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Dominance and Diversity in the Primary Human CD4 T Cell Response to Replication-Competent Vaccinia Virus¹

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Vaccination with replication-competent vaccinia protects against heterologous Orthopoxvirus challenge. CD4 T cells have essential roles helping functionally important Ab and CD8 antiviral responses, and contribute to the durability of vaccinia-specific memory. Little is known about the specificity, diversity, or dominance hierarchy of Orthopoxvirus-specific CD4 T cell responses. We interrogated vaccinia-reactive CD4 in vitro T cell lines with vaccinia protein fragments expressed from an unbiased genomic library, and also with a panel of membrane proteins. CD4 T cells from three primary vaccinees reacted with 44 separate antigenic regions in 35 vaccinia proteins, recognizing 8 to 20 proteins per person. The integrated responses to the Ags that we defined accounted for 49 to 81% of the CD4 reactivity to whole vaccinia Ag. Individual dominant Ags drove up to 30% of the total response. The gene F11L-encoded protein was immunodominant in two of three subjects and is fragmented in a replication-incompetent vaccine candidate. The presence of protein in virions was strongly associated with CD4 antigenicity. These findings are consistent with models in which exogenous Ag drives CD4 immunodominance, and provides tools to investigate the relationship between Ab and CD4 T cell specificity for complex pathogens. The Journal of Immunology, 2007, 178: 6374–6386.

Vaccinia viruses confer long-term protection from variola. Orthopoxviruses are being used to deliver heterologous Ags in the settings of vaccines and immunotherapy. CD4 T cells likely are important in primary clearance of vaccinia and in the induction and maintenance of long-term memory and protection from variola challenge. We conducted proteome-wide screens of the CD4 response shortly after primary immunization.

CD4 T cells help generate functionally important CD8 responses (1) through licensing of DC via CD40L (2). Helper functions include the priming of naive CD8 responses, the transition of primary CD8 cells into memory, the survival of CD8 memory cells, and the generation of secondary CD8 responses in specific models (3). CD4 help for CD8s is likely needed at one or more points for most viruses (4). This is especially true if viral immune evasions dampen innate signals that can mature DC, as does vaccinia (5–7).

In vivo, CD4-depleted, CD4CCR−/−, and MHC II−/− mice show delayed viral clearance and increased mortality after vaccinia infection (8, 9). Modified vaccinia Ankara (MVA)3 fails to protect MHC II−/− mice against vaccinia challenge (9). Vaccinia-specific CD8 T cells in MHC II−/− or CD4−/− mice have proliferative and functional protection defects (10–12) and cannot transition into memory cells (4). Ectromelia virus is an orthopoxvirus that infects mice in nature. Although primary CD8 responses to ectromelia virus are normal in CD4-depleted mice, helpless CD8 cells do not transition into memory or reactivate upon challenge. CD4-depleted mice fail to clear EV (13, 14).

CD4 T cells assist Ag-specific Ab synthesis. Orthopoxvirus-specific Abs are necessary and sufficient for vaccine-induced protection from orthopoxvirus challenge (15, 16). CD4-deficient mice do not make specific Abs (8, 9). The cognate T-B help theory (17) predicts that targets of specific Abs should elicit CD4 responses. IFN-γ, TNF-α, and cytotoxicity are additional antiviral functions of human vaccinia-specific CD4 T cells (18–21).

Human data are consistent with important roles for CD4 T cells. The estimated half-life for CD4 responses to vaccinia is 8–15 years (18, 22, 23). Among donors in the decay phase of memory responses, persons with intact CD8 responses always had detectable CD4 responses (18, 24). Persons with HIV-related or idiopathic CD4 deficiency may fail to clear poxvirus infection (25).

The specificity of CD4 responses to vaccinia is largely unknown. To probe the entire proteome in an unbiased fashion, we used a genomic DNA library based on a replication-competent vaccinia strain. We report that the CD4 response is very diverse, targeting up to 20 proteins per subject, and can be placed in a unique hierarchy for each person including immunodominant responses. The response emphasizes virion structural proteins and enzymes involved in viral replication. Detection of CD4 responses is highly correlated with the presence of the protein in purified virion preparations. These data are

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Abbreviations used in this paper: MVA, modified vaccinia Ankara; ICC, intracellular cytokine cytometry; WR, Western Reserve; TCM, T cell medium; ORF, open reading frame.
consistent with models of CD4 Ag presentation in which processing of extracellular Ags contributes to immunodominance.

Materials and Methods

Subjects and specimens

Adults (Table I) receiving scarification with Dryvax smallpox vaccine for occupational health care received Institutional Review Board-approved consent. Subjects 3 and 9 (subjects 1–8 correspond to our previous report (26)) had been vaccinated 35 years before whereas subjects 2, 7, and 10 were primary vaccinates. For blood Ag discovery was drawn 4–6 wk after vaccination. PBMC were cryopreserved after Ficoll enrichment. HLA typing used DNA-based methods.

Cell and viral culture

PBMC were seeded at 10^5/ml in 2 ml of T cell medium (TCM) (27) in 24-well plates. Live or UV-inactivated vaccinia was added at multiplicity of infection (MOI) 10. IL-2 (Hemagen) was started on day 5 (32–50 U/ml). Vaccinia strain NYCBH was raised and titered in BSC-40 cells (26). BSC-40 used to grow vaccinia stocks (5.4 × 10^5 TCID50/well) for informative, nonambiguous HLA restriction patterns (27, 31). Untouched CD4^+ T cells were enriched from some cultures by negative selection (Miltenyi Biotech). Screening assays were performed (see below) and cultures with activity were expanded, after enrichment of CD4^+ T cells in some cases, one cycle using anti-CD3 mAb, IL-2, and feeder cells (28). Resultant second generation bulk cultures were frozen in aliquots. For some subjects, cells in first-generation bulk cultures were cloned with phytohemagglutinin-P (RemeIl) and IL-2 (27). Cloning was done by limiting dilution, with cultures assigned as clonal coming from dilutions with <37% of cloning wells positive for growth by visual inspection, giving a >95% chance of clonality per the Poisson distribution (29). Clones were screened (day 14) by proliferation and IFN-γ assays (below). Positive clones were expanded with anti-CD3 and IL-2 (28). EBV-transformed B cells (LCL) were derived from PBMC (30). LCL 0110897 (DRB1 1401,1101;DRB3^+ ) was prepared in-house. H.W. 09123/HAYBD is HLA-DRB1 1407,1501;DRB3^+;DRB5^+. Vaccinia strain NYCBH was raised and titered in BSC-40 cells (26). BSC-40 used to grow vaccinia stocks (5.4 × 10^5 PFU/ml before inactivation) for functional assays were Mycoplasma negative (Cambrex). UV inactivation (27) eliminated detectable infectious virus.

