考にして，われわれの経験をもとに紹介する．詳細はマニュアルを参照されたい．

現在，わが国では，PACTG（Pediatric AIDS Clinical Trial Group）076 ZDV 療法を参考に予防対策が行われている．PACTG 076 ZDV療法は1994年に発表された臨床研究であり，初めて抗ウイルス薬を用いて母子感染率を低下させ，安全面でも信頼のおけるものである[5]．最新の2008年度版の米国でのPublic Health Service Task Forceの「HIV母子感染予防ガイドライン治療指針（Recommendations for Use of Antiretroviral Drugs in Pregnant HIV-Infected Women for Maternal Health and Interventions to Reduce Perinatal HIV Transmission in the United States）」でも同様の予防対策が行われている[6]．

当センターでは，過去10年間で32例のHIV感染妊婦の母子感染予防を行っているが，以下に述べる母子感染予防策にて現在のところは感染例は認めていない．

1. HIV母子感染予防対策

HIV母子感染予防対策の基本を**表1**に示す．また，具体的なことを以下に述べる．

1）母体への対応

いかに早期にHIV感染症を把握し，妊娠中の母体血中ウイルス量を管理できるかが重要である．母体の血中HIVウイルス量，免疫状態（CD4値）や臨床症状を的確に把握し，抗ウイルス薬による治療が必要かどうか検討する．出生直前にHIV感染が判明した際には，感染症科にコンサルトして的確な評価を受け，必要に応じた治療を行う．

2）分娩時期

陣痛発来前が望ましく，具体的には妊娠37週を目安に分娩時期を決定し，選択的帝王切開を行う．陣痛時には母体血（HIVウイルス陽性血液）が児に移行しやすくなり，児は分娩中に産道からの感染を受けやすい．陣痛発来前，破水前に選択的帝王切開を行うことで，これらのリスクを減少させることができる．選択的帝王切開は，母子感染の大半を占める分娩時期周辺の感染を予防する有効な方法とされている（米国では母体血のウイルス量が低い場合には帝王切開が不要だとする考えもある）．

表1 HIV母子感染予防対策の基本

1. 妊婦に抗ウイルス療法を施行し，分娩時のウイルス量を可能な限り少なくすることをめざす
2. 陣痛発来前，破水前の予定帝王切開
3. 分娩中の母体へのZDV〔ジドブジン，アジドチミジン：AZT〕の点滴投与
4. 母乳中止（止乳）
5. 出生児へのZDVの6週間投与

3）分娩中

分娩開始とともに母親にジドブジン（以下，ZDVと略す）2 mg/kgを1時間で点滴静注する．出産まで引き続き1 mg/kg/時を持続静注する．

4）母乳への対応

母乳中には多量のHIVウイルスが含まれるため，母乳を与えることで児に感染が及ぶことを両親に説明し，断乳を行う．止乳が困難な例はブロモクリプチンなどの薬剤を使用する．

5）出生児への抗ウイルス薬の予防的投与

①投与方法

すべての出生児にZDV単独療法にて6週間投与する．現在のところは，主としてZDV単独療法が行われており，他の抗ウイルス薬との併用での予防対策法はいまだ効果や安全性は証明されていない．ZDV単剤療法ではHAART（highly active anti-retroviral therapy）と比較して児に対する安全性が高いが，感染母体に対する治療効果は低く，薬剤耐性ウイルスが出現しやすい．そのため，妊娠中の母親に対する治療はCD4値やウイルス量，HIVウイルスの耐性検査などを考慮しつつ，抗ウイルス薬の選択を検討しなくてはならない．

新生児に対して，初回は出産後6～12時間までに施行する．ZDVシロップ2 mg/kgを6時間

ごとに投与し，生後6週まで続ける．経口投与できない場合は，1.5 mg/kgを6時間ごとに静脈内投与を行うZDV（別名AZT：アジドチミジン）シロップ（商品名Retrovir® syrup）を，経口で投与する．悪心・嘔吐などを認める場合は経鼻チューブから投与することもあり，それでも困難な場合は注射薬を使用する．

ZDVは腸管からの吸収後，おもにUPD-glucuronosyltransferaseによってグルクロン酸抱合を受け代謝される．新生児の場合，肝機能や腎機能の未熟性によって薬物代謝や排泄が遅延することがわかっているが，早産児ではその代謝機構はさらに未熟であり，ZDVの量を調節する必要がある．以下に例を示す．

i）30週以上35週未満で出生した場合：ZDVシロップ2 mg/kgを12時間ごとに投与し，生後2週をすぎてから同量を8時間ごとの投与に変更する．

ii）30週未満で出生した場合：ZDVシロップ2 mg/kgを12時間ごとに投与し，生後4週をすぎてから同量を8時間ごとの投与に変更する．

②ZDVの副作用

貧血の合併が報告されており，出生時にすでに貧血が認められる場合や早産児は注意して経過をみる必要がある．また，肝機能障害や好中球減少の報告もある[7]．

当センターでは過去10年間に，32例のHIV母子感染予防を行った児のフォローアップを行っているが，このうち約80%の児に貧血を認めた．輸血を必要とした症例は3例あり，その他はエリスロポエチンや鉄剤投与などの治療が必要であった．

③ZDV（AZT）シロップの入手法

陣痛発来や前期破水などの緊急時も対応できるよう早めから入手する．厚生労働省エイズ治療薬研究班から入手できる（HIV母子感染予防対策マニュアル第5版，p59参照）．

研究班ホームページ：http://www.iijnet.or.jp/aidsdrugmhw/

FAX情報サービス：03-3342-6171

事務局：東京医科大学臨床病理科

TEL：03-3342-6111（内線5086）

FAX：03-3340-5448

2. ハイリスク群に対する治療

分娩までに母体の感染が十分コントロールされていない症例，母体の感染の事実が分娩直前に判明した症例，分娩直後に母体の感染が判明した症例，母体が薬剤耐性ウイルスに感染している症例など，ハイリスク群に対する予防対策については現在のところ確立されたものはない．ZDV＋NVP（ネビラピン），ZDV＋3TC（ラミブジン），ZDV＋3TC＋NVP or LPVr（ロピナビルorリトナビル）などの併用療法開始が検討される[6]．有効性と安全性に関してはまだ十分な報告がなく，これらの使用にあたっては十分な母体の情報収集に加え，小児HIV診療専門家に相談して治療方針の検討を行うことが重要である．

3. 診療体制

当院での母体HIV感染合併妊娠，出産の際の手順を紹介する．母体のHIV感染が判明した際は，産科，内科（感染症科），小児科の連携が大切であり，また社会的，精神的支援のため，ソーシャルワーカーやコーディネーターナースなどのコメディカルスタッフの応援も必要となる．

表2に示すような連絡票を作り，産科，内科（感染症科），小児科で合同カンファランスを行って，情報共有と確認を行っている．

4. 分娩時の実際

児に付着した母体血を十分ふき取ることが重要である．

1）清拭

胎脂をふき取るオリーブオイルを準備する．インファントウォーマに防水シーツをしき，ホスピタルマットを数枚用意（児を洗浄する際の体温低下を防ぐために3～4枚用意）．温蒸留水，

表2　連絡票

患者病歴番号
氏名 ________________________　年齢____歳
夫のHIV感染（＋・－）

HIV抗体検査陽性と判明した日（ / ）
治療前（ / / ）　CD4：________/mm³
ウイルス量：________copies/mL

抗ウイルス療法（ / / ）～
内容：________________________
____

日和見感染予防________________________
____

当院産科初診日（ / / ：妊娠__週__日）
分娩予定日（ / / ）

帝王切開直前（ / / ：妊娠__週__日）　CD4：________/mm³
ウイルス量：________copies/mL

内科合併症________________________
____

妊娠経過・特記事項________________________
____

経腹超音波所見（ / / ：妊娠__週__日）
児推定体重________g
____
____

母体	帝王切開術予定日（ / / ：妊娠 週 日）
分娩前日 分娩日 分娩後 新生児	（ / ）前日夜まで抗ウイルス薬内服（ ） 手術開始3時間前からAZT点滴静注（体重 kg） 点滴用AZT 2A（400 mg/40 mL）＋5% Glu 160 mL（＝2 mg/mL）に調整 （ ： ）～ mL/時（2 mg/kg）で1時間　その後， （ ： ）～ mL/時（1 mg/kg）で継続し，手術終了まで 経口摂取可能となったら抗ウイルス薬を再開する 母乳禁：乳房冷却．プロテアーゼ阻害薬内服症例では，パーロデル®の副作用が強く出る可能性が高く，併用を避けることが望ましい
	出産後　8～12時間以内にAZTシロップ内服

*切迫早産の場合：積極的に子宮収縮を抑制するが，帝王切開予定日を早めることも検討する
*手術予定日に陣痛発来あるいは破水した場合：入院後ただちにAZT点滴静注を開始し，同時に帝王切開術の準備を進める．分娩経過が急速で，帝王切開術に比べ経腟分娩のほうが早期に児娩出可能と判断した場合には，経腟分娩とする
*その他連絡事項：________________________
____
____

生理食塩水，イソジン®付綿棒も用意する．

2）新生児の処置

小児科医は防水ガウン，フェイスシールドマスク，足袋，手袋（二重にして使用）を装着し，児を受け取ったら安全にインファントウォーマへ移送する．児の状態を確認し，必要時蘇生を行いつつ清拭を行う．吸引に際しては粘膜損傷をおこさないように注意する．全身の血液をふき取り，温蒸留水で洗浄，温オリーブオイルで胎脂の除去を行う．生理食塩水で眼と耳の洗浄を行う．皮膚に傷があるときには，傷口をイソジン®で消毒する．児の状態が落ち着いていることを確認後，新生児室に搬送する．

