CD4+ T Cells Provide Intermolecular Help To Generate Robust Antibody Responses in Vaccinia Virus–Vaccinated Humans

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CD4⁺ T Cells Provide Intermolecular Help To Generate Robust Antibody Responses in Vaccinia Virus–Vaccinated Humans

Liusong Yin,* J. Mauricio Calvo-Calle,* John Cruz,† Frances K. Newman,‡ Sharon E. Frey,‡ Francis A. Ennis,† and Lawrence J. Stern*§

Immunization with vaccinia virus elicits a protective Ab response that is almost completely CD4⁺ T cell dependent. A recent study in a rodent model observed a deterministic linkage between Ab and CD4⁺ T cell responses to particular vaccinia virus proteins suggesting that CD4⁺ T cell help is preferentially provided to B cells with the same protein specificity (Sette et al. 2008. Immunity 28: 847–858). However, a causal linkage between Ab and CD4⁺ T cell responses to vaccinia or any other large pathogen in humans has yet to be done. In this study, we measured the Ab and CD4⁺ T cell responses against four vaccinia viral proteins (A27L, A33R, B5R, and L1R) known to be strongly targeted by humoral and cellular responses induced by vaccinia virus vaccination in 90 recently vaccinated and 7 long-term vaccinia-immunized human donors. Our data indicate that there is no direct linkage between Ab and CD4⁺ T cell responses against each individual protein in both short-term and long-term immunized donors. Together with the observation that the presence of immune responses to these four proteins is linked together within donors, our data suggest that in vaccinia-immunized humans, individual viral proteins are not the primary recognition unit of CD4⁺ T cell help for B cells. Therefore, we have for the first time, to our knowledge, shown evidence that CD4⁺ T cells provide intermolecular (also known as noncognate or heterotypic) help to generate robust Ab responses against four vaccinia viral proteins in humans. The Journal of Immunology, 2013, 190: 6023–6033.

Antibody responses are essential components of protective immune responses to many pathogens, such as influenza virus (1), HIV-1 (2), smallpox virus (3, 4), and Coxiella burnetii (5). CD4⁺ T cell responses are also mediators of protective immunity to pathogens (6–8). The standard model of CD4⁺ T cell–B cell interaction can be summarized as “any helper epitope is sufficient.” In brief, during viral infection, B cells recognizing cognate Ag on the virion can internalize and process the whole virion for Ag presentation to CD4⁺ T cells specific for an epitope from any of the virion proteins. In turn, the epitope-specific CD4⁺ T cells provide intermolecular help to the B cells to generate Ab responses against any protein from the whole virion (9). This well-accepted viral intermolecular help model, in which CD4⁺ T cells provide help to B cells with different protein specificities, was established in the studies of influenza virus (10, 11) and hepatitis B virus (12), and has been confirmed in many other small virus or particle systems. Intermolecular help was also known as noncognate or heterotypic help, in which situations T and B cell determinants are present on noncovalently linked Ags (11, 13). For example, it was found that B cells producing neutralizing Abs recognizing viral surface proteins could use intermolecular help from T cells specific for an rotavirus internal protein (13), and in a study of immunization with respiratory syncytial virus Ags, covalent linkage of the B and T cell epitopes was not necessary for the generation of T cell–dependent Ab responses, although it did improve the affinity of the Ab response (14). Studies in a murine lupus model showed that Abs recognizing components of the small nuclear ribonucleoprotein particle could use T cell help from other components provided that they were present in the same particle, another example of intermolecular help in generation of Abs (15).

Despite this general concordance with the “any helper epitope is sufficient” model, several studies have identified situations where some helper epitopes function much more effectively than others. An early study of the response to influenza virus proposed a model of a hierarchy of T cell help based on the observation that B cells recognizing viral surface components could receive help from T cells specific for any of the major structural viral proteins, whereas B cells responding to internal viral components are restricted to receive help almost exclusively from T cells with the same protein specificity (16). The mechanism proposed was based on the idea that cell-surface Ab against a viral surface protein would be likely to capture intact viruses containing many different proteins able to provide helper epitopes, whereas cell-surface Ab against a core protein would be more likely to capture that protein only. The idea of a hierarchy of CD4⁺ T cell help to generate Ab responses has been investigated in other systems. In one study, B cell Ab responses to lymphocytic choriomeningitis virus surface glycoprotein were generated with help from CD4⁺ T cells against the surface glycoprotein, but not for the internal nucleoprotein, similarly to the case with influenza (17). The concept of intermolecular help has been used to design more effective subunit...
vaccines by including both the B cell and T cell epitopes in a single antigenically diverse structure (18).