Lymphocyte functional assays

Triplicate PBMC proliferation assays used 1 × 10^5 responders in 200 μl of TCM in 96-well U-bottoms and 5-day [3H]thymidine proliferation (27). For cultured responders, 3 × 10^5 – 10^6 cloned responder cells in triplicate to an HLA-DR locus. Assignment of restricting HLA class II loci used mAb against framework determinants in HLA-DR, HLA-DP, or HLA-DQ at 1/10 dilutions of culture supernatants (27). At least 40% inhibition of proliferative or IFN-γ-IgG readouts, and a consistent pattern in duplicate tests of each mAb, was required to assign restriction to an HLA locus. Assignment of restricting HLA alleles used allelogenic PBMC as APC that were partially allele-matched to the T cell donor. Results were analyzed for informative, nonambiguous HLA restriction patterns (27, 31).

PBMC and T cell replication was also tested by CFSE dilution (32). Cells were stained with anti-CD4-PE (BD Biosciences) on day 3 and 5 × 10^5 cells in the lymphoblast forward/side scatter gate were analyzed. For PBMC, IFN-γ responses were measured using 3 × 10^5 PBMC incubated with UV-mock or UV-vaccinia (1/1000) in 1 ml of TCM with anti-CD28 and anti-CD94 (BD Biosciences) (33). Brefeldin A (Sigma-Aldrich) was added after 1 h. After 6 h, cells were stained with anti-CD4-PE-cyanin 5 or 5 μl of PMA and ionomycin (34) as positive controls. IFN-γ was measured in culture supernatants by ELISA (28) on day 5 for PBMC or day 3 for T cell lines and clones.

Cytotoxicity was tested in duplicate or triplicate 4-h 51Cr-release assays with autologous mock- and vaccinia-infected (MOI 10, 18 h) LCL at 2 × 10^3/10^4 wells as targets (30) and 20 effectors/target. Percentage of specific release was calculated (30). Peptide binding to recombinant HLA molecules was performed as previously described (35).

Flow cytometry

Bulk and cloned cultures were stained for TCRαβ, CD4, and CD8 as described (26). PE-labeled tetrameric complexes of HLA-DRB4^*0101, HLA-DR4^*0101, and vaccinia peptides were prepared as described (36). Cloned T cells or PBMC were stained with 1 μl of tetramer for 60 min at room temperature in 50 μl of TCM, followed by addition of anti-CD4- FITC (Caltag), washes, and fixation. ICC and tetramer data are reported as the percentage of CD4^+ lymphocytes that stain positive. When CFSE was used in ICC protocols, we used CFSE fluorescence to exclude the CD4^+ cells in the APC population and restricted analysis to the responder cells. Data collected on FACScan (BD Biosciences) were analyzed with WinMDI 2.8 (http://facs.scripps.edu/software.html) or FlowJo 8 (TreeStar).

Vaccinia genomic library

Blunt-ended, 200–500 bp vaccinia NYCBH DNA fragments were purified as described (26) and ligated to linkers (GAGGGTTCCGACAGC) with 5’ GA overhang and blunt 3’ end. Unincorporated linkers were removed. pBAD/Myc-His/6xZ was Xhol-digested, filled in with dTTP and dCTP and gel-purified, yielding complementary overhangs. After ligation and DNA purification, libraries were created by electroporation (BTX) of E. coli DH10B (Invitrogen Life Technologies). Bacteria rinsed from ten primary 150-mm ampicillin plates were frozen in aliquots. The primary library contained ~3 × 10^5 unique colonies. Inserts in random colonies were sequenced (primers on request) with BigDye 3.0 (ABI). Vaccinia open reading frames (ORFs) are named from strain Copenhagen or Western Reserve (WR) (37). 96-well deep-dish plates (n = 3) were seeded at 30 colonies/well for the working library. Overnight cultures were frozen in aliquots.

To create β-galactosidase-vaccinia fusion protein pools, bacteria in 96-well ampicillin plates were induced with arabinose (Sigma-Aldrich) (0.2%) for 6 h after OD600 reached 0.4. In a matrix strategy, bacteria from each row and column were pooled (20 pools/plate of 240–360 bacterial variants/pool). β-Galactosidase-vaccinia fusion protein inclusions were enriched (31) and used at 1:100 in proliferation assays. In an alternative strategy, E. coli from each well (n = 288) were heat-killed (56°C, 60 min) and washed once with PBS-10 mM EDTA and twice with PBS. These were screened at 1:100–1:1000. The negative control was pBAD/Myc-His/6xZ, expressing β-galactosidase. For the inclusion body/matrix method, library “hits”

| Table I. Subjects, vaccination status, PBMC timing, and HLA class II genotypes |
|-----------------|-----------------|-----------------|
| Subjecta | Vaccination | Time (wk) | HLA Class IIb |
| 2 | Primary 6 | DRB1^*0701;DRB4^+;DBB1^*0202 |
| 3 | Revaccination 2 | DRB1 15xx/03xx;DRB3^+;DBB5^+;DBB1^*06xx;02xx |
| 7 | Primary 4 | DRB1 1403,1407;DRB3^+;DBB1 0301,0503 |
| 9 | Revaccination 4 | DRB1 1501,0401,0433;DRB4^+;DRB5^+;DBB1 0301,0602 |
| 10 | Primary 4 | DRB1 0405,1405,1445;DRB3^+;DRB4^+;DBB1 0401,0503 |

* Subjects 2, 3, and 7 were previously identified (26).
* Backslashes, allelic variants not discriminated by typing methods; (+), presence of a functional gene at DRB3, 4, or 5 loci; xx, alleles typed to low definition.
were decoded using bacteria in wells at the intersections of positive row and column pools. For either pathway, bacteria from positive pool wells were plated on ampicillin plates. Bacterial colonies (96) were picked to microtiter plates, expanded, arabinose-induced, heat-killed, and washed. Proliferation assays used 10^5–10^6 bacteria/well. Bacterial colonies stimulating H11022/1000 cpm [3H]thymidine proliferation in each of two duplicates, and with a stimulation index H11022/2.5 compared with empty vector, were considered positive. For ICC, 5 ml of transformed E. coli were arabinose-induced. Inclusion bodies resuspended in 200 l of PBS were tested at 1:1000. Bacterial colonies stimulating H11022/0.1% of CD4 H11001 cells above background were considered positive.