5. 分娩後の対応

出生後の管理は，各施設における帝王切開により出生した新生児の保育方法に準じる．母子感染の診断のための採血は，低血糖がなく呼吸状態が安定していれば出生48時間以内にRT-PCRによるHIV-RNA定量を行う．

6. フォローアップ

1）感染の有無の検査

生後15～18カ月までHIV感染母体由来の移行抗体を患児に認めることがあるため，母子感染の有無の診断にはウイルス学的検査（HIV DNA PCRやHIV-RNAアッセイ）が重要である．ウイルス学的検査にて生後1カ月までに96%以上の感染の有無が確認でき，生後6カ月までに全例で感染の有無が確定できる[8]．

HIV感染母体から生れた児は，生後48時間，生後14日目，生後1～2カ月，生後3～6カ月の計4回ウイルス学的検査を行う．さらに，生後18カ月時にウイルス抗体検査を含めウイルス学的検査を行い，HIV非感染を確定する．

感染の診断としては，2回の異なった血液検体でのウイルス学的検査で2回陽性の場合はHIV感染が疑われる．その際は再度検体を提出し，感染の診断を確定する．

生後48時間以内のウイルス学的検査が陽性（臍帯血をのぞく）の場合は，子宮内感染が示唆される．ウイルス量は生後2週間で急上昇することから，生後14日目の検査は早期診断に役立つ．生後18カ月時では，HIV感染による徴候もなく，低γグロブリン血症がなく，HIV抗体が陰性であり，ウイルス学的検査が陰性の場合には完全に感染は否定できる．しかし，なかには生後18カ月を越えても移行抗体の存在が認められることもあるため，その後も追加で検査することがある．

2）抗ウイルス薬の影響

母体が妊娠中や分娩時期に抗ウイルス薬に曝露され，また新生児期にZDVの治療を受けた児は，薬剤の影響を少なからず受ける．もっとも頻度が高いのは新生児期の貧血であり，その他に顆粒球減少症，肝機能障害などの報告がある[9]．現在，投与される抗ウイルス薬はミトコンドリアで作用するものが多く，原因不明の神経筋疾患や心疾患の発症の際はミトコンドリア機能異常による可能性があり，その原因として薬剤による可能性があることも念頭におくべきである．

米国での母子感染予防を行った児の6歳までの長期フォローアップでは，免疫学的，神経学的，成長，悪性腫瘍の有無に関してとくに有意差は認められなかった[6]．しかし，思春期から成人に至るまで，薬剤の副作用を含めた長期フォローアップが必要であると考える．

HIV感染児

小児においては年齢別に免疫能が異なるため，**表3**に示すように年齢とCD4陽性リンパ球により，まず免疫学的ステージングを評価する．また，**表4**に示すようにHIVの臨床分類が決まっている．

以上をふまえ，**表5**（米国の小児HIVガイドライン）[10]を参考に治療方針を決定する．1歳未満の乳児では病気の進行が早く，病期やCD4の値にかかわらず治療開始が推奨される．1歳以

上では臨床症状，ウイルス量，免疫学的分類を考慮して治療を選択する．

小児HIV感染症の治療においても多剤併用療法が基本である．第一選択としては，2剤のヌクレオチド系核酸逆転写酵素阻害薬（nucleoside analogue reverse transcriptase inhibitors：NRTI）と1剤の非ヌクレオチド系核酸逆転写酵素阻害薬（non-nucleoside analogue reverse transcriptase inhibitors：NNRTI），または2剤のNRTIと1剤のプロテアーゼ阻害薬（protease inhibitor：PI）があげられる．

服薬遵守は重要であり，薬剤耐性ウイルスが出現すると治療は困難になる．年齢に応じた薬剤を選択し，かつ患者および養育者の内服指導が必要である．また上記に加え，日和見感染の鑑別，診断，予防が大切である．

中でも，感染児はニューモシスチス・カリニ肺炎（*Pneumocystis carinii* pneumonia：PCP）の予防が重要であり，予防投薬を行わなければ1歳までに発症すると推定される．PCPは発症すると致死率も高いため，予防投薬は重要である[9]．

感染妊婦から出生した後，非感染と診断されていない児を対象として生後6週から開始し，HIV非感染が確認されない限り，1歳まではCD4陽性細胞数にかかわらず継続する．ST合剤をトリメトプリム（TMP）として150 mg/m^2/日を分2（分1）で3投（連続または隔日）4休の経口投与を行う．

HIVの母子感染予防策（妊娠分娩中の母体と新生児への抗ウイルス療法，選択的帝王切開，母乳禁止など）をすべて実施した場合の感染率

表4 小児HIV感染症の臨床分類（文献11）より引用）

N群（無症候）	HIV感染症によると考えられる症候がない児またはA群の症状のうち一つしかない児
A群（軽症）	以下の症状のうちの二つ以上を示すが，B群またはC群の症状を欠く児 ・リンパ節腫脹（2カ所以上で0.5 cm以上．対称性は1カ所とみなす） ・肝腫大 ・脾腫 ・皮膚炎 ・耳下腺炎 ・反復性/持続性の上気道感染，副鼻腔炎，中耳炎
B群（中等症）	A群またはC群以外の症状を示す児．この中には以下のような症状が含まれる ・貧血（＜8 g/dL），好中球減少（＜1000/μL），血小板減少（＜10万/μL） ・カンジダ症 ・細菌性の髄膜炎，肺炎，敗血症 ・心筋症 ・新生児CMV感染症 ・慢性下痢 ・単純ヘルペスの反復性口内炎 ・新生児期の単純ヘルペス気管支炎，肺炎，食道炎 ・2回以上あるいは2皮膚節以上の帯状疱疹 ・平滑筋肉腫 ・リンパ間質性肺炎（LIP）または肺のリンパ過形成 ・腎症 ・ノカルジア症 ・1カ月以上続く発熱 ・新生児トキソプラズマ症 ・播種性水痘，など
C群（重症）	AIDSの診断基準に含まれる症状（LIPを除く）

表3 小児HIV感染（13歳未満）の年齢別免疫学的分類（文献11）より引用）

免疫重症度	1歳未満		1～5歳		6～12歳	
CD4	/mm^3	%	/mm^3	%	/mm^3	%
正常	≧1,500	≧25	≧1,000	≧25	≧500	≧25
中等度低下	75～1,499	15～24	500～999	15～24	200～499	15～24
重度低下	＜750	＜15	＜500	＜15	＜200	＜15

表5　小児HIV感染症の治療開始の基準（文献10）より引用）

年　齢	クライテリア	検査値	勧告
12カ月未満	症状，CD4値によらず		治療
1歳以上4歳未満	AIDS，HIV関連症状あり		治療
	症状によらず	CD4＜25%	治療
	無～軽症	CD4≧25%　かつ HIV RNA≧100,000 copy/mL	考慮
	無～軽症	CD4≧25%　かつ HIV RNA＜100,000 copy/mL	待機
5歳以上	AISD，HIV関連症状あり		治療
	症状によらず	CD4＜350/mm^3	治療
	無～軽症	CD4≧350/mm^3　かつ HIV RNA≧100,000 copy/mL	考慮
	無～軽症	CD4≧350/mm^3　かつ HIV RNA＜100,000 copy/mL	待機

臨床所見・検査所見は3～4カ月ごとにフォローする

は1%未満であることから，生後1カ月までの間に施行された検査でHIVのウイルス量が陰性であった場合は非感染である確率が高く，この場合はPCPに対する予防投与は割愛される傾向にある．

予防接種

1．感染児

原則として生ワクチンは接種しない．つまり，わが国で頻繁に投与されるBCGや経口生ポリオワクチン（oral polio vaccine：OPV）は禁忌である．実際は，HIV感染者で問題となる日和見感染症に対して，その感染の軽減が期待できるものについて予防接種を考慮することは，CDC（Center for Disease Control and Prevention）で推奨されている．免疫不全患者では健常人と同様の免疫反応が期待できない可能性もあり，多めのワクチン量や頻回のブーストが必要となることもある[12]．

2．非感染児

OPV以外はすべて接種可能である．OPVは腸管内でワクチンウイルスが増殖し便中に排泄され，接触者が経口感染して発病する可能性があるため，家族内にHIV感染者がいる場合には接種は禁忌となっている．そのため，不活化ポリオワクチン（IPV）の接種が望ましいが，わが国ではいまだ認可されておらず，早急な対応が待たれる．

おわりに

わが国において，HIV感染症は増加傾向であり，母子感染の頻度は低いとはいえ，母子感染例に対する対応を理解しておく必要がある．

母子感染児は一生にわたって抗ウイルス薬療法を継続せねばならず，それによるさまざまな合併症をきたす可能性が大きい．集団生活や社会生活にも配慮が必要であり，思春期になると患児への告知や性教育など，児の成長とともにさまざまな問題点が出現する．上記のような母子感染例を出さないためには，まずは妊婦の早期診断が重要であり，母体HIV抗体検査の普及を100%徹底すべきである．また，早期診断後に適切な母子感染予防対策をとり，母子感染を確実に予防することが重要である．

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Guidelines for Managing Conscientious Objection to Blood Transfusion

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Parents sometimes deny their children blood transfusion because of their religious beliefs. The Japanese Joint Committee on the Refusal of Blood Transfusion on Religious Grounds asserts that the health and life of every child younger than 15 years should be guarded by the collective efforts of health, welfare, and advocacy institutions when a parent or guardian seeks to withhold transfusion therapy. Patients 18 years or older should receive treatment without transfusion after signing and submitting a "Certificate of Refusal Blood Transfusion and Exemption from Liability." For a patient younger than 18 years, but 15 years or older, essential transfusion can be performed if the patient or at least one guardian consents. Without patient's or guardian's consent, guidelines for patients 18 years or older shall apply. Health care providers should offer the best possible care that is consistent with a patient's age and competency.

