig. 3C). Most HA-E7 and E7-LAMP proteins were found in the heavy membrane fraction (lane 5 and 7, respectively) which included the cellular, mitochondrial and lysozomal membranes. Nuclear (lanes 1 and 3, respectively) and light membrane fractions (lanes 9 and 11, respectively) contained only low amounts of these proteins. Neither E7-HA nor E7-LAMP proteins were detected in the cytosolic fraction (lanes 13 and 15, respectively). Non-modified E7 protein predominated in the light membrane fraction (lane 10), though a substantial amount of E7 was found in all the other subcellular fractions (lanes 2, 6 and 14). The presence of E7 in membrane fractions could be explained by its capacity to bind to viral DNA, which is abundant in areas in the cytoplasm where VV maturation occurs. In agreement with published data, non-modified E7 was found in the nuclear and cytosolic fractions (lanes 2 and 14).

The E7-HA fusion protein induces an antibody response but not cell-mediated immunity

To determine how the fusion of the E7 protein with VV HA influenced the immunogenic properties of E7, we immunized C57BL/6 mice with one dose of 10^7 PFU of VV-E7-HA or VV-E7 virus by intraperitoneal route. Besides using these viruses prepared in our laboratory, we employed the WR-SigE7LAMP virus, which had been shown to induce a cell-mediated response and protection against tumor growth. This virus was included as positive control in immunity tests in spite of the fact that it was prepared from another parent virus strain. After the vaccinations, both humoral and cellular immune responses were examined in individual mice.

Induction of E7-specific antibodies

Groups of eight mice each were killed 6 weeks after virus inoculation. Their sera were individually examined for the presence of anti-E7 and anti-VV antibodies. The animals immunized with VV-E7-HA produced antibodies reactive with synthetic oligopeptides derived from the E7 amino-acid sequences 1–20 and 40–60 (Fig. 4A). Antibodies specific for the amino-acid sequence 20–40 were not detected in mouse sera (not shown). E7-Specific antibodies were not detected in sera of mice immunized with the VV-E7, WR-SigE7LAMP, or control viruses. To characterize the antibody response to VV-E7-HA more closely, the isotypes of anti-E7 IgGs were determined. Anti-E7 IgG2 prevailed in mice inoculated with VV-E7-HA, whereas the IgG1 isotype predominated after immunization with the purified MS2E7 fusion protein (Fig. 4B). All recombinant viruses induced anti-VV antibodies in all of the mice inoculated; however, the responses elicited by VV-E7-HA and WR-SigE7LAMP