Truncation analyses and confirmation of antigenic ORFs
Sequences of library hits typically allowed preliminary identification of antigenic regions (see Results). In some instances full-length vaccinia ORFs were cloned and expressed for confirmation. To reduce the size of some candidate regions, targeted truncations were made. Vaccinia NYCBH DNA was amplified with PCR primers containing HindIII and XhoI restriction sites (primers on request) using Pfu polymerase (Stratagene). PCR products were ligated into digested pBAD/Myc-His/lacZ and candidate clones validated by sequencing. E. coli transformed with truncations were induced to express fusion β-galactosidase-vaccinia fusions.

Ags and mitogens
UV-vaccinia stimulated maximal [3H]thymidine proliferation by PBMC from recent vaccinees at 1:1000 in preliminary assays and was used at this dilution. Vaccinia strain WR proteins L1R aa 1–185, A33R aa 58–185, B5R aa 20–275, and full-length A27L were expressed in baculovirus as polyhistidine fusions (38, 39). Proteins purified by metal chromatography were used at 2 μg/ml. Synthetic peptides (CPC or Mimotopes) were dissolved (10 mg/ml) in DMSO. PHA-P was used at 1.6 μg/ml and anti-CD3/CD28 beads (Invitrogen Life Technologies) at 10 beads/cell.

Statistics
The proportions of vaccinia ORFs that are CD4 Ags and that are structural virion components were compared in a 2 × 2 table using Fisher’s exact test, two-tailed (Instat 3.06; GraphPad).

Results
CD4 responses to vaccinia in PBMC
Vaccinia infection causes acquisition of virus-specific CD4 responses, assessed by both [3H]thymidine incorporation and CFSE dilution. The vaccinees also acquired CD4+ lymphocytes that are IFN-γ.

FIGURE 1. CD4 responses to human vaccinia NYCBH infection in PBMC. Top, Net proliferative responses to UV-vaccinia or anti-CD3/CD28 beads before and after vaccination. Data are mean ± SD of 8 reported (26) subjects. Middle, CFSE dilution after PBMC stimulation with UV-vaccinia, before and after primary vaccination. Bottom, IFN-γ accumulation by PBMC before and after primary vaccination. Data are the proportion of CD4+ lymphocytes that are IFN-γ.

FIGURE 2. Vaccinia-specific CD4 clones. Top, Representative clones (n = 36) from subject 3 made by nonspecific cloning of PBMC stimulated one cycle with live vaccinia. Data are stimulation indices (27) and raw OD450 IFN-γ ELISA values. Middle, HLA restriction of clones from subject 10 used for determination of fine specificity. Autologous PBMC were used as APC with anti-HLA class II mAbs. Readouts are (top) mean of triplicate net [3H]thymidine incorporation, and mean of triplicate IFN-γ in 72 h supernatants (OD450). Bottom, HLA restriction of clones 10.80 and 10.91 using autologous or partially HLA-DR-matched PBMC as APC.
IFN-γ upon Ag stimulation (Fig. 1), with integrated frequencies of vaccinia-specific cells on the order of 1% at 2–4 wk after primary vaccination. We therefore enriched vaccinia-reactive cells before probing them with fragments of the vaccinia proteome. PBMC were cultured one cycle with live- or UV-treated vaccinia strain NYCBH. Either treatment led to medium acidification and increases in cell number over 10–14 days, with support from exogenous IL-2. The outgrowing cells were mostly TCRβ. The proportions of CD4 and CD8 single-positive cells varied between subjects (not shown).

Vaccinia-specific CD4 clones

We used CD4 T cell clones for initial rounds of expression cloning-based Ag discovery. Vaccinia-specific CD4 clones could be derived from bulk cell lines made by live or UV-vaccinia re-stimulation of PBMC, with or without CD4 selection before cloning (Figs. 2 and 3). Of note, some CD4+, TCRαβ+ clones proliferated, but did not make IFN-γ in response to vaccinia Ag (Fig. 2). Clones 10.80 and 10.91 from subject 10 (Table I) were chosen for antigenic specificity analyses. Both were restricted by HLA-DR, and proliferated in response to vaccinia plus allogeneic APC that were matched to subject 10 at HLA-DRB1*0405 (Fig. 2). Vaccinia-specific clones typically displayed selective cytotoxicity toward autologous, vaccinia-infected LCL, but not toward uninfected LCL. Alloreactivity toward partially HLA-DR-matched LCL was noted for a clone from subject 7 (Fig. 3). This clone was DR-restricted based on mAb inhibition (not shown). Subject 09123 was matched with subject 7 at DRB1*1407, whereas subject 10897

FIGURE 3. Alloreactivity of CD4+ clone 39 from subject 7. Left, Cytotoxicity toward infected or uninfected LCL at an E:T ratio of 20 in triplicate 51Cr release. Right, Proliferative responses to infected, irradiated, washed LCL. Data are mean ± SD [3H]thymidine incorporation.

FIGURE 4. Fine specificity of vaccinia-specific CD4+ clones 10.80 and 10.91. Top, Clone 10.80. Left are mean of duplicate proliferative responses to killed E. coli expressing the indicated vaccinia protein fragment fusions to β-galactosidase, or UV-mock or UV-whole vaccinia controls. Right are reactivity of clone 10.80 (upper) or bulk vaccinia-reactive T cells (middle) to controls or vaccinia peptides (10 μM) using IFN-γ ICC readout. Bottom, Clone 10.91. At left is proliferation as for clone 10.80. At right is IFN-γ reactivity to E. coli expressing defined fragments of ORF D13L. Numbers are percentage of CD4+ cells expressing IFN-γ.
expressed DRB1*1401, a near match for DRB1*1407. Neither expressed DRB1*1403, a rare allele which is divergent from *1401 and *1407 at the amino acid level (40). Neither allogeneic cell line consistently presented vaccinia. Proliferation and CTL assays showed clear-cut reactivity with the allogeneic cell line 09123 in the absence of vaccinia.
Vaccinia Ags recognized by CD4 T cell clones

To determine fine specificity, we adapted the inducible prokaryotic protein expression strategy from previous work on HSV-2 (31, 41). Vaccinia virus and HSV-2 both have large DNA genomes (42). Vaccinia-fragment fusion proteins were expressed that form insoluble inclusions. These remain antigenic within killed bacteria, or after enrichment by lysis, when PBMC are used as APC (31). Overall, 3 × 10^4 independent colonies were obtained in the primary library. Library quality was checked by sequencing insert DNA in 36 random clones. Single vaccinia inserts, 305 ± 206 bp long (mean ± SD), were present in 72% of clones. The remaining clones were empty (14%), or contained a primate DNA sequence, likely from BSC-40 cells (11.1%). One clone (0.3%) contained a Mycoplasma insert followed by a vaccinia fragment ligated end-to-end. The primary library screen used 288 library pools with a complexity of 30 independent transformed E. coli clones per pool. Considering library purity, fragment length, and the vaccinia genome size (191,738 bp for strain Copenhagen; see Ref. 37), the genome was over-sampled 9.9 times.