PATIENTS, OR THEIR legal guardians, may object to blood transfusion for various reasons. Reasons arising from misinformation or fear can be approached as with other medical interventions. Informed reasons arising from religious belief or personal conviction are legally and ethically challenging and warrant special consideration.

Concepts of human rights and personal autonomy have expanded in modern society where people with different value systems must coexist. Jehovah's Witnesses, who number more than 200 000 active members in Japan and 6 million worldwide, embrace conservative values and avail themselves of modern medical care except transfusion of whole blood and the 4 major components of red blood cells, platelets, plasma, and white blood cells.[1]

A Japanese national survey revealed that in 2003, 0.8% (4 of 541 cases) of deaths attributed to surgical bleeding were related to religious refusal of blood transfusion.[2] Although competent adults have the right to refuse blood transfusion for themselves,[3,4] laws or judicial precedents in Japan have not totally established whether parents have the right to refuse necessary medical care, including transfusion for their children, even though there are 2 cases in which such parental rights were clearly denied by lower courts.[5,6] The US Supreme Court made this clear in 1944, "parents may be free to become martyrs themselves. But it does not follow they are free, in identical circumstances, to make martyrs of their children before they have reached the age of full and legal discretion—."[7]

With regard to patients who object to blood transfusion, in 1998, the Japan Society of Blood Transfusion (currently the Japan Society of Transfusion Medicine and Cell Therapy [JSTMCT]) reported that patients 18 years or older should be allowed to submit a "Certificate of Refusal of Blood Transfusion and Exemption From Liability" as a personal human right.[8] For patients younger than 12 years, transfusion therapy is deemed appropriate when necessary, even against the wishes of the parent or guardian. However, the Japan Society of Blood Transfusion did not address the patients between 12 and 18 years old, leaving these cases for hospitals to address independently, because clear guidance was lacking at the time.[8]

An investigation of child fatalities in the United States between 1975 and 1995 revealed that when faith healing was used in lieu of conventional medical treatment, a substantial number (>140 cases) of child fatalities and associated suffering could have been prevented.[7] Existing laws in 1998 may have been inadequate in the United States to protect children from this form of medical neglect.[9]

The United Nations Children's Fund found that almost 3500 children younger than 15 years die every year from child abuse and neglect in 27 developed countries.[10] Japan appears to have a higher incidence of child maltreatment deaths

From the Japanese Joint Committee on Refusal of Blood Transfusion on Religious Grounds; Japan Society of Transfusion Medicine and Cell Therapy; Japanese Society of Anesthesiologists; Japan Pediatric Society; Japan Society of Obstetrics and Gynecology; Japan Surgical Society; Waseda Law School, Waseda University; School of Law, Waseda University; The Institute of Medical Science, the University of Tokyo, and The Asahi Shimbun Company.

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(1.0/100 000 children a week) than Spain (0.1) and Italy (0.2), comparable with Germany (0.8), UK (0.9), and Canada (1.0), and lower than the United States (2.4) and Mexico (2.7). Some parents/guardians will seek healing through religion rather than medical care. Medical neglect evaluations should focus on the child's needs rather than the caregiver's motivations or justifications. Religious objections should not be granted fundamentally different status from other types of objections.[9]

Because second-generation believers are born into and grow up in a religion chosen by their parents or guardians, these second-generation believers can, in principle, leave the religion of their upbringing and move to another one; young children in this category should be protected by society as a whole from caregivers who abuse and/or neglect them.

The Joint Committee guidelines, discussed below, are officially sanctioned by JSTMCT, Japanese Society of Anesthesiologists, Japan Pediatric Society, Japan Society of Obstetrics and Gynecology, and Japan Surgical Society. They clearly define when to accommodate the wishes of competent patients and when to protect younger children in situations where there is an objection to blood transfusion.

BASIC POLICIES REGARDING THE ADMINISTRATION OF BLOOD TRANSFUSION

Patients likely to benefit from blood transfusion, or suffer without transfusion, should be considered as belonging to 1 of 3 age categories: 18 years or older, 15 to 17 years, and younger than 15 years (Fig 1). Age 18 marks the transition from childhood to adulthood in Article 4 of Japan's Child Welfare Law, and age 15 has been considered a threshold of competence in several Japanese legal sources, including Article 797 of Japan's Civil Code, as the age of valid consent to adoption without proxy; Article 961 of the Civil Code, in reference to testamentary capacity; and Japan's Organ Transplant Law, as the age of valid consent to donate organs. Patients should also be considered as competent to make medical decisions, or not, as assessed by more than one doctor, including the doctor in charge of the patient's care.

For Patients 18 Years or Older and Competent to Make Medical Decisions, the Following Apply:

If the medical provider consents to treat without blood transfusion, the patient shall sign and submit a Certificate of Refusal of Blood Transfusion and Exemption From Liability (see Appendix 1, Note 1) to the medical provider.

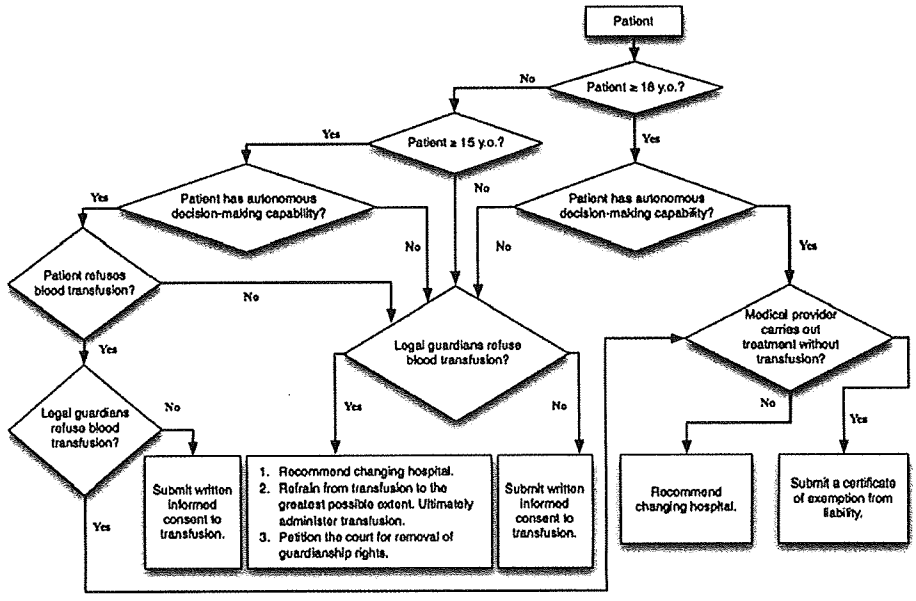

Fig 1. Flowchart of consent and refusal of blood transfusion. The flowchart shows that objection to blood transfusion should be resolved according to the age and autonomous decision-making capability of the patient.

If the medical provider cannot consent to treat without blood transfusion, the medical provider shall, at the earliest possible time, recommend transfer to the care of another provider.

For Patients Younger Than 18 Years or Not Competent to Make Medical Decisions, the Following Apply:

Patients 15 years or older and competent to make medical decisions:

a. When a legal guardian objects to blood transfusion but the patient wishes to receive it, the patient's informed consent shall be documented.
b. When a legal guardian approves of blood transfusion but the patient objects, the medical provider shall avoid transfusion to the greatest possible extent but may transfuse according to medical necessity. The guardian's informed consent shall be documented.
c. When all legal guardians and the patient object to blood transfusion, guidelines for patients 18 years or older shall apply.

Patients younger than 15 years (see Appendix 1, note 2) or not competent to make medical decisions, and a legal guardian objects to transfusion:

d. When both legal guardians object to transfusion, the medical provider shall seek consent from the guardians and shall carry out treatment without blood transfusion to the greatest possible extent but may transfuse according to medical necessity. If consent is not granted, to the detriment of patient care, the medical provider shall report the situation as a case of child abuse to a governing authority. The governing authority shall place the child under protective custody and petition a family court to suspend the rights of guardianship. When the petition is granted, transfusion shall proceed according to medical need with the consent of a court-appointed proxy guardian.
e. When one legal guardian consents to blood transfusion but the other objects, the medical provider shall make an effort to obtain the consent of both guardians but, in the case of an emergency, shall administer blood transfusion with the consent of the guardian who consents to blood transfusion.

FLOWCHART OF CONSENT TO BLOOD TRANSFUSION AND CERTIFICATE FOR EXEMPTION FROM LIABILITY

Figure 1 presents a flowchart showing the procedures that a medical provider should follow in cases where objection to blood transfusion is asserted by a patient and/or legal guardian(s). Form 1 (see Appendix 2) is a Certificate of Refusal of Blood Transfusion and Exemption From Liability.

BLOOD TRANSFUSION THERAPY AND INFORMED CONSENT

The Ministry of Health, Labor and Welfare released the *Guidelines for Performing Blood Transfusion Therapy* (revised version) and the *Guidelines for the Use of Blood Products* (revised version) in September 2005.[11] The responsibilities for medical professionals appear in these guidelines. Regarding the requirements on the effectiveness and safety of blood products and the proper use of the said products, the guidelines specify that medical professionals shall provide appropriate and adequate explanation to patients and/or their family members in an effort to obtain informed consent and that a medical professional shall decide whether or not to perform blood transfusion therapy after adequately weighing the potential benefits against the risks. Transfusion shall be kept to the minimum required for the desired effect, and excess transfusion shall be avoided. Every effort shall be made to relieve clinical symptoms while avoiding blood transfusion to the greatest possible extent, if suitable alternatives are available. In addition, the statement on explanations and informed consent specifies that the following items shall be adequately explained in a manner easily understood by the patient and/or his or her family members:

(1) need for blood transfusion therapy,
(2) type and amount of blood product to be used,
(3) risks involved in transfusion,
(4) remediation available to those who experience adverse effects and infectious diseases arising from transfusion,
(5) availability of autologous blood transfusion,
(6) screening for infectious diseases and storage of specimens,
(7) retention of medical records and their use in retrospective surveys, and
(8) other transfusion therapy precautions.