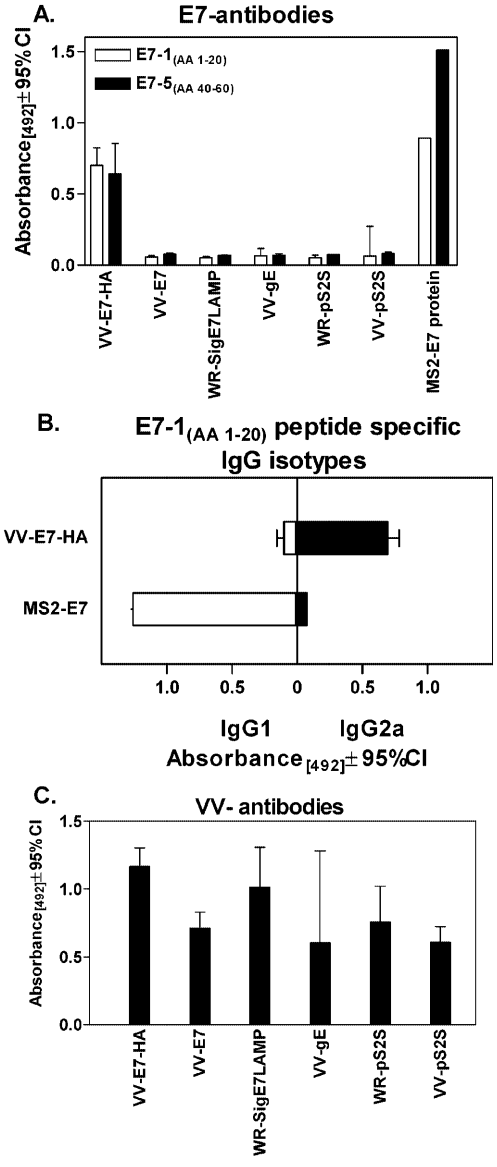

Fig. 4 E7-specific antibody response of mice inoculated with recombinant VVs. **A** Individual sera of six immunized mice were assayed by ELISA for antibodies specific for E7 oligopeptides E7-1 and E7-2. **B** Anti-E7-1 specific IgG1 and IgG2a isotypes were determined in individual positive sera of animals inoculated with VV-E7-HA. Anti MS2-E7 was a pool of sera of mice immunized with recombinant protein. **C** The same mouse sera as in **A** were examined for the presence of VV-specific antibodies

were the highest. The similar anti-VV antibody responses to these two recombinants suggest that the viruses multiplied at a similar rate, and that consequently the differences in anti-E7 antibody response to them cannot be ascribed to a difference in their replication.

Cell-mediated immunity

In testing the E7-specific cellular response to the different viruses, groups of two mice were used. Each spleen

was processed separately. Immunization with either of the E7-expressing viruses resulted in a primary response undetectable by ELISPOT or the tetramer assay in freshly isolated splenocytes (not shown). However, when the splenocytes were restimulated in vitro by E7 peptides for 6 days, IFNγ- and IL4-secreting splenocytes were detected (Fig. 5A and B, respectively). The binding of the $2D^b/E7_{(49–57)}$ tetramer by $CD8^+$ lymphocytes (Fig. 5C) was also found. Specific responses in IFNγ-ELISPOT and the tetramer assay were detected in mice immunized with VV-E7 or WR-SigE7LAMP but not with VV-E7-HA or the control virus (VV). In contrast, immunization with VV-E7-HA but not with VV-E7 or WR-SigE7LAMP resulted in the presence of IL4-secreting splenocytes. Similar results were obtained in three independent experiments.