To determine the specificity of CD4^+ clones, a combinatorial library screen of row/column matrices of pooled inclusion body preparations was performed. Single bacteria from pools at the

FIGURE 5. Examples of the deduction of the specificity of vaccinia-reactive CD4 T cells from subject 2. See text for details. Each shows, at left, mean of duplicate proliferative responses of bulk T cells to E. coli expressing the indicated vaccinia protein fragments fused to β-galactosidase. Background response to E. coli transformed with empty pBAD/Myc-His/β-lacZ was <1000 cpm. At right, bulk responders were stimulated with peptides and stained for IFN-γ. Other peptides tested, stimulated less than 0.4% of CD4^+ cells. For E4L, all library hits (left) were in-frame fusions with β-galactosidase. For H3L, each library hit was an out-of-frame fusion and the indicated starting amino acid numbers are the first internal methionine residues. For H2R (third from top), the library hit is the upper bar (aa 63–163). Full-length H2R is below, followed by the indicated truncation constructs. For A7L, two library hits are shown at top. Constructs expressing overlapping regions were negative (third and fourth bars). Peptides in the C-terminal nonoverlap region were positive (right, see text).
intersections of positive rows/columns were assayed as arabinose-induced, heat-killed E. coli. Single active bacterial clones were identified and their DNA was sequenced. For T cell clone 10.80, sequencing revealed a single insert predicted to encode aa 103–192 of ORF A48R. However, the fusion polypeptide was predicted to be out of frame with predicted vaccinia Copenhagen ORF. The first predicted methionine codon in the insert was aa 160. We compared full-length A48R, the original library hit (103-M160-192), and PCR-generated truncations. Proliferative responses confirmed reactivity with ORF A48R and mapped an epitope to aa 160–192 (Fig. 4, top). A48R peptides 160–174 and 166–180 were strongly positive by IFN-γ ICC assay, assigning the epitope to aa 166–174, IHWQIISSE (Fig. 4, top). Other peptides in the 160–192 region were negative (not shown). Bulk vaccinia-specific T cells from subject 10 also showed CD4 reactivity to both peptides. For T cell clone 10.91, the positive library hit contained, in-frame with β-galactosidase, DNA predicted to encode aa 288–383 of vaccinia ORF D13L. Truncation analysis using heat-killed E. coli as Ag mapped the epitope to aa acids 318–348 (Fig. 4, bottom).

High throughput epitope discovery

We previously adapted CD8 expression cloning from virus-specific CD8 T cell clones to polyclonal T cell responders (26, 28), saving resources and time. One cycle of vaccinia stimulation enriches CD4+ IFN-γ responses to whole virus Ag by 20- to 30-fold (Figs. 1 and 4), yielding polyclonal lines that are sufficiently reactive for Ag discovery. In this format, we use easily prepared heat-killed E. coli from library pool wells (complexity, 30/well) rather than inclusion bodies. Every positive well (stimulation index >4 compared with empty vector) was submitted for reduction to positive single bacterial colonies. Sequencing the inserts revealed a plethora of diverse vaccinia DNA fragments. The “hits” were assembled into overlapping regions and subjected to further analyses to derive candidate antigenic regions, as summarized in Table II. Examples of deductive processes are outlined below for subject 2.

Overall, we detected 22 unique antigenic regions for subject 2, 12 for subject 7, and 23 for subject 10 using this strategy. Some regions were antigenic for more than one subject (Table II), for a total of 43 unique antigenic zones in 34 separate ORFs uncovered with the library strategy. Assignment of the probable antigenic ORF and region from the sequence(s) of library hit(s) was straightforward in some cases, but less so in others (Fig. 5). For vaccinia ORF E4L, bulk T cells reacted with independent library fragments containing aa 111–244, aa 173–237, and aa 209–259, respectively, each expressed as in-frame fusions with ORF H3L, 5 unique library hits were each expressed as out-of-frame fusion fragments with β-galactosidase. The shortest overlapping region was aa 209–237. Peptide 215–229 (NKEITEILPDNNPSP) was positive (Fig. 5, top row), with 0.91% of CD4 T cells accumulating IFN-γ compared with 23.4% for whole UV vaccinia Ag, whereas overlapping/adjointing peptides from this region were negative (<0.1%, not shown). For vaccinia ORF H3L, 5 unique library hits were each expressed as out-of-frame fusion fragments with β-galactosidase. Four shared a common internal methionine codon at residue 223 (197–276, 209–267, 199–271, and 184–263), while in the fifth (123–293) the first internal methionine was at residue 144. The predicted in-frame overlap, assuming initiation at an internal methionine, is aa 223–267. Peptides 223–237 and 229–241 both stimulated IFN-γ responses, localizing an epitope to aa 229–237 (ILDNAAKYV) of H3L (Fig. 5, second row).

Some predicted antigenic regions were long and were therefore truncated before peptide synthesis. For example, a library hit encoded aa 44–163 of vaccinia ORF H2R as an out-of-frame fusion with β-galactosidase, with a first internal methionine codon at aa 63. Truncations narrowed the original candidate region, 63–163, to aa 137–163. Peptide 149–163 (QYLIKHKSNVITCG) was positive in IFN-γ ICC (Fig. 5, third row); neighboring peptides were negative (not shown). One complexity is illustrated for ORF A7L. Fragments encoding aa 44–297 (as an in-frame fusion with β-galactosidase) and aa 243–310 (out of frame, internal methionine at aa 251) led us to detailed study of the predicted overlap region of aa 251–297. However, PCR-generated fragments of this region were negative (Fig. 5, bottom). We then made peptides covering aa 289–310, within the library hit 44–297 but extending C terminus

FIGURE 6. Hierarchy of CD4 responses to vaccinia antigenic fragments among bulk vaccinia-reactive T cells. The readout throughout was IFN-γ accumulation among gated CD4+ responder cells. The total response was evaluated by stimulating bulk T cell with UV-killed vaccinia at 1:100 and subtracting the value for UV mock Ag (<1.0% for each subject). The response for each indicated library fragment was obtained by stimulating bulk T cells with an inclusion body preparation (1:1000) and subtracting the value for inclusion bodies of β-galactosidase derived from E. coli transformed with empty vector (<0.5% for each subject). Responses were normalized by considering the whole vaccinia response to be 100%. Insets show responses to whole vaccinia and most stimulatory fragment for each subject. Data are percentage of CD4+ responders that are IFN-γ."
to the 251–310 overlap. Peptides 289–303 and 295–310 were positive, locating an epitope to aa 295–303 (ETSFIFIET). Likely, there is a separate epitope within aa 44–251, which is currently under investigation. The epitope region assignments based on shortest overlapping region analyses without peptide confirmation should be considered provisional, but antigenic ORFs are still considered to be accurately assigned by our data.