Upon informed consent of the patient and/or his or her family members, documentation of consent shall be executed. A copy shall be given to the patient, and a copy shall be attached to the medical record (physically or electronically, as applicable). If consent of the patient or of his or her family is not obtained, blood transfusion should not, in principle, proceed.

ACTIONS TO BE TAKEN BY MEDICAL PROVIDERS

On the basis of judicial precedents, published guidelines set forth a way to allow blood transfusion under special circumstances, such as a situation that is life threatening without transfusion, even if patient and/or guardian consent is not obtained. It is also feasible for medical institutions to adapt guidelines to particular circumstances after due diligence and with the approval of an ethics committee and/or other relevant body. In addition, it is desirable to have procedures in place to secure the consensus of more than one doctor, including the doctor in charge of a patient's care, to judge a patient's capacity to make medical decisions.

COMMENTARY ON THE GUIDELINES FOR MANAGING CONSCIENTIOUS OBJECTION TO BLOOD TRANSFUSION

Japan Society of Blood Transfusion (currently JSTMCT) published the *Report on informed consent in blood transfusion*[8] in 1998. Regarding objection to blood transfusion on the basis of one's religious beliefs, this report states that such patients should be required to submit a Certificate of Refusal of Blood Transfusion and Exemption From Liability and/or to change hospitals according to the rights of self-determination in medical care. From judicial precedents described below, refusal of blood transfusion is regarded as a personal human right in the case of competent adult patients. However, for patients younger than 18 years, objection to blood transfusion has been a matter for hospitals to address independently.

More recently, however, local family courts have issued provisional orders to affirm petitions for the temporary suspension of parental rights and the appointment of a proxy guardian from the directors of child consultation offices in cases of surgery with high levels of urgency.[12] Although intervention in parental rights must follow court proceedings, which generally take time, the judiciary has expressed understanding toward hospitals having difficulties in the case of surgery for children for whom parental consent is not obtained, and this can be said to have prompted local family courts to issue provisional orders pending final determination. Discussions on the revised Child Abuse Prevention Law, passed on May 25, 2007, included one on allowing children to be treated without parental consent under the stipulation that only the "custodial rights" by which children are protected and supervised (see Appendix 1, note 2) could be temporarily suspended. This was not included in the present revised law but was incorporated in an appendix as the "temporary termination of parental rights" to be discussed in future revisions of the law.

The concept of medical neglect has contributed to an increase in such discussions. Medical neglect means not giving children the health care necessary or appropriate for them in light of medical standards and conventional wisdom. It includes not only parents not taking children to a hospital but also parents taking children to a hospital but not consenting to treatment. There is also the view that parental objection to blood transfusion for their children, for example, on the basis of religious beliefs, is child endangerment and a form of child abuse.[13] However, it cannot be denied that, depending on their age or stage of mental development, the children themselves may have internalized their parents' religious beliefs and established the refusal of blood transfusion as their own belief. It is, therefore, also difficult to make a sweeping judgment that all cases of the refusal of blood transfusion are cases of child abuse.

On the basis of the above-mentioned recent trend, Japanese Guidelines recognize a duty to provide the best treatment, including blood transfusion, to persons younger than 15 years or, otherwise, lacking the capability to make medical decisions by giving special consideration while respecting the right of self-determination to the greatest extent possible. Regarding adults older than 20 years who are incapable of making medical decisions, at present, the refusal of blood transfusion can only be left as a future issue to be addressed in view of the relevant legal and social

trends because ethical, medical, and legal standpoints are not yet fully established.

Assertion of People Refusing Blood Transfusion on Religious Grounds and Consideration of Their Psychologic Characteristics

People who refuse blood transfusion on the basis of their religion assume an attitude of being unequivocally against blood transfusion, asserting the superior value of not receiving blood transfusion over maintaining life, in accordance with their faith. However, it is assumed that they accept alternatives to blood transfusion and, indeed, seek them out actively. From this viewpoint, the medical provider should explain the availability of alternative treatments and the likelihood of successful surgery without blood transfusion at the hospital concerned.

Consideration should also be given to differences in the mental characteristics between first-generation followers who themselves chose to follow the religion and second-generation followers who have been greatly influenced, through their parents, by the doctrine and the organization of the religious group since childhood. It is pointed out that the second-generation followers are likely to acquire their parents' faith on the basis of their upbringing, and their feelings of fear and guilt at disobeying both their faith and their parents may be stronger than those of first-generation followers. The possibility should therefore be taken into consideration that children still under the care of persons with parental rights may encounter a negative psychologic effect from choosing blood transfusion treatment of their own will or upon being administered such treatment against their will, which may affect their future faith or family relationships. The medical provider is responsible for encouraging the parents of children who were administered blood transfusion against their will to nurture their children with the same care and responsibility as before the treatment. Measures should also be taken to obtain understanding and support from the religious organization if possible. In addition, counseling by persons specializing in pediatric/adolescent psychiatry should be provided to the patients during and after hospitalization to minimize the negative emotions that may arise as a result of having received blood transfusion contrary to their faith or against the parents' will. If blood transfusion is given under the temporary termination of parental rights, the parental rights should be reinstated quickly after the blood transfusion and continuous support be given so that the persons with parental rights will fully nurture the children after transfusion.

Judicial Precedents

Judicial decisions in which patients or their legal guardians objected to blood transfusion or treatment are described below. These are very important cases in understanding the right to refuse transfusion and health care neglect.

Case 1. A male patient in his 30s was hospitalized in university hospital A for bone sarcoma surgery in 1984. The patient desired surgery without blood transfusion for religious reasons. His parents filed a provisional disposition with the court to the effect that the hospital could be entrusted with operating on their son, the blood transfusion needed for it, and other medical intervention. Oita District Court ruled it impossible to conclude that the refusal of blood transfusion was an illegal violation of parental rights because the patient was an adult of normal mental ability, including understanding and decision-making capacity, and dismissed the provisional disposition (see Appendix 1, note 3) (December 2, 1985).

Case 2. A 10-year-old male patient was injured in a car accident in 1985. His parents refused blood transfusion, and the patient died at university hospital B without receiving blood transfusion. Ultimately, only the driver was charged with an offense causing death and was found guilty and fined ¥150 000, although this criminal case was a summary order (see Appendix 1, note 4; Kawasaki summary order, August 20, 1988).

Case 3. A 63-year-old female patient underwent surgical excision of a hepatic tumor at university hospital C in 1992. The patient was given blood transfusion against her will. She claimed damages, and the Supreme Court ruled that the right to refuse blood transfusion was a human right (see Appendix 1, note 5; February 29, 2000).

Case 4. For an infant (born in 2005) in whom a brain abnormality had already been detected in the fetal stage, the doctor explained the need for surgery because leaving the infant without treatment was very likely to lead to serious psychomotor retardation or death. However, the infant's parents (persons with parental rights) did not

consent to surgery on the basis of their religion. The hospital reported this as child abuse to the child consultation office. The director of the child consultation office petitioned a family court to effect the determination of the removal of parental rights, take preservative measures before determination to temporarily suspend parental rights until final determination, and appoint a doctor specializing in the patient's disease, a former university medical professor, as the surrogate to exercise parental rights during that period. The Kishiwada Branch of the Osaka Family Court affirmed this petition on February 15, 2005, and stated that it was necessary to temporarily terminate the father's and mother's exercise of parental rights because the refusal of surgery, even on the basis of religion or personal conviction, was an immediate danger to the infant, with a high likelihood of impeding healthy development, which are fundamental to a child's welfare. In addition, it was decided that because "waiting for the results of determination on the merits of this case risks being hazardous to life or causing serious impairment, proper treatment, including surgery, must be performed as quickly as possible." With regard to the surrogate guardian, the court-appointed doctor was considered a specialist in the said disease and "to have the ability to choose the most appropriate medical procedures" after carefully evaluating various conditions including the patient's medical condition and the appropriateness and risks of surgery (see Appendix 1, note 6).

Case 5. An infant with serious heart disease (born in 2006) required emergency surgery. However, the infant's parents (persons with parental rights) did not consent to surgery on the grounds of their religion. The director of a child consultation office asked a family court to enforce adjudication of the removal of parental rights on the merits of this case and to take preservative measures to provisionally terminate the exercise of parental rights before final and conclusive determination and to select a suitable lawyer as legal guardian during that period. The Nagoya Family Court accepted this request in an adjudication on July 25, 2006, and stated that leaving the situation as it was would certainly have risked the infant's life and that the parent's refusal to consent to surgery was an abuse of parental rights in the absence of a rational reason (see Appendix 1, note 6).

APPENDIX 1. END NOTES

Note 1

A Certificate of Refusal of Blood Transfusion and Exemption From Liability (see Appendix 2) is desirable. In an urgent case, however, a similar document completed by the patient himself/herself is also considered valid.

Note 2

Children or infants refer to persons younger than 15 years in these guidelines.

Note 3

The decision of case 1 can be considered as having had considerable impact on the subsequent theoretical and practical development of the blood transfusion refusal issue in Japan.

Note 4

Case 2 suggests that even a patient's parents may face criminal charges, such as for abandonment of a vulnerable dependent person or for causing death by misconduct (involuntary manslaughter). This may also apply to the doctor who treats such patients. Other questions arise: Was there any causal relationship between the driver's behavior and the child's death? Can parents be allowed to refuse blood transfusion to their children on the grounds of their own religious beliefs? Are parents not criminally responsible? Is the doctor who withheld blood transfusion not criminally responsible for the child's death? Asserting the parents' religious beliefs against the best interests of the child's life may also be considered an abuse of parental rights. The child should not be prevented from establishing his/her own religious beliefs in the future.

