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ORFeome approach to the clonal, HLA allele-specific CD4 T-cell response to a complex pathogen in humans

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Abstract

The CD4 T-cell response to vaccinia promotes antibody and long-term CD8 responses. HLA class II molecules present microbial epitopes to CD4 T-cells. In humans, at least 3 loci encode cell-surface peptide-binding HLA class II heterodimers. Using intracellular cytokine cytometry (ICC) assays, we determined that HLA DR had the strongest contribution to vaccinia antigen presentation. Among panels of vaccinia-restricted T-cell clones, most were DR-restricted but rare DQ-restricted clones were also recovered. Vaccinia has over 200 open reading frames (ORFs), providing a significant bottleneck to assigning fine specificity. To overcome this, we expressed each predicted vaccinia ORF using *in vitro* transcription and translation. Array-based pool proteins were used to rapidly assign fine specificity to each DQ-restricted clone and to a sample of HLA DR-restricted clones. Reactivity was confirmed using synthetic peptides for selected CD4 T-cell clones. This method should be broadly applicable to the study of large-genome, sequenced pathogens, and could also be used to investigate T-cell responses to cDNAs expressed in neoplastic and autoimmune disorders in which CD4 responses might be adaptive or harmful.

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T-cell; CD4; antigen; epitope; specificity; combinatorial

1. Introduction

In this report, we present tools we have developed while performing a detailed analysis of the CD4 response to vaccinia virus in humans using peripheral blood mononuclear cells (PBMC) from persons vaccinated with Dryvax™, a live, replication-competent, non-clonal cow-derived vaccinia preparation. Dryvax was replaced in 2007 by a similar, clonal, cell culture-derived vaccine from the same vaccinia strain NYCBH genetic background (Monath and Frey, 2008). We adapted direct multi-cytokine ICC and observed that the CD4 response seemed to be predominantly restricted by HLA DR, in contrast to smaller contributions from HLA DP and DQ. Evaluation of HLA locus restriction of panels of vaccinia-reactive CD4 clones lead to a similar conclusion. An efficient screening method using blocking mAb and partially matched antigen-presenting cells (APC) is presented that allowed rapid preliminary assignment of HLA restriction during the screening process of clones at the microculture stage, and therefore selective enrichment of rare clones restricted by HLA DQ.

We then desired to know the fine specificity of the infrequent DQ-restricted T-cell clones, as well as of representative HLA DR-restricted clones. Vaccinia contains over 200 predicted ORFs. As such, synthesis of overlapping peptides covering the entire proteome (Sylwester et al., 2005) would be very expensive. Analysis of T-cell clones with such a reagent set would additionally require the resource-intensive expansion of clones of interest to very high number. The related tetramer-guided epitope mapping approach requires pre-made panels of overlapping peptides (Reijonen and Kwok, 2003). We previously described a method of decoding CD4 clonal specificity using genomic DNA libraries expressed in bacteria. While useful, this procedure requires careful library optimization and sequencing and contig assembly of library “hits” (Koelle et al., 1998; Koelle, 2003; Jing et al., 2007). Computational methods can narrow down epitopes once HLA restriction is definitively proven but typically require empiric testing of many peptides per final epitope (De Groot et al., 2008).

To rapidly and inexpensively determine the specificity of a panel of CD4 T-cell clones at the ORF level, we expressed each predicted vaccinia ORF using in vitro transcription and translation. The resulting proteins were extraordinarily potent in driving proliferative responses by CD4 T-cell clones, proving effective at dilutions up to 1:12 000 and in pools of up to 24 proteins each. We used arrayed matrices of vaccinia proteins to determine the fine specificity of CD4 T-cell clones that were only expanded to a modest level (a few million cells per clone), allowing inexpensive, parallel assessment of many clones. Reactivity at the ORF level was confirmed in repeat assays, and in selected cases with synthetic peptides and HLA-peptide binding studies. The use of irradiated PBMC as APC and cloned CD4 cells as responder cells with the expressed ORF set lead to very robust data sets. Combination of the techniques of selection of CD4 clones restricted by an allele or locus of interest followed by use of an ORFeome panel should be useful to investigators interested in disease states linked to a particular HLA class II locus or allele, and in which responses to an infectious, neoplastic, or “self” antigen may contribute to pathogenesis or therapy and for which data concerning fine antigenic specificity are desired.

2. Materials and Methods

2.1 Subjects and specimens

Persons requiring vaccinia vaccination for employee health purposes signed an IRB-approved consent form. Subject numbers conform to our previous publications (Jing et al., 2005; Jing et al., 2007; Jing et al., 2008) with new subjects outlined in Table 1. HLA types were obtained at the Puget Sound Blood Center. PBMC were cryopreserved and thawed for use.

2.2 Cell and viral culture

PBMC were re-stimulated with UV-killed, cell-associated vaccinia and IL-2 as described (Jing et al., 2007) to yield first-generation bulk cultures (B1). B1 were expanded with anti-CD3 and IL-2 (Koelle et al., 2001) to yield B2 polyclonal cultures. B1 cells were also cloned by limiting dilution using the mitogen PHA (Koelle et al., 1994b). Clones meeting screening criteria were expanded once with anti-CD3 and IL-2 (Koelle et al., 2001). Vaccinia strain NYCBH was raised in BSC40 cells, which were recovered by scraping when they showed full cytopathic effect at 3 days, sonicated, frozen in aliquots, and titered using BSC40 cells (Jing et al., 2005). NYCBH is the parent strain for Dryvax™ vaccine. BSC40 and Cos-7 cells were cultured in MEM, 10% FCS, penicillin/streptomycin, and L-glutamine. Murine hybridoma cells secreting inhibitory antibodies to framework determinants shared by all HLA DR (clone L243), HLA DP (clone B7.21), or HLA DQ (clone SPVL3) allelic variants were cultured as described (Koelle et al., 1994a; Koelle et al., 1994b). Supernatants stored at −80 °C had titers of at least 1:100 for maximal staining of wild-type EBV-transformed B-cell lines (B-LCL) using flow cytometry. B-LCL were cultured from PBMC (Tigges et al., 1992)