Similar pathways defined two more peptide epitopes. The polyclonal line from subject 2 detected a library hit encoding aa 237–412 of ORF A3L. Truncation analysis narrowed an antigenic region to aa 296–355. Peptide 314–328 (MNIRMGFYCNDDDA) was positive in IFN-γ ICC with subject 2 responders. Also for subject 2, overlapping in-frame hits encoding aa 227–298 and 232–282 of ORF C10L were reactive. Peptide 258–272 (DDNR LI1R 118–137 by HLA DR. Boxed overlap) was HLA-DQ restricted at the level of proliferative responses to L1R 127–137 by HLA DR. Boxed inset at right: bulk T cells from subject 2 show staining of CD4+ cells with tetramer of HLA DRA1*0101/ DRB1*0401 complexed to L1R 127–137, whereas control PBMC do not.

Within-subject hierarchies of CD4 T cell responses to vaccinia Ags

The CD8 response to vaccinia (43, 44) and other large genome viruses (45, 46) show high degrees of immunodominance in mice, with single responses to single epitopes accounting for a large proportion of total responses. Less is known about humans or about immunodominance in CD4 responses. To investigate this topic, we isolated inclusion bodies from E. coli clones containing the vaccinia ORF fragments identified (above) as antigenic. Polyclonal T cells responder cells from three primary vaccinees were each probed with the set of vaccinia ORF fragments matched to that subject by expression cloning. The total response was defined with whole UV vaccinia, used at a high concentration (1:100, corresponding to 36 PFU/cell before inactivation) that gives maximal responses (not shown). Single cell reactivity was enumerated with IFN-γ ICC. CFSE labeling excluded the autologous PBMC used as APC from analysis.

Each subject shows a clear hierarchy of CD4 reactivity (Fig. 6). For subjects 2 and 10, the largest single response accounted for ~13% of the total. Summing the 14 to 19 detectable IFN-γ responses in these subjects, we could account for 49–54% of the overall reactivity. Subject 7 had fewer Ags eliciting IFN-γ reactivity. A single fragment of ORF F11L accounted for 30% of the whole virus response, and overall we could reconstruct 81% of the total response. ORFs F11L and H3L were among the
top three Ags for two subjects, and other ORFs including A3L and L4R were also potent in this assay. Some vaccinia fragments that were positive in our proliferation readout-based library screen did not stimulate IFN-γ/H9253 responses above baseline. This was especially notable for subject 7, who had 9 antigenic fragments positive for proliferation (Table II), but only six by IFN-γ/H9253 ICC (Fig. 6).

**CD4 epitopes in purified neutralizing Ags**

Protection from heterologous orthopoxvirus challenge is dependent upon Abs (15). Several potent vaccinia neutralizing membrane Ags have been described (38, 39, 47–49). We expressed and purified three proteins (L1R, A33R, B5R) as extracellular domains and one (A27L) as a full-length construct. Using the polyclonal vaccinia-reactive T cell lines discussed above, we detected CD4 T cell reactivity to ORF L1R in subjects 2 (Fig. 7) and 9 (not shown), and to A33R in subject 2 (Fig. 7). Reactivity to L1R, but not A33R was independently detected in the library screen (Table II). The A33R data bring our aggregate CD4 Ag discovery to 44 antigenic regions in 35 vaccinia ORFs. We were able to define peptide epitopes in A33R (aa 160–173, TKTTSDYQDSDVSQ) and L1R (aa 127–137, KIQNVIIDECY) after obtaining preliminary positives with overlapping 20-mers (not shown). The A33R response was HLA-DQ restricted, whereas the L1R response was HLA-DR restricted. The L1R 127–137 peptide sequence (KIQNVIIDECY) did not have characteristics of the index subject’s DRB1 allele (*0701) (40). We made fluorescent tetrameric complexes of HLA-DRB4*0101 (with DRA1*0101) and the peptide. Specific staining of 0.92% of CD4+/H11001 lymphocytes in the polyclonal vaccinia-specific was noted (Fig. 7), similar to IFN-γ/H9253 ICC (Fig. 7). L1R 127–137 also bound with high affinity (162 nM) to recombinant DRB4*0101 in a competition assay (35). Subjects 2 and 9 each had a functional DRB4 gene, reacted with peptide 127–137, and did not share DRB1 alleles or supertypes. Taken together these data suggest restriction by DRB4*0101.

**HLA restriction**

We sought to determine the HLA-restricting loci for polyclonal CD4 T cell responses. Restriction by DR, DP, and DQ loci were each frequently observed. In some cases, the subjects were homozygous at the restricting locus, allowing for assignment of the restricting locus. CTL assays with partially HLA-matched, peptide-pulsed LCLs (31, 50, 51) were also used to assist analyses of HLA restriction (Table II). T cell lines from subjects 7 and 10 each reacted with the same protein fragment of ORF WR206 predicted to encode aa 11–190 for ORF WR206 (Table II). The response was HLA-DQ restricted, whereas the L1R response was HLA-DR restricted. The L1R 127–137 peptide sequence (KIQNVIIDECY) did not have characteristics of the index subject’s DRB1 allele (*0701) (40). We made fluorescent tetrameric complexes of HLA-DRB4*0101 (with DRA1*0101) and the peptide. Specific staining of 0.92% of CD4+/H11001 lymphocytes in the polyclonal vaccinia-specific was noted (Fig. 7), similar to IFN-γ/H9253 ICC (Fig. 7). L1R 127–137 also bound with high affinity (162 nM) to recombinant DRB4*0101 in a competition assay (35). Subjects 2 and 9 each had a functional DRB4 gene, reacted with peptide 127–137, and did not share DRB1 alleles or supertypes. Taken together these data suggest restriction by DRB4*0101.