Note 5

The court decision for case 3 is more definitive than that for case 1 in that the refusal of blood transfusion was explicitly acknowledged as a human right. The hospital adopted the policy that if patients refuse blood transfusion on the basis of their religion, their refusal of blood transfusion is to be respected to the greatest possible extent, but that blood transfusion shall be administered regardless of the consent of the patient and the family members if there is no other lifesaving procedure than blood transfusion. The Supreme Court stated, "It is right for doctors engaged in the profession of managing

human life and healthcare to perform appropriate surgery in accordance with medical standards to remove the liver tumor from the patient. However, if the patient expresses a definite intention to refuse medical treatment including blood transfusion on the grounds that receiving blood transfusion violates his/her religious beliefs, the right to make such a decision must be respected as a human right. Since the patient had a strong determination to refuse blood transfusion under all circumstances in accordance with his/her religious beliefs and entered hospital C expecting to receive surgery without blood transfusion, the doctors should have explained that they would follow their hospital policy of giving transfusion if a situation arose during surgery in which no lifesaving means other than blood transfusion was available, and should have left the decision on whether or not to undergo surgery to the patient, while continuing the patient's hospitalization. Furthermore, it is undeniable that the doctors deprived the patient of decision-making rights regarding whether or not to undergo surgery that was likely to involve blood transfusion, and that, from this point of view, the patient's human rights were violated and the doctors should be liable to compensate the patient who suffered emotional distress [partially omitted]."

Note 6

Cases 4 and 5 are not cases of religious belief per se but concern the temporary termination of parental rights and the appointment of surrogate guardians by preservative measures before determination because of the refusal of the parents to allow surgery. Case 4, in particular, was the first case of this type of decision in Japan. Regarding these cases, it should be noted that the framework of child abuse prevention was used in which upon the receipt of the hospital's report of the parents' refusal to consent to surgery as a case of child abuse, the director of the child consultation office filed the petition to family court (Article 6 of the Child Abuse Prevention Law and Article 25 of the Child Welfare Law). This indicates that the irrational refusal of treatment should be taken to be medical neglect, even if arising from religious beliefs. It is also noted that the doctor and the lawyer were appointed as guardians in cases 4 and 5, respectively, during the temporary termination of parental rights. The system adopted in these determinations is such that the court does not directly enforce medical treatment of a child but excludes irrational judgment by the persons with parental rights and leaves medical care decisions to persons able to act rationally. It can be said, therefore, that case 4, which determined that a person able to choose the most appropriate medical treatment should be selected as the surrogate guardian, provided a decision that can serve as a beacon in the future. In general, it takes time to legally intervene in parental rights, but it was recently indicated that in a very urgent case involving human life, the court can take preservative measures for the temporary termination of parental rights in a short time (October 21, 2006, Osaka District Court).

APPENDIX 2. FORM 1: CERTIFICATE OF REFUSAL OF BLOOD TRANSFUSION AND EXEMPTION FROM MEDICAL LIABILITY (EXAMPLE)

Regarding the treatment (surgery, etc.) of _____
Date of explanation:
Explained by:
Doctor in charge (signature) _____
Doctor in charge (signature) _____
Department: _____

Director of (blank) Hospital

I have received an explanation of the possibility and/or need to receive transfusion of the following blood product(s) for the procedure described below:
(Specify the type and dosage of the blood product)
_____
_____

In accordance with my personal convictions, however, I request that blood transfusion be withheld regardless of the risks or disadvantages that may arise.

I will not, in any way, hold responsible the medical professionals concerned, including the doctor in charge, any situation caused by my refusal of blood transfusion.

I refuse the following types of blood transfusion (circle all items that apply): whole blood, red blood cells, white blood cells, platelets, blood plasma, autologous blood (preoperative blood storage/perioperative dilution/perioperative recovery/postoperative recovery), and blood plasma fraction products (albumin, immunoglobulin, blood coagulation factor, and other products (please specify) _____).

I have no objection to treatment involving fluid infusion or a plasma expander.

Date of signature
Name of patient (signature) _____
Name of proxy (signature) _____
Relationship with the patient _____

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Different Vaccine Vectors Delivering the Same Antigen Elicit CD8$^+$ T Cell Responses with Distinct Clonotype and Epitope Specificity[1]

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Prime-boost immunization with gene-based vectors has been developed to generate more effective vaccines for AIDS, malaria, and tuberculosis. Although these vectors elicit potent T cell responses, the mechanisms by which they stimulate immunity are not well understood. In this study, we show that immunization by a single gene product, HIV-1 envelope, with alternative vector combinations elicits CD8$^+$ cells with different fine specificities and kinetics of mobilization. Vaccine-induced CD8$^+$ T cells recognized overlapping third V region loop peptides. Unexpectedly, two anchor variants bound H-2D^d better than the native sequences, and clones with distinct specificities were elicited by alternative vectors. X-ray crystallography revealed major differences in solvent exposure of MHC-bound peptide epitopes, suggesting that processed HIV-1 envelope gave rise to MHC-I/peptide conformations recognized by distinct CD8$^+$ T cell populations. These findings suggest that different gene-based vectors generate peptides with alternative conformations within MHC-I that elicit distinct T cell responses after vaccination. *The Journal of Immunology,* **2009, 183: 2425–2434.**

Whereas protective immune responses against viral infections have been achieved by vaccination, HIV-1 has proven recalcitrant to preventive vaccination. Although humoral and cellular immunity contribute to the control of HIV-1 (1–3), it has not been possible to elicit the broadly neutralizing Abs required to prevent infection by diverse strains, prompting the development of T cell vaccine approaches. Recently, gene-based vaccines, including naked DNA and replication-defective viral vectors, have been used to stimulate antiviral T cell immunity in both nonhuman primates (4, 5) and humans (6, 7). Furthermore, in nonhuman primates, vaccine-induced T cells directed against processed immunodominant peptides can provide a degree of protection against acute and chronic immunodeficiency virus infections (8–11). However, immunodominance profiles and variation in the T cell responses elicited by vaccination are not yet well understood (9, 10, 12), and the differences in immunogenicity of alternative prime-boost vaccine regimens are ill defined. Further explorations of vaccine efficacy are required in human clinical studies. At the same time, murine models allow better analysis of genetic and immunological factors that regulate vaccine responses and are more amenable to mechanistic studies.

Virus-specific CD8$^+$ CTL recognize peptide epitopes that are generated by an intracellular processing pathway and are presented at the cell surface bound to MHC class I molecules (MHC-I). Because recognition of MHC-I/peptide complexes by the $\alpha\beta$ TCR is based on both MHC and peptide specificity, strategies for improving CTL immune responses have included the identification of immunodominant viral peptides, improvement of immunogenic peptide affinity for MHC-I, and efforts to maximize the functional affinity of the TCR for MHC-I/peptide complexes (13–15).

The immunogenicity of HIV-1 envelope (Env)[3] has been analyzed in animals (16) and humans (6). The third V region (V3) of the HIV-1 gp120 envelope glycoprotein is essential for co-receptor binding upon HIV entry (2, 17), and thus, Env has been a focus for the study of immunogenicity in experimental animals and humans. In mice, this region also serves as an immunodominant epitope, recognized both by CTL and by neutralizing Abs (17, 18). The V3 loop peptide in the CXCR4-tropic HIV-1 strain IIIB is an immunodominant CTL antigenic determinant in mice of several different H-2 haplotypes (19), and MHC-I tetramers have been used to analyze specific immunity to HIV-1_{IIIB} (20). However, because HIV-1_{IIIB} is not commonly found among natural isolates, it may have limited value as a target sequence in Env-directed HIV vaccines. To explore a potentially more clinically relevant virus (9, 10), we have selected HIV-1_{BaL} as a vaccine candidate that represents a CCR5-tropic virus and is more closely related to the strains responsible

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[3] Abbreviations used in this paper: Env, HIV-1 envelope; Ad, adenovirus; β_2m, β_2-microglobulin; BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; CM, central memory; EM, effector memory; V3, third V region.

for HIV-1 transmission, in contrast to the CXCR4-tropic HIV-1_{IIIB} (9, 16). We have used Env as the substrate for recombinant vector-based vaccines and have studied prime-boost combinations with DNA or recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG) priming, followed by recombinant adenovirus (rAd) boosting.

In this study, we first identified functional peptides related to the immunodominant V3 loop peptide of HIV-1_{BaL} that bind well to the H-2D^d restriction element. These peptides were used to make a set of H-2D^d/peptide tetramers that enabled the detection and characterization of disparate subpopulations of HIV-specific CD8$^+$ T cells induced by DNA or rBCG priming before rAd boosting compared with rAd Env vector immunization alone. Structural analysis and TCR sequencing were used to examine the molecular basis for differential recognition of specific H-2D^d/peptide complexes by distinct populations of CD8$^+$ T cells.

Materials and Methods

Cell culture and peptide induction of surface MHC-I expression

A TAP-defective cell line, LKD8, expressing H-2D^d (21), was propagated in DMEM supplemented with 10% FCS, 10 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 50 μM 2-ME. Cell cultures were incubated with indicated peptides overnight either with or without the addition of human β_2-microglobulin (β_2m). Cells for flow cytometric analysis in all studies were incubated with the viability dye ViVid (Molecular Probes) (22). Subsequently, cells were stained with mAb 34-5-8S, which binds a peptide-dependent, but not peptide-specific H-2D^d epitope, or with the conformation-independent anti-H-2D^d mAb 34-2-12S, followed by anti-mouse IgG-PE (Sigma-Aldrich) (23). Stained cells were analyzed using a modified BD LSR II flow cytometer with FlowJo software (Tree Star), and the results are shown as Δ mean fluorescence intensity.