2.3 Antigens

For the ORFeome approach, each predicted ORF in vaccinia WR was expressed as described (Davies et al., 2005a; Davies et al., 2005b; Sundaresh et al., 2006; Crotty, 2007; Davies et al., 2007a; Davies et al., 2007b; Eyles et al., 2007; Jing et al., 2008; Sette et al., 2008). Briefly, each predicted ORF was PCR-amplified. PCR fragments were checked for appropriate size and ligated in vitro in a vector with a T7 promoter suitable for *in vitro* transcription and translation using an *E. coli* lysate. Protein products after *in vitr*o protein synthesis were each checked using a fusion tag and selected ORFs with low intensity were re-synthesized. Final reaction volumes of 50 μl per run using the RTS system (Roche, Nutley, NJ) per the manufacturer's protocol. We used two work plans to determine the fine specificity of vaccinia-reactive T-cell clones using the ORFeome panel. In the first, *in vitro* transcribed/translated proteins (n=288, including some empty vector and no-DNA negative control reaction products) were each assayed in duplicate in ^{3}H thymidine T-cell assays (section 2.4) at final concentrations of 1:1 000. This required about six 96-well plates per T-cell clone. In the second, high-throughput format, the proteins were arrayed as a 12 × 24 matrix. Pools corresponding to rows and columns contained 12 or 24 proteins each. Individual proteins were present at final concentrations of 1:12 000. The presence of reactivity to a pooled row or pooled column indicated that one or more of the constituent proteins were antigenic. When a single row and single column were reactive, the ORF at their intersection was identified as the putative antigenic protein. These single ORFs were tested in confirmatory assays at final concentrations of 1:1 000.

For some ORFs scoring positive as full-length proteins, eukaryotic expression was used to narrow down antigenic regions by expressing partial-length proteins. Truncated fragments of candidate antigenic ORFs were inserted into peGFP-C1 (Clontech, Mountain View, CA) using polymerase chain reaction with vaccinia NYCBH DNA as template, Platinum-Taq polymerase (Invitrogen) and primers (sequences available on request) with distal *Xho* I and *Hind* III restriction sites. In-frame PCR product ligation into the C-terminal end of eGFP was sequence-

confirmed. Antigens were transiently transfected into Cos-7 cells (Jing et al., 2005), harvested by scraping and freeze-thaw at 48 hours, and used at 1:100.

Peptides covering selected vaccinia ORFs or sub-ORF-length regions were 13-mers with 9 amino acid overlap (Sigma, St. Louis, MO, or Mimotopes, San Diego, CA) based on the vaccinia WR genome (Lefkowitz et al., 2005). Peptides dissolved in DMSO were used at 1 μg/ml for cultured responder cells and 5 μg/ml for PBMC. Whole virus/positive control was UV-treated vaccinia NYCBH (above) with a titer of 10^9 pfu/ml prior to UV irradiation and zero after and was used at 1:1 000 dilution. Mock UV-virus prep was BSC40 cells used the same dilution. For direct PBMC intracellular cytokine cytometry (ICC), virus was double sucrose gradient-purified (Gomez et al., 2007) prior to titration and UV treatment.

2.4 Lymphocyte functional assays

Interferon-gamma (IFN-γ) ICC was performed on cultured responder T-cells as described (Jing et al., 2005). Briefly, an equal number of responder cells and autologous PBMC, the latter labeled with carboxyfluorescein succinimidyl ester (CFSE) (Gonzalez, 2005), were co-cultured for six hours in the presence of antigen, co-stimulatory anti-CD28 and anti-CD49d mAb, and 1.25 μg/ml brefeldin A (Becton Dickinson) (the latter starting at 1 hour). Surface CD4 and intracellular IFN-γ were stained at six hours. Analysis used a FacsCanto II cytometer (Becton Dickinson) and Flowjo. CFSE-positive cells were dump-gated. Direct PBMC ICC was modified from a published protocol (Horton et al., 2007). Briefly, PBMC were thawed and rested overnight in R10 (RPMI 1640, 10% FCS, L-glutamine and penicillin/streptomycin), and stimulated in 96-well plates with UV-killed vaccinia, media, or staphylococcal enterotoxin B for 20 hours. Co-stimulatory antibodies were used as above and brefeldin A added after 6 hours. Anti-HLA mAb (above) were added at 1:10 prior to antigen (Koelle et al., 1994b). Cells were stained with violet live/dead stain (Invitrogen, Carlsbad, CA), permeabilized with FacsPerm 2 (Becton Dickinson), and stained with anti-CD3-ECD (Coulter, Fullerton, CA, UCH11), anti-CD4-allophycyanin-H7 (SK3), anti-CD8 peridinin chlorophyll protein-Cy5.5 (SK1), anti-IFN-γ-phycoerythrin-Cy7 (4S.B3), anti-TNF-α-FITC (Mab11), and anti-IL-2-phycoerythrin (MQ1-17H12) (all Becton Dickinson). After washes and fixation, cells were analyzed by LSR II (Becton Dickinson) and Flowjo. Data are the proportion of live, CD3(+), CD4(+) cells expressing the indicated cytokine, minus the proportion that test positive for media. Inhibition by antibodies is reported as percentage reduction. Double- and triple-cytokine-expressing cells were analyzed using PESTLE software (gift of Dr. Mario Roederer).

IFN-γ enzyme-linked immunosorbent spot assay (ELISPOT) used modifications of our published procedures (Koelle et al., 2008). For cultured responder cells, 8×10^4 to 1×10^5 B2 and 1–1.2×10^5 autologous or allogeneic PBMC (total, 2×10^5 cells/well) and peptides were incubated in duplicate for 18–20 hours. Anti-HLA class II mAb supernatants were added at 1:10 prior to antigen. Plates were read on a CTL brand (Shaker Heights, OH) ELISPOT reader.