**CD4 RESPONSE TO VACCINIA IN HUMANS**

**Table III. Simplified summary of kinetic, functional, structural, and antigenic characteristics of vaccinia proteins identified as human CD4 antigens**

<table>
<thead>
<tr>
<th>ORF&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Function&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Functional/Structural Class&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Present in Virions&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Kinetic Class&lt;sup&gt;c,d&lt;/sup&gt;</th>
<th>Human CD8&lt;sup&gt;e&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>A3L</td>
<td>Core</td>
<td>Core</td>
<td>yes</td>
<td>L</td>
<td>Yes</td>
</tr>
<tr>
<td>A7L</td>
<td>Transcription factor</td>
<td>Transcription factor</td>
<td>yes</td>
<td>L</td>
<td>No</td>
</tr>
<tr>
<td>A10L</td>
<td>Core</td>
<td>Core</td>
<td>yes</td>
<td>L</td>
<td>Yes</td>
</tr>
<tr>
<td>A33R</td>
<td>EEV membrane</td>
<td>Membrane</td>
<td>yes</td>
<td>L</td>
<td>Yes</td>
</tr>
<tr>
<td>A34R</td>
<td>EEV membrane</td>
<td>Membrane</td>
<td>yes</td>
<td>L</td>
<td>Yes</td>
</tr>
<tr>
<td>A42R</td>
<td>Profilin-like; movement</td>
<td>Other</td>
<td>yes</td>
<td>L</td>
<td>No</td>
</tr>
<tr>
<td>A48R</td>
<td>Thymidylate kinase</td>
<td>Enzyme</td>
<td>no</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>A50R</td>
<td>DNA ligase</td>
<td>Enzyme</td>
<td>yes</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>B19R</td>
<td>IFN-αβ binding</td>
<td>Immune evasion</td>
<td>no</td>
<td>E</td>
<td>No</td>
</tr>
<tr>
<td>C3L</td>
<td>Complement regulatory</td>
<td>Immune evasion</td>
<td>no</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>C10L</td>
<td>no</td>
<td>E</td>
<td>Yes</td>
<td>E</td>
<td>Yes</td>
</tr>
<tr>
<td>D3R</td>
<td>Nucleotide triphosphatase</td>
<td>Enzyme</td>
<td>no</td>
<td>E, L</td>
<td>Yes</td>
</tr>
<tr>
<td>D8L</td>
<td>IMV membrane</td>
<td>Membrane</td>
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<td>L</td>
<td>No</td>
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<tr>
<td>D13L</td>
<td>Rifampicin target</td>
<td>Assoc. inner IMV</td>
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<td>L</td>
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<tr>
<td>E3L</td>
<td>RNA binding; kinase</td>
<td>Immune evasion, enzyme</td>
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<td>E</td>
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<td>E4L</td>
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<td>L</td>
<td>No</td>
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<tr>
<td>F11L</td>
<td>RhoA-binding</td>
<td>Perinuclear virus factory, cell motility</td>
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<td>E</td>
<td>No</td>
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<td>Membrane</td>
<td>yes</td>
<td>L</td>
<td>No</td>
</tr>
<tr>
<td>G3L</td>
<td>Entry/fusion</td>
<td>yes</td>
<td>L</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>G7L</td>
<td>Core</td>
<td>Core</td>
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<td>L</td>
<td>Yes</td>
</tr>
<tr>
<td>H2R</td>
<td>Entry fusion</td>
<td>Membrane</td>
<td>yes</td>
<td>L</td>
<td>No</td>
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<tr>
<td>H3L</td>
<td>Membrane</td>
<td>Membrane</td>
<td>yes</td>
<td>L</td>
<td>Yes</td>
</tr>
<tr>
<td>I3L</td>
<td>ssDNA binding</td>
<td>Regulatory</td>
<td>yes</td>
<td>E, I</td>
<td>Yes</td>
</tr>
<tr>
<td>I8R</td>
<td>Helicase</td>
<td>Enzyme</td>
<td>yes</td>
<td>I</td>
<td>No</td>
</tr>
<tr>
<td>J3R</td>
<td>Methyltransferase</td>
<td>Enzyme</td>
<td>yes</td>
<td>E</td>
<td>No</td>
</tr>
<tr>
<td>J6R</td>
<td>DNA polymerase</td>
<td>Enzyme</td>
<td>yes</td>
<td>E, L</td>
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<td>K4L</td>
<td>Phospholipase-like</td>
<td>Enzyme</td>
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<td>Membrane</td>
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<td>L</td>
<td>Yes</td>
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<tr>
<td>L4R</td>
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<td>Core</td>
<td>yes</td>
<td>L</td>
<td>No</td>
</tr>
<tr>
<td>O1L</td>
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<td>No</td>
<td>Yes</td>
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<tr>
<td>WR148&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>WR206</td>
<td>no</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

<sup>a</sup> Blank, No data or literature consensus.

<sup>b</sup> ORF names and amino acids from Copenhagen (GenBank M35027) except for ORFs differentially present in WR (GenBank NC006998) (37). Sequences for three derivatives of Dryvax are also available (GenBank DQ377945, AY313847, and AY313848).

<sup>c</sup> From proteomics reports and reviews (54–56, 64).

<sup>d</sup> E, early; I, intermediate; L, late.

<sup>e</sup> Drives CD8<sup>e</sup> responses in humans and/or HLA class I transgenic mice (26, 53, 58, 62, 73–75, 77).
from subject 10 was HLA-DP restricted, whereas the response from subject 7 was DR restricted. Most likely, this difference is due to recognition of separate epitopes. Similar differences in HLA-restricting loci were also noted when other protein fragments were assayed with T cell lines from separate subjects (not shown), again consistent with the presence of multiple epitopes.

Structural, functional, and replication kinetic features of CD4 Ags

Some data indicate that the CD8 response may be biased to early proteins and transcription factors (26, 46, 52, 53). In contrast, only 1 of the 35 CD4 Ags (A7L) we detected is a transcription factor, and more than half are late proteins (Table III). The CD4 responses emphasized membrane proteins (10 ORFs), enzymes, and core proteins. Immune evasion proteins (IFN-binding, complement regulatory, and double-stranded RNA-binding proteins) were also represented, as were proteins of unknown function. Three recent mass spectroscopy surveys of the proteome of purified vaccinia virions, typically intracellular mature virions, are generally in agreement. Overall, these studies found that 27 (77%) of the 35 CD4 Ags disclosed in this report are present in virions (54–56). A total of 63 to 80 proteins of 218 predicted vaccinia proteins (29 to 37%) were identified in virions. The association between ORF CD4 antigenicity and presence in virions is nonrandom (p < 0.0001, assuming 80 structural proteins). The largest kinetic category (17 of 31 CD4 antigenic proteins with data in the literature) were late vaccinia proteins.

We elected to use the traditional vaccinia strain Copenhagen nomenclature (37), based on restriction endonuclease fragments, for antigenic vaccinia proteins. Sequence recently became available for three clones of NYCBH (GenBank DQ37795, AY313847, AY313848). Our subjects were vaccinated with Dryvax, a product related to NYCBH. We cannot judge which of the sequenced clones is closest to Dryvax. For those ORFs not predicted to be present in Copenhagen, we followed literature precedent and used strain WR nomenclature (44, 54). The three WR ORFs we report as CD4 Ags, WR148, WR149, and WR206, are each present in Copenhagen, we followed literature precedent and used strain WR nomenclature (37), based on restriction endonuclease fragments, for antigenic vaccinia proteins. Sequence recently became available for three clones of NYCBH (GenBank DQ37795, AY313847, AY313848). Our subjects were vaccinated with Dryvax, a product related to NYCBH.