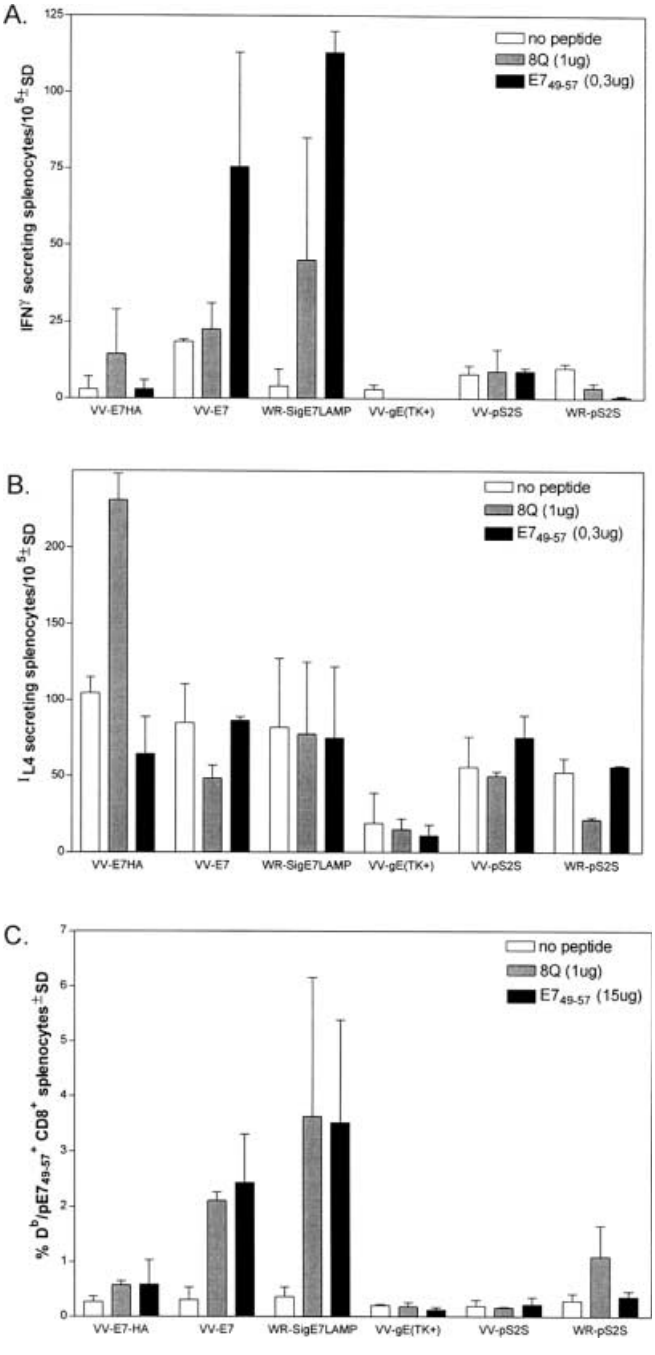

Fig. 5 Cell-mediated response of mice inoculated with recombinant VVs. Splenocytes isolated 12 days after virus inoculation were restimulated with indicated oligopeptides for 6 days and then analyzed by ELISPOT for cytokine production. Frequency of E7 specific cells secreting: **A** IFNγ, **B** IL4. **C** Frequency of $D^b/pE7_{(49–57)}{}^+$ $CD8^+$ cells among restimulated splenocytes detected by flow cytometry

Vaccination with VV-E7 but not with VV-E7-HA protects against E7-expressing tumor challenge

In order to determine whether the recombinant E7-expressing viruses induced antitumor immunity, vaccinated mice (eight per group) were challenged with a dose of $2{\cdot}10^4$ TC-1 cells/animal. Statistically significant protection against tumor growth was found after immunization with VV-E7 or WR-SigE7LAMP. Vaccination with VV-E7-HA, similarly to control viruses, did not induce any significant delay of tumor growth as compared with a group of animals inoculated with PBS only (Fig. 6A). Comparison of the growing tumors by size (Fig. 6B) showed that their mean size in mice vaccinated with VV-E7-HA and in animals given no VV (PBS controls) was similar. The inoculation of control viruses (VV-gE, VV-pS2S or WR-pS2S) enhanced the growth of tumors, which was in line with our previous unpublished results.

Discussion

In the present study we changed the subcellular location of the E7 protein expressed by human papillomavirus type 16 (HPV16) to the surface of infected cells, intending thereby to increase the immunogenicity of this tumor antigen. For this purpose, a recombinant VV with the E7 gene fused into the transmembrane sequence of the VV HA gene was prepared. The E7-HA fusion protein, as shown by immunofluorescence and subcellular fractionation, is found in the heavy membrane fraction and is localized on the surface of VV-E7-HA infected cells. Our approach was motivated by a previous report by Galmiche et al. [7], but our method of preparation of recombinant viruses differed in certain details. We inserted the E7 DNA fragment in a different region of HA and removed part of the HA chain. Consequently, our virus was HA^-. Still, the construct induced a high expression of the fusion protein on the cell surface. We were interested in finding out whether this targeting of the antigen would influence its immunogenicity, as reported previously by Andrew et al. and Langford et al. [2, 18]. In these authors' experiments, the cell-surface anchoring of antigen expressed by recombinant VV increased both the cellular and humoral immune responses to foreign antigen. Moreover, we presumed that vaccination with the E7-HA fusion