^{3}H thymidine cell proliferation assays were performed as described (Koelle et al., 1994b). For screening of clones, one-tenth (~ 10^4 cells) of microcultures were incubated with autologous irradiated PBMC and whole vaccinia antigen (3 wells) or mock antigen (one well), or allogeneic irradiated PBMC with mock or vaccinia antigen (one well each). Of the 3 wells with autologous PBMC and vaccinia antigen, one had anti-DR and one had anti-DQ (see below) mAb (1:4). Clones with a stimulation index (^{3}H counts per minute for autologous PBMC/UV vaccinia divided by the value for mock antigen) > 5.0 were considered vaccinia-specific. Clones with >50% inhibition by either anti-DR or anti-DQ, but not both, were considered locus-restricted, while clones with any other pattern (restriction by both, or neither, mAb) were not considered to have diagnostic information. Clones with a stimulation index of > 5.0 when allogeneic PBMC were used as APC were considered to react to vaccinia epitopes plus HLA present on the allogeneic cells, while clones with >5 000 CPM of ^{3}H thymidine incorporation in response

to allogeneic PBMC plus mock antigen were considered to be allo-cross-reactive and were not worked up. Follow-up assays (duplicate to triplicate) used 1×10^4 cloned responder cells/well, admixed with APC ($7–10 \times 10^4$ PBMC) and antigen. APC were γ-irradiated (3,300 rads). Assays used 200 μl T-cell medium (Koelle et al., 1994b) in 96-well U-bottom plates with addition of ^{3}H thymidine (0.5 μCi/well) at 72 hours and harvesting 7–20 hours later.

3. Results

3.1 Vaccinia-specific CD4 T-cells detected *ex vivo* are primarily HLA DR-restricted

Memory CD4 T-cells typically express one or more cytokines upon exposure to recall antigen. Human CD4 Th1 cells can secrete IFN-γ, IL-2, and/or TNF-α. While these may all be co-expressed, single or double cytokine-expressing vaccinia-reactive cells have also been detected (Hawkridge et al., 2008). PBMC from four subjects vaccinated 1 to 4 years prior to sampling (Table 1) had a range of 0.43% to 0.83% of CD4 T-cells secreting IFN-γ, 0.19 to 0.75% secreting IL-2, and 0.27% to 1.12% secreting TNF-α in response to purified, UV-killed virions (Table 2). In this assay, responses were predominantly CD4(+), with live, CD3(+), CD8(+) lymphocyte size/scatter-gated cells not showing responses above background (not shown). Single, double (representative subject, Fig. 1), and triple (not shown) cytokine-producing cells were observed. Vaccinia-specific CD4 responses for each cytokine (Table 2, representative subject in Fig. 1) or combination of cytokines (not shown) were decreased by anti-DR antibody to a greater extent than by anti-DP or anti-DQ. For each of the 4 subjects with PBMC available for testing, CD4 responses were about 75% HLA DR-restricted for each cytokine. Inhibition was only on the order of 20%–25% for anti-HLA DP, with relatively equal inhibition of each cytokine. Inhibition by anti-HLA DQ mAb was slightly less consistent between cytokines, but in each case showed the least reduction of cytokine (+) cells. Similar results were noted when direct PBMC IFN-γ ELISPOT was performed in the absence or presence of anti-HLA class II mAb (representative subject, Fig. 1). We therefore became interested in the rare DQ-restricted cells.

3.2 Cultured vaccinia-specific CD4 T-cells are primarily HLA DR-restricted

Based on the above data, we hypothesized that most vaccinia-specific CD4 cells derived from PBMC would also be DR-restricted. The frequency of vaccinia-reactive CD4 T-cells in PBMC declines from high levels soon after primary vaccination (Miller et al., 2008) to memory levels of < 1 % (Table 2, Fig. 1). We therefore selectively enriched and expanded vaccinia-specific CD4 T-cells prior to T-cell cloning. The resultant polyclonal bulk-cultured cells were enriched for vaccinia-reactive T-cells that were primarily CD4 (+) and DR-restricted using IFN-γ ELISPOT (representative subject, Fig. 2). Polyclonal vaccinia-reactive cells from two donors were cloned with a non-specific mitogen using an efficient cloning protocol (Moretta et al., 1983). The majority of the clones were vaccinia-specific (113 of 192, 58% for subject 3; 98 of 144, 68% for subject 9). Inhibition of proliferation of each clone was tested using anti-DR and anti-DQ. HLA DR-restricted clones were predominant: 61 of 113 (54%) for subject 3 and 53 of 98 (54%) for subject 9. The minority of clones were HLA DQ-restricted: 8 of 13 (7%) for subject 3 and 9 of 98 (9%) for subject 9. The remaining vaccinia-reactive clones did not give clear locus-specific restriction patterns with the anti-HLA DR and anti-HLA DQ mAb.

3.3 HLA class II allele-level restriction by T-cell clones

Combining mAb inhibition data and data obtained using single-haplotype matched allogeneic APC allowed preliminary assignment of HLA restriction to the allele level for a subset of clones from donor 9. We excluded four HLA DR-restricted clones from donor 9 that were also alloreactive with donor 15 PBMC (data shown). Within the 49 DR-restricted clones, 13 (27%) were reactive to vaccinia antigen presented by allogeneic APC from donor 15 (Table 1). These clones were thus restricted by either HLA DRB1*1501 or the product of the HLA DRB5 locus,

which is in linkage disequilibrium with DRB1*1501 (Marsh et al., 2000). The remaining 36 HLA DR-restricted clones (73%) were not presented by donor 15 PBMC, and were thus tentatively assigned to restriction by HLA DRB1*0401 or the product of the linked HLA DRB4 locus In contrast, presentation at the DQ locus was skewed toward one allele. 9 of 9 (100%) evaluable DQ-restricted clones were also stimulated by donor 15 PBMC plus vaccinia and thus likely restricted by DQB1*0602 (which forms a heterodimer with DQA1*0102 (Marsh et al., 2000)).

The above data indicated that DQ-restricted responses might be relatively infrequent in terms of abundance and weighted towards HLA DQB1*0602 restriction. To specifically target HLA DQB1*0601, we also studied the DQ-restricted response from subject 3 (Table 1), who shares the DQA1*0102/DQB1*0602 haplotype. During the initial screening of candidate clones from subject 3, we again combined evaluation of each clone with self APC, and anti-DR or anti-DQ mAb, with testing of allogeneic APC matched at DQB1*0602 but not DQB1*0201 (in the presence or absence of vaccinia). All 8 DQ-restricted clones from subject 3 appeared to be DQB1*0602-restricted on the basis of this test.