The present studies expand limited data on orthopoxvirus-specific CD4 T cell responses (19). Recently, CD4 reactivity was documented with vaccinia ORFs A27L, L1R, B5R, and A33R, which are each targets of neutralizing Abs (58). Three epitopes were identified in A27L. Vaccinia-immune H2-2 mice have splenocyte proliferative (likely CD4+ T cells) responses to ORFs D8L and A27L (59). We detected responses to D8L. The HLA alleles restricting responses to A27L were not stated (58), so we cannot know if we missed A27L-specific responses in HLA-appropriate subjects.

We studied persons 2–6 wk after primary vaccination. Zaunders et al. (60) tracked PBMC CD4 responses in similar subjects. Activated (CD38+) cells peaked on day 14 at 5–7% of CD4+ cells. Using whole viral Ag, CD4+ cells up-regulating CD134 in response to vaccinia reached similar levels. IFN-γ+ CD4 cells peaked at ~1%, as observed in this study and by others (18, 61). To investigate the difference between assays, Zaunders et al. sought, but did not detect, bystander CD4 T cell activation. It is therefore possible that some vaccinia-specific CD4 cells are IFN-γ negative, and in fact IL-2, CD40L, or TNF-α cells that were IFN-γ were detected. We observed clones with proliferative but not IFN-γ responses (Fig. 1). This implies that the overall CD4 magnitude, Ag dominance hierarchies (Fig. 6), and diversity could vary by T cell readout, an issue we can now address with specific vaccinia Ags and epitopes.

Using in vitro cell lines, we were able to account for 50–80% of IFN-γ CD4 reactivity to whole killed virus. This level of completeness is unusual for T cell studies in humans or mice. In some previous studies of CD8 responses to vaccinia, the summation of peptide-specific responses actually exceeded the response to whole virus, in at least some donors (53, 62, 63). We titrated the whole virus preparation, and used it at the concentration giving maximal CD4 IFN-γ responses (Fig. 6). Although we believe the relative magnitudes and rank orders of the peptide/Ag region-specific responses we measured are relevant in vivo, future direct ex vivo studies will be required to confirm our findings.

It is possible that our secondary in vitro re-stimulation with UV-killed vaccinia produced cell lines biased toward responses to certain proteins, if some vaccinia polypeptides were missing or under-represented in our whole virus preparation. As mentioned, mass spectroscopy studies detected 80 vaccinia gene products in purified virions. We know our preparation, made from and specifically designed to include infected cell material as well as virions, also contained nonstructural proteins. For example, enough A48R protein, a nonstructural protein (54–56), was present to both enrich A48R-specific T cells before cloning, and drive strong proliferation by an A48R-specific CD4 T cell clone (Fig. 3). In addition, responses to several other nonstructural proteins (B19R, C3L, C10L, C14L, F11L, and O1L, Table III) were detected in T cell lines created by PBMC stimulation with our whole virus Ag.

The diversity estimates from our studies are less susceptible to artifacts from in vitro cell expansion and are likely to be minimal estimates. We stopped decoding low-positive library pools when the pool breakdown process failed to yield antigenic single bacterial colonies and omitted borderline responses from our final analyses. For example, single bacterially expressed fragments of H7R and B2R were reproducibly, weakly (Δcpm <2000 cpm) positive in proliferation assays and also low-positive in IFN-γ ICC (<0.3% of CD4 T cells above background) in all replicates. As overlapping genomic hits were not obtained for these regions, we did not assign these ORFs CD4 reactivity.

Each response that we investigated in detail yielded an antigenic peptide. We have prioritized for future work determining the peptide specificity of the responses to F11L and other protein fragments that drove abundant CD4 IFN-γ responses. Combined with CD8 work from our laboratory and that of others, it is now rational to select candidate immunodominant ORFs for complete sets of overlapping peptides for examination of both CD4 and CD8 responses in wider specimen sets.

Viral proteins can be classified by structural (capsid, envelope, etc.), functional (transcription, replication, immune evasion, entry, etc.), and kinetic (early vs late corresponding to before and after genome replication) criteria. Viral proteins usually have multiple functions, complicating simple, yet meaningful classification (Table III). Poxviruses replicate in the cytoplasm and encode unusually complete DNA replication machinery, some of which is performed in virions (64). It is notable that enzymes are well represented among CD4 Ags (Table III). Extracellular enveloped
virions are a special form of virions. At least three extracellular enveloped virion-specific proteins (A34R, A33R, and F13L) are CD4 Ags. 17 of the 35 Ags (54, 64) are late vaccinia protein, with 8 early, 1 early intermediate, 1 intermediate, 3 early late, and 5 without data. Protein expression kinetics are related to structure and function, with most virion components synthesized with after DNA replication (late) (64). The relationship between CD4 antigenicity and late structural gene products diverges from CD8 Ags, among which transcription factors and other early nonstructural proteins appear to be dominant (46).

Immunodominance occurs when T cells reactive with specific epitopes comprise a large numerical proportion of the total number of T cells reactive with a pathogen (65). CD8 responses to the vaccinia, and large-genome herpesviruses, display moderate to extreme immunodominance in inbred mice (43, 44, 66). CD4 immunodominance is noted in the MHC class II-restricted response to LACK in *Leishmania*-infected H-2d mice (67). Several factors influence CD4 immunodominance, including HLA-DM, the susceptibility of epitope-flanking regions to proteolysis, trafficking of proteins to various membranous compartments, especially in the setting of intracellular Ag synthesis, and innate activation of APC (68). MHC allelic variation of course plays a role, but probably less so than for CD8 responses due to the greater promiscuity for peptide binding of MHC class II compared with MHC class I.

The priming of CD4 responses in vivo is thought to occur principally after phagocytosis and processing of Ag by professional APC, rather than as a consequence of endogenous synthesis. In this setting, Ag abundance has been hypothesized to influence the magnitude of CD4 responses (68). In our data set, proteins that are present in purified virion preparations were recognized significantly more often than proteins that are not detected in virions. There was no obvious relationship, however, between immunodominance, as assessed by hierarchies of IFN-γ responder cell numbers (Fig. 6) or the presence of multiple epitopes per ORF (Table II) and protein occurrence in virions (55). A3L, for example, was dominant in subject 2 and contains at least 3 epitopes. In contrast, a fragment of ORF F11L showed within-subject dominance in subjects 7 and 10, but F11L has not been detected in virions.