Production and preparation of rBCG, rAd, and plasmid DNA-expressing modified HIV-1 Env

We used a previously characterized vector encoding gp140ΔCFIΔV1V2 and prepared a rBCG vaccine expressing this modified Env gene.

Immunization

BALB/c mice purchased from The Jackson Laboratory were maintained in the Vaccine Research Center Animal Care Unit, National Institute of Allergy and Infectious Diseases, National Institutes of Health, under pathogen-free conditions. The animal studies were approved by the Vaccine Research Center Animal Care and Use Committee and conducted in accordance with all federal and National Institutes of Health policies and regulations. Mice were immunized with 10^8 viral particles of rAd in saline i.m., 50 μg of DNA in saline i.m., or 0.5 mg of rBCG in saline intradermally (supplemental Table S-II).[4]

Flow cytometric analysis of tetramer staining and intracellular cytokine production

PBMC and spleen cells (10^6) were simultaneously and sequentially incubated with PE- and/or allophycocyanin-conjugated H-2D^d tetramers containing human β_2m for 15 min at room temperature; cells were then stained for CD3 (BD Pharmingen), CD8 (BD Pharmingen), CD16/32 (Beckman Coulter), CD44 (BD Pharmingen), CD62L (eBioscience), CD127 (eBioscience), KLRG-1 (Southern Biotechnology Associates), and CCR7 (Biolegend). The following synthetic peptides were used for tetramer production: Env-modified PA9 (IGPGRAFYA), Env-modified PI10 (IGPGRAFYTI), native PT10 (IG PGRAFYTT), native PT9 (IGPGRAFYT), P18I10 (RGPGRAFVTT), and motif control (AGPARAAAL) (National Institute of Allergy and Infectious Diseases Tetramer Core Facility). In some experiments, immune spleen cells were incubated with the peptide (2 μg/ml), anti-CD28 (2 μg/ml; BD Pharmingen), anti-CD49d (2 μg/ml; BD Pharmingen), anti-CD107a (10 μl; BD Biosciences), and anti-CD107b (5 μl; BD Biosciences) for 6 h and stained with H-2D^d/V3 tetramers, as described previously (24). Optimal concentrations of all Abs and tetramers used in this study were determined in pilot titration experiments. Intracellular cytokine production was quantified, as described previously (22).

[4] The online version of this article contains supplemental material.

TCR clonotype analysis

Small, live CD16$^-$/CD19$^-$/CD32$^-$CD8α^+ H-2D^d tetramer-positive spleen cells (10,000 per condition) were sorted to greater than 98% purity using a modified FACS DIVA (BD Biosciences) (25). Unbiased analysis of *TCR* gene expression was conducted, as described previously, using a strand-switch anchored RT-PCR with *TCRA* and *TCRB* C region primers (26). All sequences were analyzed with reference to the international ImMunoGeneTics information system website V-align (http://imgt.cines.fr).

Protein expression, structure determination, and crystallographic refinement

The soluble extracellular segment (aa 1–275) of H-2D^d was expressed in *Escherichia coli* as inclusion bodies, solubilized, and refolded in vitro with similarly expressed murine β_2m and either the PA9 or PI10 peptide, essentially as described previously for the H-2D^d/β_2m/P18I10 complex (27). Crystals were frozen in liquid nitrogen after dipping in paratone oil and examined by synchrotron radiation at beamline X29A at the National Synchrotron Light Source at Brookhaven National Laboratory. Data were collected from single crystals in a nitrogen stream at 100 K, and were indexed, scaled, and merged using HKL2000. The PA9-containing complex crystallized in space group $P2_12_12$ with one complex (H chain, β_2m, and peptide) in the asymmetric unit, and a Matthews coefficient of 2.50. The PI10 complex in space group $P2_12_12_1$ also had one complex per asymmetric unit, and a Matthews coefficient of 3.14. Data collection and refinement statistics are reported in Table S-III. The structures were readily solved by molecular replacement with MOLREP of the CCP4 suite, using the H-2D^d/β_2m complex from 1QO3 from which both peptide and Ly49A had been removed. Refinement was conducted in CNS 1.2, manual fitting of each of the peptides was accomplished with Coot (28), and molecular graphics figures were prepared with PyMOL (http://pymol.sourceforge.net/). The PA9 and PI10 complexes were determined to 2.4 and 2.1 Å, respectively, with corresponding R_{work}/R_{free} of 22.8/27.5 and 21.9/25.1. Coordinates of the refined models and structure factors have been deposited in the protein data bank (D^d-PA9, 3E6F and D^d-PI10, 3E6H). Side chain accessibility was calculated with AREAIMOL of the CCP4 suite.

Data analysis and statistics

All comparisons between recombinant and control groups and between immunization groups were conducted using ANOVA tests assuming variances with the JMP program (SAS Institute). Data are expressed as the mean $\pm$ SD.

Results

Identification of variant V3 peptide epitopes elicited by HIV-1_{BaL} Env immunization

Because previous studies of the immune response in BALB/c mice to HIV$_{IIIB}$ Env revealed that a decamer peptide spanning the V3 loop, RGPGRAFVTI (P18I10), was immunodominant for the H-2D^d-restricted CD8$^+$ T cell response (20), we asked whether the HIV$_{BaL}$ Env (29) was cross-reactive, and whether the two responses were of comparable magnitude (Fig. 1*A* and Table S-I). Mice primed with HIV-1_{BaL} expressed in a DNA plasmid vector and boosted with rAd showed a recall response to the pool of overlapping 15-mer peptides representing the entire BaL Env protein and to the specific BaL-derived peptides PT9 and PT10, but did not respond to the P18I10 peptide. Mice immunized with the corresponding HIV-1_{IIIB} vaccine responded to the cognate peptide pool as well as to P18I10, but failed to respond to either PT9 or PT10. However, the recall response (as measured by the percentage of CD8$^+$ T cells producing intracellular IFN-γ) to either native PT9 or PT10 in HIV-1_{BaL}-immunized mice was consistently weaker than the response to the P18I10 peptide following HIV-1_{IIIB} immunization (5.6 $\pm$ 2.8% for PT9 and 4.6 $\pm$ 2.5% for PT10 as compared with 15.3 $\pm$ 1.5% for P18I10). Thus, responses to each of the vaccines were specific for the delivered peptide epitopes.

Inspection of the amino acid sequence of the HIV-1_{BaL} V3 loop suggested that processed peptides derived from this region might not bind to the H-2D^d-presenting element to the same degree as the immunodominant peptide derived from the HIV-1_{IIIB} isolate.

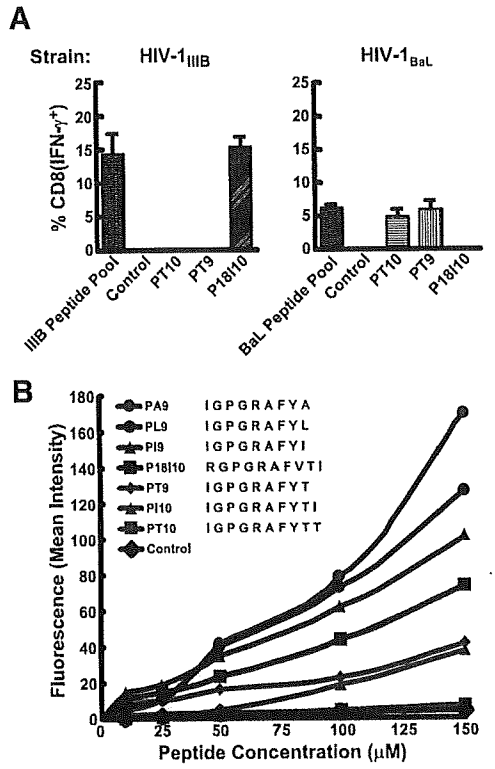

FIGURE 1. $CD8^+$ T cells from HIV Env-immunized mice show specificity for individual virus isolates, exhibit distinct potencies, and recognize variant antigenic peptides that associate differently with H-2D^d. *A*, Mice were primed with DNA encoding either HIV-1_{BaL}- or HIV-1_{IIIB}-modified Env and boosted with rAd expressing either HIV-1_{BaL} or HIV-1_{IIIB} Env, as described in *Materials and Methods* and Table S-II. Splenocytes were harvested 14 days after boosting, stimulated in vitro for 6 h with the V3 epitope peptides (2.5 μM) P18I10 (RGPGRAFVTI), PT10 (IGPGRAFYTT), or PT9 (IGPGRAFYT); pools of overlapping 15-mer peptides spanning HIV-1_{BaL} Env (BaL peptide pool) or HIV-1_{IIIB} Env (IIIB peptide pool); or an irrelevant Ebola Env peptide as a control (29); and then stained for intracellular IFN-γ production, as described previously (22). Functional profiles of $CD8^+$ T cell responses to native and variant peptides in HIV-1_{BaL} Env-vaccinated mice are shown in Fig. S1. *B*, Peptide-induced surface expression of H-2D^d with V3 loop-related peptides. TAP-negative LKD8 cells were incubated with the indicated concentrations of each peptide and stained with mAb 34-5-8, as described in *Materials and Methods*. Control peptide is WKEATTTLLCASDAK. Results are shown as the mean fluorescence intensity over background (Δ mean fluorescence intensity). Red lines indicate peptides used for tetramer construction.