3.4 High-throughput determination of CD4 clone specificity

We determined the ORF-level specificity of each available HLA DQ-restricted clone from donors 3 and 9 and of a random sample of 10 HLA-DR-restricted clones from donor 3. Among the 9 DQB1*0602-restricted clones from donor 9, 6 expanded to usable numbers (~10^7 cells), while among the 8 such clones from donor 3, 7 expanded sufficiently. Clones were tested in duplicate with each individual vaccinia full-length ORF. Variance between positive duplicates was usually <20%. A single ORF was reactive for each clone (sample clones, Fig. 3). For subject 9, 3 clones reacted with ORF M2L, 2 with ORF E3L, and 1 with ORF D8L.

Definition of reactive peptides is frequently required for detailed studies such as HLA-peptide binding, functional avidity for T-cell activation, and comparison of sequences among related viruses. To determine fine specificity while economizing on peptide synthesis, in some cases we performed initial molecular truncation studies (Fig. 3). Protein prepared by the procedurally simple approach of transiently transfecting Cos-7 cells with cloned ORF fragments and preparing sonicates of scraped cells has proven adequate to map epitopes to ORF sub-regions when CD4 clones are used as responder cells. Use of an eGFP fusion at this step allows protein expression in the transfected cells to be monitored visually.

Follow-up assay with overlapping peptides allowed determination of fine specificity in one additional step (Fig. 3). For each clone studied, assignments of specificity made using the ORF panel (and truncations in some cases) were confirmed with peptides. Testing of overlapping 13-mers covering full-length E3L, or regions of M2L and D8L specified by preliminary truncation analyses (Fig. 3), allowed definition of one antigenic epitope for each ORF. All three clones recognizing M2L each recognized the same internal peptide, amino acids 53-65. Of the two E3L-specific clones, one recognized two overlapping peptides (AA 49-61 and AA 53-65), while the other recognized only AA 49-61 (data not shown).

For subject 3, we analyzed the 7 DQ-restricted clones with sufficient cell availability. One clone (not shown) reacted with D8L, peptide 61-73, previously detected in subject 9 (Fig. 3). Of the remaining 6 clones, two reacted with ORF B19R, one with ORF F13L, one with ORF E3L, one with ORF C12L (Fig. 3), and one with J1R (not shown). Thus there are at least 8 distinct HLA DQB1*0602-restricted epitopes in vaccinia, despite the relative paucity of DQ-restricted CD4 T-cells overall compared to HLA DR. The diversity of the DQ-restricted response was 3 to 5 epitopes per subject. Workup of even relatively small panels of clones (n= 6 or 7) showed redundant epitope detection and also identified one epitope in common between two subjects sharing DQB1*0602.

To reduce the time and expense of antigen definition, a combinatorial approach was used to study the ORF-level specificity of 10 vaccinia-specific clones from subject 9. We chose clones that we preliminarily assigned to DRB1*1501 (or the linked DRB5) restriction based on mAb inhibition and allo-presentation (Fig. 4, bottom). The panel of vaccinia ORFs and negative control vector reaction products (288 antigens) were arrayed in a 12 × 24 matrix to yield 36 pools. The final dilutions of individual *E. coli* lysates were 1:12 000. Each T-cell clone had a single, highly positive row and column in cell proliferation assays (sample clone, Fig. 4 upper left), representing a single vaccinia ORF. The candidate reactive protein at the intersection of the positive rows and columns was validated in each case in a follow up assay (sample clone reactive with ORF E3L, Fig. 4, right). Overall, diverse ORFs drove proliferation of DR-restricted clones, with some redundancy noted at the ORF level (Fig. 4, bottom).

4. Discussion

The CD4 T-cell response to infectious pathogens is important for optimal CD8 CTL responses and for helping microbe-specific antibody responses. These effects have been proven for vaccinia and protection from poxvirus infection (Bennett et al., 1998; Janssen et al., 2003; Shedlock and Shen, 2003; Ashton-Rickardt, 2004; Bevan, 2004; Janssen et al., 2005; Castellino and Germain, 2006; Castellino et al., 2006; Ekkens et al., 2007). Priming of naive CD4 T-cells is accomplished by specialized APC such as monocytes, macrophages, and dendritic cells. These cells have specific phagocytic and protein-degrading machinery and constitutively express HLA class II molecules that can present peptides to T-cell receptors. In humans, the protein products of the polymorphic HLA DRB1 locus and of the less variable DRB3, DRB4, and DRB5 loci form a heterodimer with the invariant HLA DRA1 protein. Not all individuals have DRB3, DRB4, or DRB5 genes. Therefore, the complement of unique DR molecules per person ranges from one (in the instance of homozygosity at DRB1 and the absence of DRB3, DRB4, and DRB5) to four. There is a single DQB locus per chromosome, but both the HLA DQA1 and DQB1 loci are polymorphic. Various combinations of DQA1 and DQB1 proteins can form stable heterodimers. In some individuals, chimeras between the proteins encoded within haplotypes are possible, leading to a maximum complement of four distinct DQ heterodimers per person (Kwok et al., 1993). In addition to DR and DP molecules, DP heterodimers are encoded by the polymorphic HLA DPB1 and DPA1 loci such that up to four productive pairings are possible per person.

Recently, it has been shown that replication-competent vaccinia modulates cell surface HLA class II levels and antigen presentation to CD4 T-cells in various experimental systems (Li et al., 2005). It is not known if these effects differentially influence distinct HLA class II molecules. Poxvirus vectors, both replication-competent and –incompetent in human cells, that express heterologous antigens are being increasingly used for infectious disease and malignancy indications to boost specific immunity. These vectors might differ in modulation of class II antigen presentation and thus HLA class II-restricted T-cell responses. The reason that HLA DR appears to dominate the response in terms of HLA locus restriction for CD4 T-cells remains unknown but could be related to cell surface density of HLA molecules or the nature of the APC responsible for priming and amplifying the CD4 response.