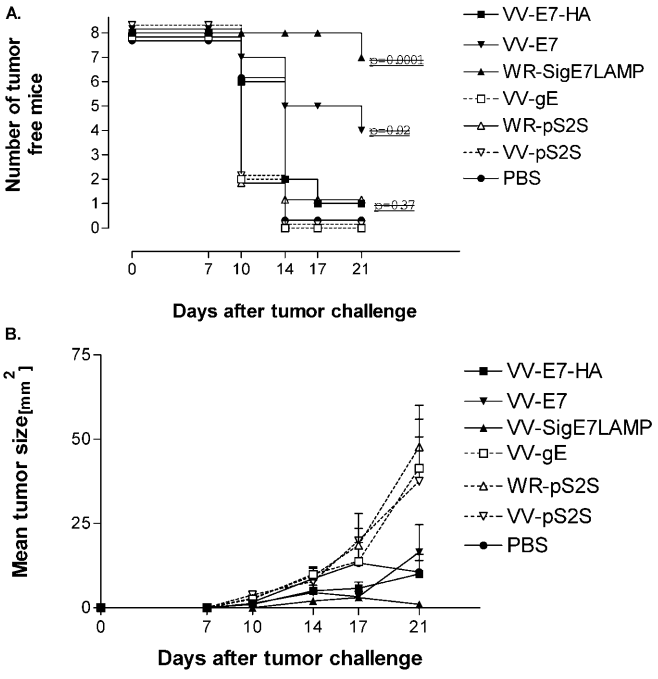

Fig. 6 In vivo tumor protection and growth of tumors in mice inoculated with recombinant VVs. Eleven days after virus inoculation groups of mice were challenged with $2{\cdot}10^4$ of TC1 tumor cells administered s.c. *Graph A* shows numbers of tumor-free animals; *graph B* demonstrates the mean size of growing tumors >1 mm^2. The results indicate that VV-E7-HA was not able to protect mice from the growth of E7 expressing tumor. Immunization with VV-E7 induced protection of a portion of animals. WR-SigE7LAMP was included as a positive control virus that is able to induce full protection of animals. The in vivo protection test was repeated three times with similar results. Statistical significance was determined using the log-rank test. All groups were compared with the group that received PBS only

protein expressed under the HA (A56R) late promoter might protect mice against challenge with HPV16-induced-tumor cells. Bronte et al. [3] have shown that vaccination with viruses expressing tumor-associated antigen under late promoters mediates protection against tumor growth, albeit a combination of early/late promoters was more effective.

In our model, immunization with VV-E7-HA provided no protection against the growth of E7-expressing tumors. Analysis of the immune responses showed that E7-HA did not induce a response of $CD8^+$ T lymphocytes or Th1 cells, which are the effectors of protection against HPV-induced tumor [6]. On the other hand, our VV-E7-HA recombinant was a good inducer of anti-E7 antibodies, as well as of Th2 cells, the producers of IL4. An analysis of the IgG isotypes revealed a Th1-like response, with predominance of the IgG2a isotype. Presumably, the switch to IgG2a might be related to an increased production of IFNγ as a result of the infection with VV and is not a consequence of the E7-specific Th1 response [20]. We demonstrated that the fusion of E7 with the signal peptide and transmembrane domain of HA increased the stability of the E7 protein. Unmodified E7 is a short-lived molecule [25] and its rapid degradation by the ubiquitine-proteasome pathway may contribute to its ability to induce CTLs [29]. However, the increased stability of E7-HA cannot wholly explain its different immunogenicity, because WB analysis indicated that the SigE7LAMP fusion protein accumulated in recombinant VV-infected cells at similar levels to E7-HA (not shown).

The predominance of the antibody response over CTL might be ascribed to the concurrent activity of several factors. It is possible that soon after the inoculation of VV-E7-HA, the infected cells were able to present E7 on their surface mainly to B cells but not to T lymphocytes, because the expression of MHC molecules might at this time (late phase) be downregulated owing to the viral infection [31]. Activated B cells soon start to play the role of antigen-presenting cells, and their interaction with T cells results in a proliferation of Th2 cells. Under these conditions the activation of type 1 helper lymphocytes by cross-priming in secondary lymphoid organs might be suppressed by an already established response of Th2 lymphocytes [26]. The viruses VV-E7 and VV-E7-HA induced in mice opposite polarized type 1 and type 2 responses, characterized by proliferation of IFNγ- and IL4-producing lymphocytes, respectively. It is well known that IL4 has the potential to modulate the function of cytotoxic and IFNγ-producing T cells, and that polarization of the immune response is usually established soon after exposure to antigen and is sustained by immunological memory. Since polarization of the immune response is a critical parameter for the outcome of virus infection [27] and protective antitumor immunity [5], we plan to use the recombinants described in this paper for a study of the detrimental effect of the type 2 response on protective immunity to HPV-associated tumors.

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