Therefore, we examined whether peptides from this region of the BaL V3 loop would bind to H-2D^d in an epitope stabilization assay. The H-2D^d peptide-binding motif, determined first by analysis of peptides that copurify with H-2D^d (30, 31) and further characterized in x-ray structures of H-2D^d complexed with the IIIB-derived peptide P18I10 (27), consists of G at position 2, P at position 3, R at position 5, and a C-terminal hydrophobic residue at position 9, 10, or 11. Because H-2D^d is known to bind well to both nonamer and decamer peptides, and because the C-terminal anchor residue strongly influences peptide binding, we evaluated a set of synthetic 9-mer and 10-mer peptide variants for their ability to bind to H-2D^d (Fig. 1*B*). Using LKD8, a TAP-deficient H-2D^d-positive cell line, as an indicator (21), we observed a hierarchy of binding, as follows: PA9 > PL9 > PI9 > P18I10 > PT9 > PI10 > PT10. Thus, several nonamer and decamer variants of the PT9 and PT10 sequences found in the BaL Env immunogen bind H-2D^d with higher apparent affinity than either the native 9-mer or 10-mer. Because PA9 binds H-2D^d better than the putative endogenously generated PT9, and because PI10 binds H-2D^d better than the putative endogenously generated PT10, we expected that tetramers prepared with these variant peptides would have greater stability and would be more effective reagents with which to monitor specific T cells. However, it remained possible that subtle differences in either the proportion of molecules bound by the higher affinity peptides or the conformations of the epitopic residues of these peptides when bound to H-2D^d might influence either the specificity of the T cells elicited or the ability of such T cells to be detected with specific tetramers.

We analyzed the fine specificity of the HIV-1_{BaL} response to PA9, PT9, PT10, and PI10 using intracellular cytokine staining for IFN-γ, IL-2, and TNF-α (Fig. S1). In all vaccine vectors, the amino acid sequence encoded in the functional epitope was IG PGRAFYTT, which includes both PT9 and PT10. The native PT9 and PT10 peptides, which have apparently lower affinities for H-2D^d, elicited no triple cytokine-positive $CD8^+$ T cells in HIV-1_{BaL} Env-immunized mice. However, the higher affinity, anchor-variant peptides, PA9 and PI10, elicited a significant proportion of triple-positive cells (46 and 47%, respectively). All the V3 peptides specifically stimulated immune $CD8^+$ T cells and not $CD4^+$ T cells (data not shown), whereas a pool of HIV-1_{BaL} Env gp120 peptides stimulated both $CD4^+$ and $CD8^+$ T cells.

Diversity of $CD8^+$ T cells reactive with H-2D^d tetramers elicited by different prime-boost combinations

Having established that the two native and two variant peptides could stimulate cytokine production in DNA-primed/rAd-boosted $CD8^+$ T cells, we proceeded to explore the responses elicited by various vaccines and their prime-boost regimens with respect to T cell specificity using H-2D^d tetramers prepared with PA9, PT9, PI10, and PT10. To determine whether these different tetramers reacted with distinct T cell subsets or the same subsets bearing cross-reactive TCRs, double-staining experiments were performed. As controls, vaccine-elicited cells were double stained with the same tetramers labeled with PE or allophycocyanin (Fig. 2*A*). As expected, in each case, most of the positive cells clearly stained simultaneously with both the PE and allophycocyanin tetramers. Other controls, using P18I10 and a motif peptide known to bind H-2D^d, but lacking epitopic side chains (Table S-I), showed no reactivity (Fig. 2*A*, *right two panels*). For some tetramer combinations, D^d-PA9 and D^d-PI10, D^d-PT10 and D^d-PI10, and D^d-PT9 and D^d-PI10, minimal double staining was observed (colored panels in Fig. 2*B*). Furthermore, the differential staining patterns were not affected by performing the staining sequentially in either direction (Fig. 2*C*, paired with matching colored panels in Fig. 2*B*). These results indicate that these MHC-I/peptide complexes were recognized by distinct T cell subsets. In contrast, D^d-PT9/D^d-PA9, D^d-PT10/D^d-PT9, and D^d-PT10/D^d-PA9 double staining revealed that 82, 65, and 6% of the positive cells bound both tetramers, respectively (nonhighlighted panels in Fig. 2*B*). $CD8^+$ T cells from unimmunized mice did not react with any of the tetramers (data not shown). Thus, the Env V3 tetramers were specific for immune $CD8^+$ T cells of mice immunized with HIV-1_{BaL} Env and also could detect distinct populations of immune T cells. Several MHC tetramer pairs reacted with the same T cells, whereas D^d-PI10 tetramers detected a distinct T cell population.

Differential fine specificity of BaL Env V3-specific $CD8^+$ T cells after immunization with rAd alone, priming with DNA, or priming with rBCG

Because of the high degree of cross-reactivity between D^d-PA9 and D^d-PT9 tetramers (Fig. 2*B*), their distinct reactivity from that

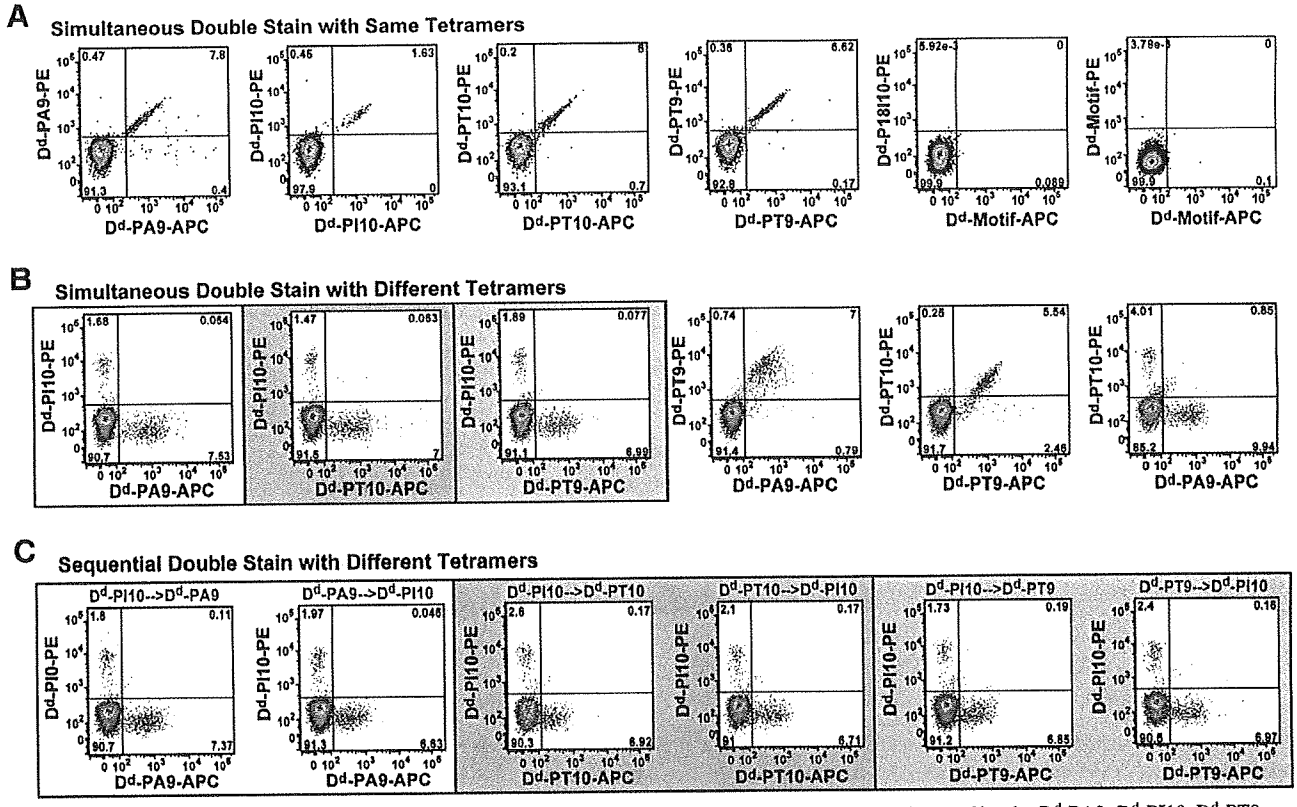

FIGURE 2. Reactivity of H-2D^d/V3 peptide tetramers in immune cell populations showing double-staining profiles for D^d-PA9, D^d-PI10, D^d-PT9, and D^d-PT10 tetramers. Spleen cells from BALB/c mice immunized with rBCG/rAd were analyzed for reactivity with the H-2D^d/peptide tetramers conjugated to PE or allophycocyanin. *A*, Simultaneous double staining of immune spleen cells with each of the four tetramers. D^d-P18I10 and D^d-motif tetramers were used as controls that bear the P18I10 and the binding motif peptide AGPARAAAL, respectively (Table S-I). Percentage of positive cells is indicated in each quadrant. Plots are gated on live CD16$^-$/CD19$^-$/CD32$^-$/CD3$^+$/CD8$^+$ T cells, as described in *Materials and Methods*. *B*, Simultaneous double staining with different tetramers. *C*, Pairs of tetramers highlighted in *B* were also used in sequential double-staining experiments. The differential staining profiles of CD8$^+$ T cells between D^d-PI10 vs D^d-PA9, D^d-PI10 vs D^d-PT10, and D^d-PI10 vs D^d-PT9 were similarly detected in either DNA/rAd or rAd immunizations (data not shown).

of the D^d-PI10 tetramer (Fig. 2, *B* and *C*), and their ability to produce functional responses after immunization with HIV-1_{BaL} vector vaccines (Fig. S1), we further analyzed immune responses with the two tetramers that were not cross-reactive, D^d-PA9 and D^d-PI10. Responses were measured following different immunization schemes with rAd, DNA, and rBCG vectors, either alone or in DNA/rBCG prime-rAd boost combinations (Fig. 3). rAd elicited higher frequency responses than either DNA ($p < 0.001$) or rBCG ($p < 0.0001$) as detected by the percentage of D^d-PA9 tetramer-positive cells (Fig. 3*A*). A similar result was observed, at lower magnitude, with the D^d-PI10 (Fig. 3*B*) and D^d-PT10 tetramers (data not shown).