Using multi-cytokine ICC, we found that the majority of vaccinia-specific CD4 T-cells in direct *ex vivo* assays were DR-restricted. Polyclonal responder lines contained components of DP- and DQ-restricted responder cells, but DR-restricted responses were dominant at the bulk and clonal levels. By studying one subject in detail, we determined that multiple DRB gene products were involved in his response.

Because DQ-restricted responses were numerically rare and exclusively restricted by HLA DQB1*0602 in heterozygous persons, we focused attention on determining the fine specificity

of DQB1*0602-restricted responses. HLA DQB1*0602 has been associated with several autoimmune diseases (Gersuk and Nepom, 2009; Miyagawa et al., 2009), and the database of DQB1*0602-restricted peptides is small, such that the DQ*0602 binding motif is not well defined. We used a panel of each predicted vaccinia ORF, expressed in vitro using linked transcription and translation, in a one-step assay configuration to determine the fine specificity of the minority DQ-restricted clones. The vaccinia ORFeome panel, previously used to interrogate polyclonal responders (Jing et al., 2008), was readily able to decode clone specificity to the ORF level. This approach required almost 6 plates of ^{3}H thymidine incorporation assays per T-cell clone, but was able to interrogate in a single step the entire predicted proteome.

The proteome set used was based on the vaccinia strain WR and may be missing a few proteins encoded by the Dryvax™ vaccine used to immunize the test subject. Dryvax™ is genetically related to strain Copenhagen. Sequence comparisons (available at http://www.poxvirus.org/vaccinia_orthologs.asp) indicate T-cell antigenic genes such as Copenhagen B22R that do not have homologs present in our protein set. We did not detect any clones reactive with whole vaccinia that failed to react with our ORFeome set, so we do not believe omission of these few ORFs has significantly influenced our results.

Our study of a small subset of DR-restricted clones also showed that many different vaccinia ORFs were recognized. To accomplish this, we performed a more economical, higher-throughput ^{3}H proliferation screen, again using the ORFeome protein panel but this time as pools. The proteins were strongly biologically active at final dilutions of up to 1:12 000, and in pools of up to 24 proteins (Fig. 4). In each case, the candidate antigenic ORF, identified from the pattern of positive pools in a matrix array, was confirmed as antigenic when tested as a single protein.

The pool process used only 38 wells of ^{3}H thymidine per clone in the primary screen followed by a few more wells for confirmation. We still had to expand the T-cell clones of interest after they passed initial screening, which consumed time and resources. In ongoing work we are downscaling and speeding up proliferation assays at the screening, ORFeome interrogation, and confirmatory stages to allow us to complete the assignment of T-cell clone specificity from the initial T-cell clone.

Our methods provide one pathway to rapid assignment of CD4 T-cell reactivity to the ORF level. Once reactive proteins are determined, additional approaches are required to define peptide epitopes. We chose to use “brute force” testing of overlapping peptides in antigenic regions, in some cases preceded by truncation analyses. Alternative and complementary methods can independently discover peptide epitopes, and some are also suitable once an antigenic ORF or sub-region has been defined. In the field of orthopoxvirus research, computational algorithms have been applied to discover CD4 epitopes (Moutaftsi et al., 2007). Recently, a consensus approach using several algorithms proved very efficient for a specific HLA class II allele, leading to a high ratio of “hits” to peptides tested (Calvo-Calle et al., 2007). Mass spectroscopy of peptides eluted from antigen-loaded APC has also been successful for vaccinia (Johnson et al., 2005; Strug et al., 2008). The method discussed in this report is similar to algorithm and mass spectroscopy-driven approaches in that specific HLA loci and alleles of interest can be targeted, but may offer efficiencies of scale and cost.

The methods reported may be applicable in other situations in which CD4 T-cells are of biological interest. Indeed, the entire predicted ORFeome for *P. falciparum* and *F. tularensis* has been expressed using the same platform reported here, and CD4 T-cell responses are an important component of the immune response to these organisms (Sundaresh et al., 2006; Doolan et al., 2008). In the fields of cancer and autoimmunity, it may be rational to screen

blood- or tissue-derived T-cell clones for reactivity with whole tumor or inflamed tissue, and then investigate fine specificity with a collection of suitable host organism cDNAs such as those available from several commercial and non-profit agencies. Several human autoimmune diseases are linked with specific HLA class II alleles. The CD4 clone screening pathway that is outlined in the present report in which yes/no reactivity to whole antigen plus self APC and candidate HLA restriction are determined simultaneously can be used to speed up isolation of CD4 clones restricted by the disease-linked allele.

We recognize that CD8 T-cell responses are vital in many infectious and non-infectious conditions and that the *E. coli*-derived ORFeome panel described in this report will be difficult to apply to CD8 T-cells given the differences in antigen processing and presentation between T-cell subsets. We have previously reported genomic DNA library approaches to CD8 antigen discovery (Koelle, 2003; Jing et al., 2005), and are currently shifting to a virtual library of full-length ORFs. An outstanding challenge is to determine an efficient, inexpensive method to transducer suitable, abundant APC such as autologous, EBV-transformed B-cells with such an ORF collection in a high-throughput, non-toxic fashion for CD8 antigen discovery.

The present report contributes to the collection of known vaccinia epitopes. Knowledge concerning vaccinia antigens and epitopes recognized by vaccinia-specific CD4 T-cells is accumulating at an accelerating pace. On-line databases emphasizing T-cell epitopes in pathogens of biodefense concern (Vita et al., 2008) and review articles (Kennedy and Poland, 2007) capture the complexity of this field. Several fundamentally different approaches have been used, with ours having been termed T-cell driven (Yewdell, 2006). T-cell lines or clones reactive with whole vaccinia are paired with genomic libraries or ORF sets to resolve fine specificity. Using this approach, we previously reported peptide epitopes in ORFs A3L (Jing et al., 2007; Jing et al., 2008), L4R, and F11L (Jing et al., 2008) and in A7L, A33R, A4R, C10L, E4L, H2R, H3L, and L1R (Jing et al., 2007), including examples of restriction by HLA DR, DP, and DQ molecules. In common with Tang *et al*. (Tang et al., 2006), we also specifically targeted vaccinia proteins that are known IMV neutralizing antibody targets, and found a discrete, novel epitope in ORF A27L. At the ORF level, we previously showed that more than 50% of known vaccinia ORFs are CD4 antigens within a relatively small set (11) of subjects. Overall, we can anticipate an almost overwhelming complexity of CD4 responses both within individuals and within the population.