F11L is generally conserved in orthopoxviruses, but in the candidate vaccine/vector, MVA, the F11L region is split into two potential ORFs. The first is predicted to express a 96 aa long polypeptide that is 100% homologous to vaccinia Copenhagen in the antigenic region we identified (37, 69, 70). It is not known whether this truncated form is transcribed or translated. Full-length F11L (348 aa) is expressed early (71), effects cell motility via RhoA, and is required for productive infection of primate cells (69). MVA F11L expression can be addressed by testing F11L-specific clones with MVA preparations.

H3L was strongly recognized by subjects 2 and 10. This protein induces neutralizing Abs (49, 72). H3L 229–237 (ILDNAAKYV) accounted for 7% of reactivity to whole virus (Fig. 5) in subject 2. Restriction by DBR4*0101 is implied from mAb inhibition and the HLA-DR alleles expressed by these subjects. Direct binding assays and binding of HLA peptide tetramers to reactive cell lines, confirmed binding of an epitope in another neutralizing Ag, L1R to DBR4*0101.

We compared CD4 Ags to those that drive human or murine HLA-transgenic CD8+ responses (26, 53, 58, 62, 73–77). Overall 16 ORFs (A3L, A10L, A33R, A34R, A48R, A50R, B19R, C3L, C10L, D5R, E3L, G7L, H3L, I3L, J6R, and O1L) are both CD4 and CD8 Ags. ORFs A3L and D5R contain multiple CD4 and CD8 epitopes. Within subject 2, we found 15 ORFs with only CD4 responses, 9 with only CD8 responses, and 4 (A3L, A48R, D5R, and E3L) with both (26). In the future, we hope to extend our studies to within-person comparison of Ab and CD4 responses. This issue is of practical interest for subunit vaccine design, and of basic interest with regards to theories of cognate vs nonspecific CD4-B cell help (78).

Diverse methods are used to investigate T cell responses to complex pathogens (46). Complete sets of synthetic peptides can detect both CD4 and CD8 T cell responses (79). A related approach uses peptides that are predicted to tightly bind relevant MHC molecules. A distinct minority of peptides is hits, but clear epitope hierarchies can be observed. The majority of the total virus-specific response can be accounted for in inbred mice (43). The method we use probes vaccinia-specific T cell clones or lines that have responded once to vaccinia in vitro before we probe them. This may add specificity, as T cells can react to many peptide-MHC combinations (80). Enrichment could distort the hierarchy of responses, due to differential re-stimulation or expansion. We use a crude mixture of sonicated infected cell debris as Ag, rather than purified virions or supernatant, to include all viral proteins. Future work will examine dominance hierarchies among unmanipulated responder cells.

For this study, we modified previous HSV-2 CD4 technology (31) to improve throughput. Polyclonal CD4 cell lines gave an adequate signal-to-noise ratio, sparing resources from making T cell clones. We used random viral DNA fragments, derivatized with adaptors, to make one library, rather than using libraries generated in each fusion reading frame. Random fragmentation, in place of restriction enzymes, reduced the chance of bisecting epitopes. Ag preparation was fast and simple by heat-killing *E. coli* containing inclusion bodies of recombinant proteins. APC present in PBMC process dead bacteria. It is possible some vaccinia proteins were poorly expressed in our *E. coli* library. Gao et al. (81) used a different fusion partner and Coomassie blue staining, an insensitive method, and found 16% of 50 full-length vaccinia ORFs were not expressed. One of their nonexpressed ORFs, E4L, was definitely expressed by our library (Tables II and III). Our system expresses fragments rather than full-length vaccinia ORFs. These are less likely to be toxic in *E. coli* due to enzymatic activity, and our oversampling of the proteome gives each epitope several independent opportunities for expression. In addition, we fuse vaccinia fragments with a form of β-galactosidase known to be highly expressed in *E. coli*. T cells are sensitive to low levels of protein (82), but likely no library can be truly complete.

Our work was also assisted by fortuitous translation initiation at internal methionine codons within plasmids. We observed many examples in which fragments of vaccinia genes fused out of frame to lacZ were still antigenic as proven with synthetic peptides or PCR-amplified genes expressed in-frame with β-galactosidase. A related occurrence is initiation, in *E. coli*, at vaccinia AUG start codons. For example, an antigenic *E. coli* colony contained lacZ sequences in fusion with 162 backward bp from the N-terminal region of gene D9R (83). This is predicted to encode 8 aa followed by a stop codon. The insert continues with intergenic DNA and the initial ATG and the next 176 aa of DBL. DBL fragment 1–177 was expressed and found to be antigenic.

The CD4 antigenic regions identified in this report should be useful in comparing the CD4 responses of different products currently under evaluation for smallpox prevention. Most, such as MVA and NYVAC, are not replication competent in human cells, potentially altering the quantity and/or quality of specific immuinity. Many of the Ags and epitopes eliciting CD4 responses are preserved in these alternative vaccines. Defined epitopes may also be useful in comparing poxvirus vectors being studied as backbone to express heterologous Ags, in which a strong anti-insert response is desired and an immunodominating anti-vector response is disadvantageous.
Dryad is a rare complication of vaccinia vaccination with infiltrating leukocytes and no virus in affected hearts (84, 85), consistent with a possible autoimmune component. We detected allo-reactivity (Fig. 3), in which a CD4+ T cell clone recognizes self-HLA plus vaccinia peptide, and also allogeneic cells. This demonstrates that vaccinia-specific T cells can show promiscuous APC recognition, typical of many virus-specific T cells (86, 87). Self-reactive T cells are not entirely eliminated during development (88), and it is possible that CD4 T cells reactive with vaccinia epitopes could also cross-react with self-HLA and peptides derived from cellular proteins.

In summary, live, replication-competent orthopoxvirus infection stimulates a broad CD4 response in humans. Several vaccinia ORFs contain multiple discrete epitopes, and some ORFs elicit both CD4 and CD8 responses in the same subject. The CD4 response appears to emphasize late, structural virion proteins, and some ORFs elicit a broad CD4 response in humans. Several vaccinia proteins as vaccines, filed by their employer, Univ. of Washington-Koelle and Jing are co-inventors on a preliminary patent concerning use of cross-reactivity (Fig. 3), in which a CD4+ T lymphocyte response appears to emphasize late, structural virion proteins, and also allogeneic cells.

Acknowledgment
Dr. Elaine Jong assisted with recruitment.

Disclosures
Koelle and Jing are co-inventors on a preliminary patent concerning use of vaccinia proteins as vaccines, filed by their employer, Univ. of Washington-Koelle. No final application has been filed and no royalty payments or licensing activity has occurred.

References
CD4 RESPONSE TO VACCINIA IN HUMANS

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