To study the effects of prime-boost immunization, we next monitored the CD8$^+$ T cell responses after DNA/rAd and rBCG/rAd (Fig. 3*C*). Boosting of DNA- or rBCG-primed mice with rAd (Table S-II) resulted in differential timing of the peak response, as detected with either the D^d-PA9 or D^d-PI10 tetramers. At 4 days after the boost, the D^d-PI10-responsive CD8$^+$ T cell subset in the rBCG/rAd group was preferentially elicited (mean value, 5.8 ± 1.5%; blue lines in *left panel* in Fig. 3*C*), and lower levels were achieved with DNA/rAd or rAd alone without priming (red and green lines in *left panel* in Fig. 3*C*, respectively; both $p < 0.001$). This D^d-PI10 tetramer-binding CD8$^+$ T cell population decreased by day 8 postboost and remained stable until day 90. A switch in the dominance of CD8$^+$ T cell populations from D^d-PI10 to D^d-PA9 specificity in mice immunized with rBCG/rAd was observed 14 days after rAd vector boosting. Although the peak D^d-PI10 response occurred earlier than the peak D^d-PA9 response (day 4 as compared with day 14), the magnitude of the D^d-PA9 response was significantly greater than the maximal D^d-PI10 response (24.6 ± 10.5% as compared with the D^d-PI10 response described above). The rBCG-vector control/rAd group showed results very similar to those for the rAd-alone vector group (data not shown).

The maturation and differentiation status of D^d-PA9 and D^d-PI10-specific CD8$^+$ T cells was compared between DNA/rAd and rBCG/rAd regimens at the peak of the immune response 14 days after rAd boosting (Fig. 4). The majority of the D^d-PA9-specific CD8$^+$ T cells in the spleen showed an effector cell CD127^{low} CD62L^{low}CD44^{high} phenotype in both the DNA/rAd and the rBCG/rAd immunization protocols (57.6 and 73.5% of gated cells, respectively; Fig. 4*A*). The remaining D^d-PA9-specific CD8$^+$ T cells were CD127^{high}CD62L^{low}CD44^{high} effector memory (EM; 33.7 and 16.4% in DNA/rAd and rBCG/rAd, respectively) and CD127^{high}CD62L^{high}CD44^{high} central memory (CM; 0.76 and 3.98% in DNA/rAd and rBCG/rAd, respectively). In contrast, of the D^d-PI10-specific CD8$^+$ T cells analyzed at the same time, the majority were EM (66.7 and 31.6% in DNA/rAd and rBCG/rAd, respectively) and CM (11.6 and 28.9% in DNA/rAd and rBCG/rAd, respectively). Thus, at the peak of the immune response, D^d-PA9-specific CD8$^+$ T cells were substantially skewed toward more differentiated effector phenotypes relative to the contemporaneous D^d-PI10-specific CD8$^+$ T cell populations (Figs. 4*C* and

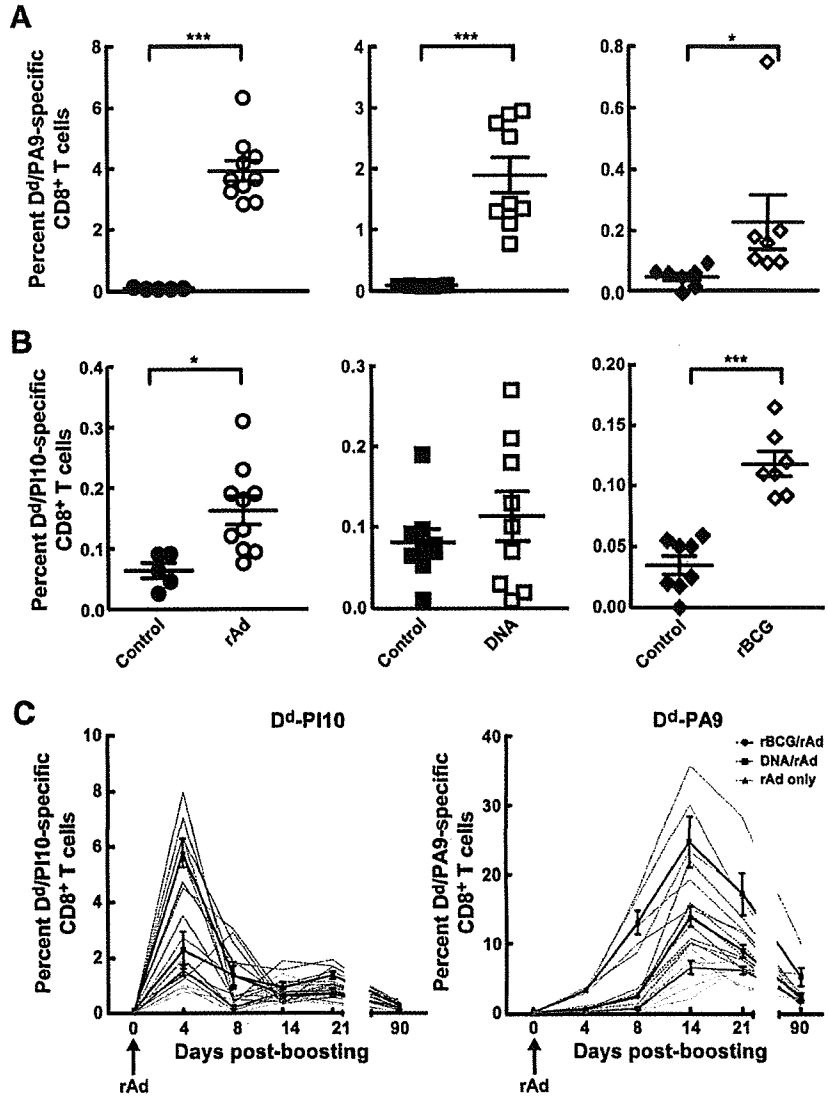

FIGURE 3. H-2D^d/V3 peptide tetramer-positive CD8$^+$ T cell responses in mice immunized with rAd, DNA, rBCG, DNA/rAd, or rBCG/rAd. *A*, Mice were immunized once with rAd (*left*), three times with DNA (*middle*), or once with rBCG (*right*), as described in Table S-II, and Ag-specific CD8$^+$ T cells were detected with D^d-PA9 (*A*) and D^d-PI10 (*B*) tetramers. Control refers to immunization with vector alone. Response patterns similar to those observed with D^d-PA9 were also observed with the D^d-PT10 tetramer (data not shown). *C*, After DNA priming (Table S-II), animals were boosted with rAd (DNA/rAd, red dotted lines). Animals were also immunized with rBCG/rAd (blue dotted lines) or rAd without priming (green dotted lines), as shown in Table S-II, and analyzed for the generation of CD8$^+$ T cells specific for D^d-PI10 (*left panel*) and D^d-PA9 (*right panel*). Dotted lines show data for each animal, and solid lines show mean values of five animals in each group. D^d-PT10 responses were lower, but showed a similar pattern to those specific for D^d-PA9 (data not shown).

S2), indicating clear differences between the two distinct CD8$^+$ T cell subsets according to MHC-I/peptide specificity.

Analysis of TCR *gene expression in Env V3-specific CD8$^+$ T cell populations*

To characterize the *TCR* gene usage of these tetramer-positive cells at 14 days postboost, we analyzed *TCRA* and *TCRB* gene expression at the clonotypic level. In rBCG/rAd-immunized mice, the tetramer-positive populations were clonotypically distinct (Fig. 5). Remarkably, D^d-PA9-specific CD8$^+$ T cells sorted from two different mice immunized with rBCG/rAd contained dominant *TCRB* sequences that were identical at the nucleotide level, representing 100 and 80% of the sequences; the corresponding *TCRA* sequences were distinct. In one mouse, the *TCRA* sequences were very restricted, whereas in the other five different sequences were observed. In contrast, the CD8$^+$ T cell population specific for D^d-PI10 was more diverse, although the distinct tetramer-positive CD8$^+$ T cells exhibited similar effector potential, as determined by CD107 expression (data not shown). Thus, D^d-PA9-specific and D^d-PI10-specific CD8$^+$ T cells, in addition to representing discrete tetramer-staining populations, also exhibit distinct TCR usage.

MHC-peptide structures suggest a basis for recognition by different T cell populations

To gain further insight into the nature of the MHC-I/peptide epitopes that constituted these different H-2D^d/peptide tetramers, we determined the high resolution x-ray crystal structures of H-2D^d complexed with PA9 and with PI10, and compared these with the previously published structure of H-2D^d bound to the related HIV-1_{IIIB} envelope peptide P18I10 (Figs. 6 and S3). Details of the structure determination and crystallographic refinement are provided in *Materials and Methods* and in Table S-II. The structures of PA9 and PI10, each complexed with H-2D^d and murine β_2m, were determined to a resolution of 2.4 and 2.1 Å, respectively. P18I10 bound to H-2D^d has been structurally characterized as the trimeric H chain/β_2m/peptide complex (27) and also with the same peptide in complex with the murine NK cell receptor Ly49A (32). We compared the two newly determined D^d-PA9 and D^d-PI10 structures with D^d-P18I10. The comparisons are focused on the $\alpha1\alpha2$ domain and bound peptide to illustrate the conformational differences of the three different bound peptides. For all three structures, the N-terminal five residues of the peptides superpose precisely (root mean square deviation of 0.053 to 0.127 Å for the three pairwise superpositions), but there is considerable