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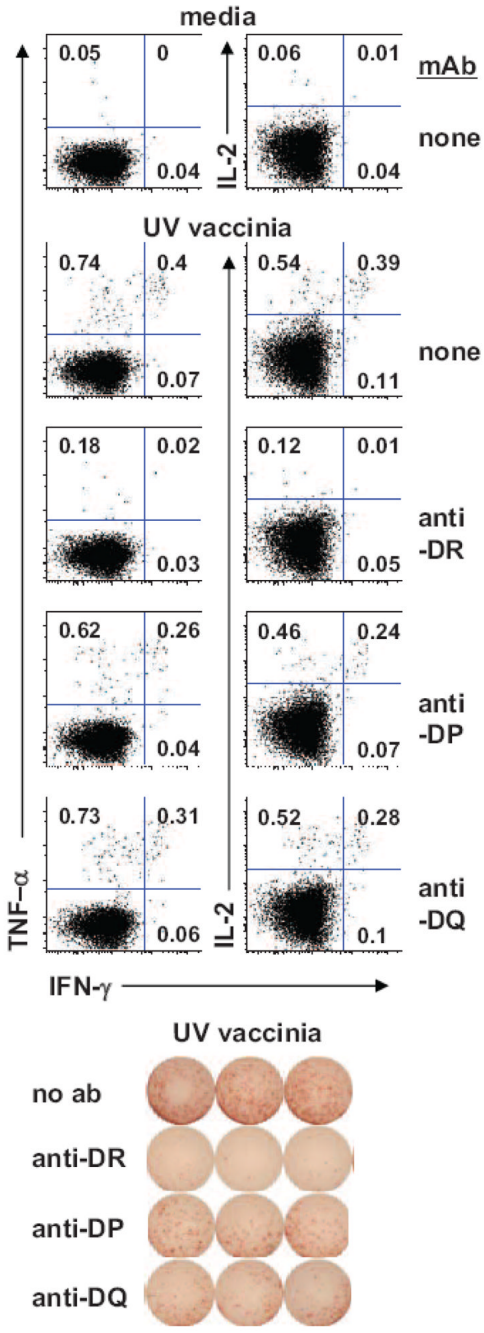

Fig. 1.

Top: *ex vivo* subject 9 PBMC cytokine responses to vaccinia in the absence or presence of anti-HLA mAb amongst gated CD3(+), CD4(+), live lymphocytes. Numbers are percentages of cells in quadrants. Bottom: *ex vivo* subject 9 PBMC IFN-γ ELISPOT responses (triplicate) to vaccinia with the indicated mAb.

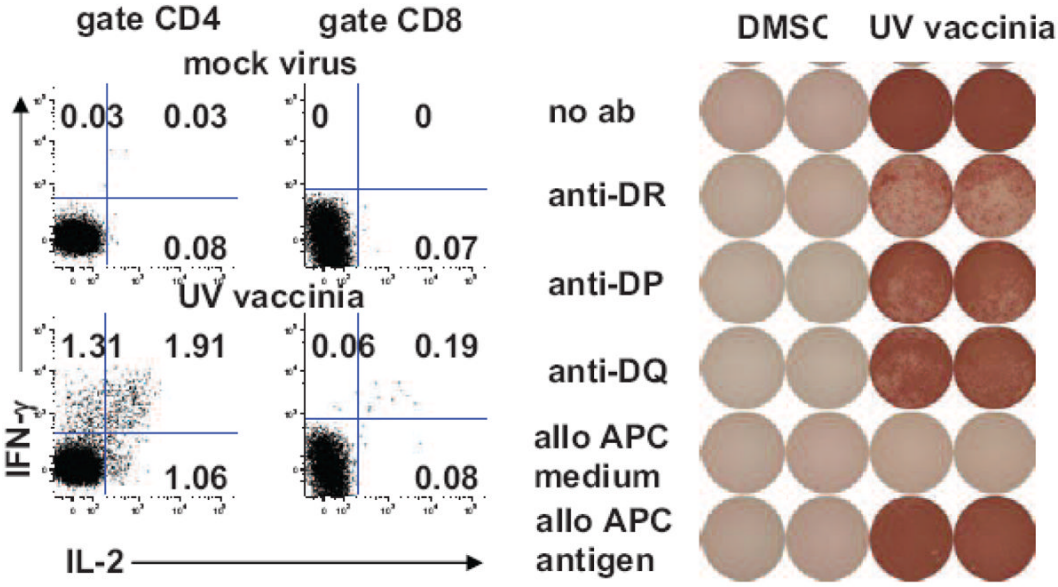

Fig. 2.

Left: cytokine responses of subject 9 polyclonal in vitro responder cells to UV vaccinia. Gated CD4 (+) or CD8 (+) lymphocytes are shown after dump-gating antigen presenting cells (see text). Right: duplicate IFN-γ responses of the same responder cells to vaccinia or a defined vaccinia peptide using autologous PBMC as APC (top 4 rows) with the indicated mAb. UV-treated cell-associated vaccinia was used as antigen. Allogeneic PBMC (bottom 2 rows) were from subject 15 (Table 1) and were thus a one-haplotype match at HLA class II for subject 9.

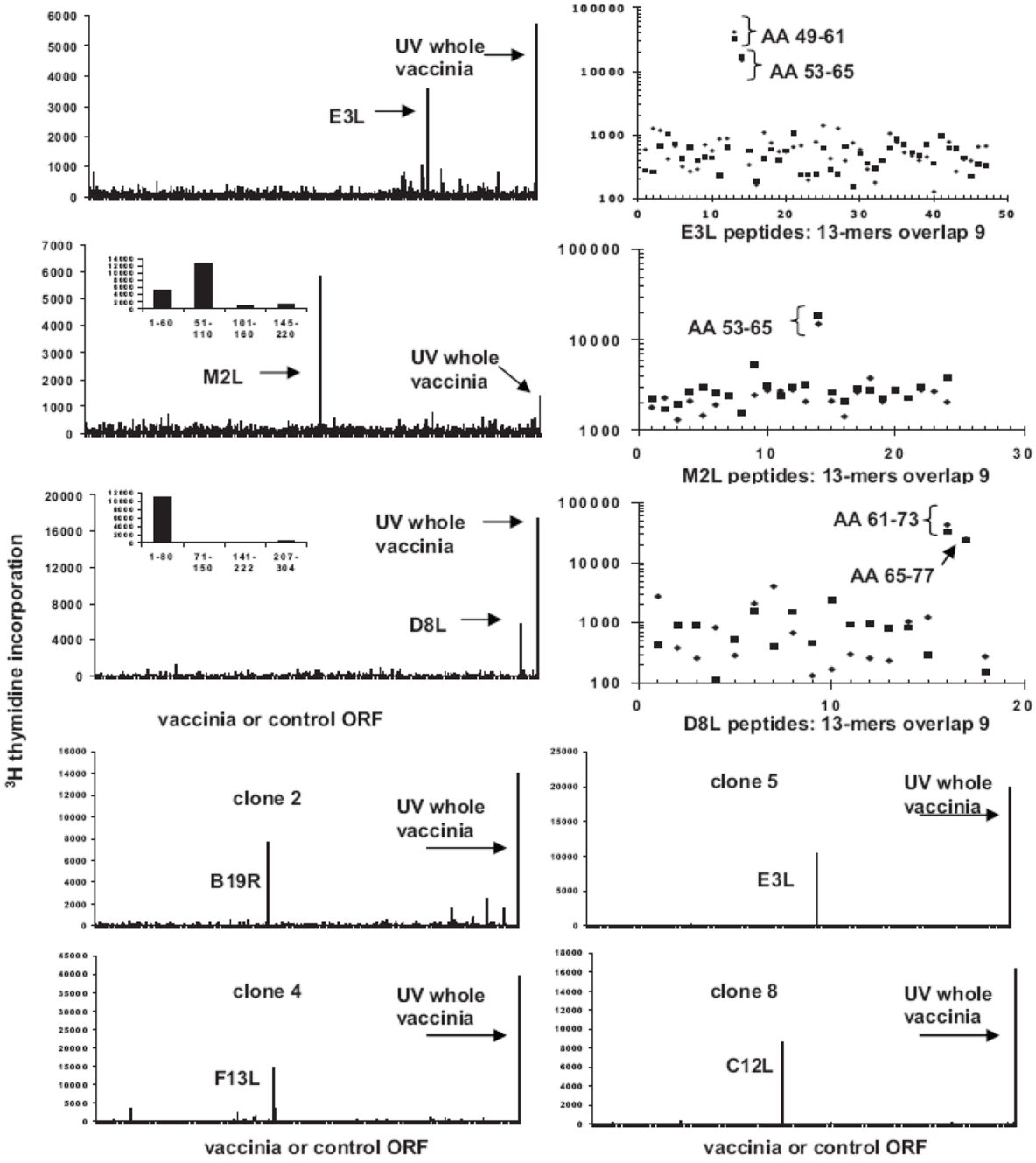

Fig. 3.

Determination of peptide epitopes for HLA DQA1*0102/DQB1*0602-restricted clones. Top left 3 panels: raw ^{3}H thymidine values (duplicate) for representative clones from subject 9 exposed to the panel of vaccinia and negative control microbial ORFs. Positive ORFs and responses to whole vaccinia are labeled. For M2L and D8L, insets show the mean of duplicate responses to molecular truncations containing the indicated amino acids. Top right 3 panels: epitope mapping using overlapping synthetic peptides. Raw ^{3}H thymidine values (duplicate) for the clones used at left. Bottom 6 panels: mean of duplicate ^{3}H thymidine incorporation for 6 clones from subject 3 exposed to the same ORF panel. Positive ORFs and responses to whole vaccinia are labeled.

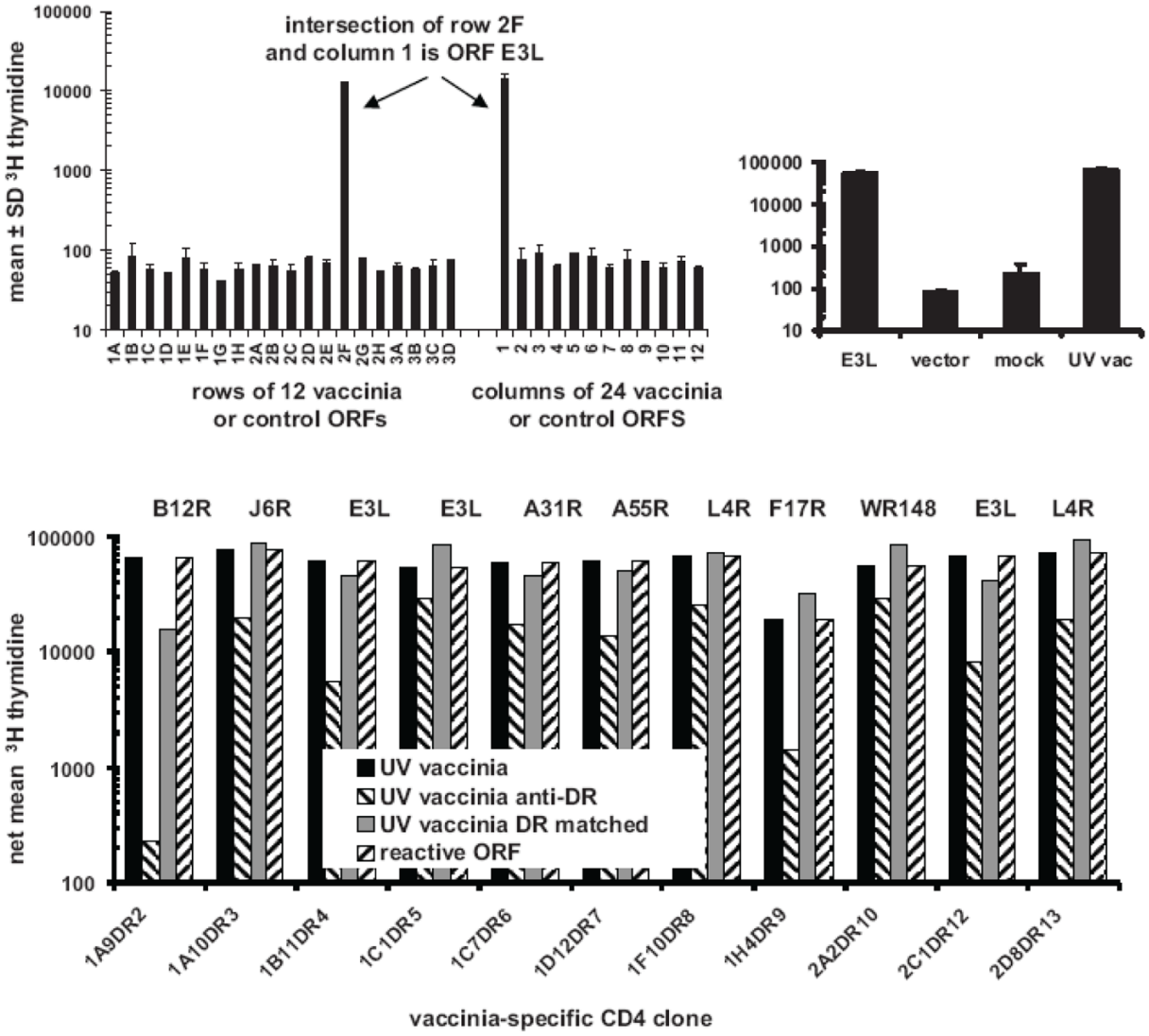

Fig. 4.

Determination of the ORF-level specificity of HLA DR-restricted clones from subject 9. Top left: *E. coli*-derived proteins were pooled in rows and columns and tested in duplicate ^{3}H thymidine proliferation assays. Data are mean values. Top right: the imputed positive ORF, E3L, and indicated control antigens were tested in triplicate ^{3}H thymidine assays. Data are means and standard deviations. Bottom: net mean ^{3}H thymidine incorporation for 11 distinct CD4 clones (clone names on × axis). Thymidine incorporation for mock virus or mock recombinant protein antigen and relevant APC were < 300 counts per minute in each case (not shown) and were subtracted to give net values. Autologous PBMC were used as APC with whole vaccinia in the absence or presence of anti-HLA DR mAb (first two bars), or with single recombinant proteins deduced from row/column matrix assays (ORF names at top for each clone) in the absence of mAb (4th bar for each clone). To confirm HLA DR restriction, allogeneic PBMC matched at DRB1*1501 and DRB5 were used as APC with whole vaccinia as antigen (3rd bar for each clone).

Table 1

Subjects participating in the study.

subject[a]	number of vaccinations (timing)[b]	months between vaccination and sample[c]	HLA DRB1	HLA DRB3, 4, 5	HLA DQA1	HLA DQB1
1	2 (43)	60	*16xx, *11xx	3,5	not done	*03xx, *05xx
3	2 (32)	65	*1501, *0301	3, 5	*0102, *0501	*0201, *0602[d]
9	2 (35)	44	*1501, *0401[e]	4, 5	*0102, *0303	*0301, *0602
12	1	60	not done	not done	not done	not done
15	0	NA	*1501, *0301	3, 5	*0102, *0501	*0201, *0602

[a] Subject numbers < 15 correspond to our previous publications (Jing et al., 2005; Jing et al., 2007; Jing et al., 2008).

[b] The number of total lifetime vaccinations is followed in parentheses by the interval(s) in years between the current and past vaccinations. All vaccinations were clinical “takes” (Wharton, 2003).

[c] Months between most recent vaccination and first blood sample for T-cell studies. NA, not applicable.

[d] Typing cannot exclude HLA DQB1*0633, a rare allele.

[e] Typing cannot exclude HLA DRB1*0433, a rare allele.

Table 2

CD4 T-cell cytokine expression in response to whole sucrose-purified UV-killed vaccinia and inhibition by blocking locus-specific anti-HLA class II antibodies.

		cytokine		
subject	**mAb**	**IFN-γ**	**IL-2**	**TNF-α**
1 %(+) CD4[a]		0.083	0.190	0.270
	anti-DR[b]	75.9	72.1	77.0
	anti-DP[b]	30.1	36.8	37.0
	anti-DQ[b]	38.6	0.0	3.7
12 %(+) CD4		0.430	0.750	1.120
	anti-DR	85.3	82.7	79.5
	anti-DP	27.9	8.0	19.6
	anti-DQ	11.6	0.0	5.4
9 %(+) CD4		0.137	0.250	0.473
	anti-DR	72.6	78.2	89.5
	anti-DP	15.9	2.0	2.8
	anti-DQ	15.9	0.0	0.0
3 %(+) CD4		0.295	0.545	0.510
	anti-DR	66.1	67.0	62.7
	anti-DP	25.4	39.4	21.6
	anti-DQ	8.5	28.4	7.8
mean ± SD, 4 subjects	anti-DR	75.0 ± 8.0	75.0 ± 6.9	77.2 ± 11.1
	anti-DP	24.8 ± 6.3	21.6 ± 19.3	20.3 ± 14
	anti-DQ	18.6 ± 13.6	7.1 ± 14.2	4.2 ± 3.3

[a] Percentage of lymphocyte forward/side scatter, live, CD3(+), CD4 (+) cells with specific accumulation of the indicated cytokine in response to vaccinia after subtraction of values for mock stimulation.

[b] Percentage inhibition of the number of similarly gated cells by the indicated mAb.