However, the studies on linkage between CD4+ T cell responses and Ab responses for large and complex pathogens, such as poxvirus and bacteria, remain very limited. Recently, this linkage for vaccinia virus was evaluated in mice by Sette and colleagues (19). Using a set of previously identified CD4+ T cell epitopes (20), they found that the Ab response to each particular protein target needs to be accompanied by a matched CD4+ T cell response against the same protein, as if the virion were perceived as a collection of individual protein specificities. Vaccinia virus is a large and complex virus with ~200 viral proteins (21) and two infectious forms called intracellular mature virus (IMV) and extracellular enveloped virus (EEV), which are different structurally, antigenically, and functionally (22). Sette et al. suggested that the large size of vaccinia virions, ~360 nm in diameter, relative to B cell endocytotic vesicles, ~150 nm in diameter (23–25), would complicate the linkage between CD4+ T cell and B cell targets because of the possibility that B cell might endocytose viral fragments but not whole virions (19). This new model of intramolecular help in responses to large and complex Ags like poxviruses and bacteria, with CD4+ T cells providing help to B cells only with the same protein specificity, is essentially an extreme variant of the hierarchy of help concept developed in studies of small viruses like influenza (80 nm) or hepatitis B virus (25–40 nm), with every protein behaving as if it were a viral core Ag. Intramolecular help was also termed as cognate or homotypic help, which requires the antigenic determinants recognized by T and B cells to be covalently linked on the same Ag (11, 13). The model has received great attention for its academic and practical implications in studies of the nature of T cell help for Ab generation (26–32), in the strategy of CD4+ T cell epitope identification approaches that focus only on targets eliciting strong Ab responses (33–39), and in vaccine design studies that include proteins targeted strongly by CD4+ T cells (40–45). Despite this interest and multiple citations, few studies have experimentally attempted to establish the linkage between CD4+ T cell and Ab responses in the response to large and complex Ags. Two follow-up studies analyzed the human allergic response to Timothy grass Ags (38), and in rodents to a bacterial pathogen, C. burnetii (46). Unlike the original study, these two publications did not observe a strong correlation between the targets of Ab and T cell responses.

In humans, CD4+ T cell (47–49) and Ab responses (50, 51) against vaccinia virus are extremely diverse and heterogeneous, targeting both IMV and EEV early and late proteins. Although the idea of deterministic CD4+–Ab correlation has been applied to the identification of CD4+ epitopes by focusing on targets with strong Ab responses (36), to date, the linkage of CD4+ T cell and Ab specificities for vaccinia virus in humans has yet to be evaluated experimentally.

In this study, we evaluated the linkage between CD4+ T cell and Ab responses against vaccinia virus proteins A27L, A33R, B5R, and L1R in human donors. A27L and L1R are IMV membrane proteins, whereas A33R and B5R are EEV membrane proteins. A DNA vaccine composed of four genes encoding A27L, A33R, B5R, and L1R showed significant protective immunity in mice (52) and nonhuman primates (53, 54). Corresponding recombinant proteins also provided protective immunity in mice (55), whereas a combination of DNA prime followed by a protein boost seemed more efficacious in nonhuman primate (54). Strong Ab responses against A27L, A33R, B5R, and L1R were observed in humans after vaccination (50, 51, 56). CD4+ T cell epitopes for these four proteins in humans have also been mapped (36, 57). In this study, we measured the Ab responses and CD4+ T cell responses against A27L, A33R, B5R, and L1R in 90 recently vaccinia virus–vaccinated healthy donors and 7 long-term vaccinated donors. We concluded that there is no direct linkage between CD4+ T cell and Ab responses against each individual protein, and thus that the conventional intermolecular help model applies to the human immune response against vaccinia virus, at least for the four proteins tested in a vaccination trial.

Materials and Methods

Human donors

Sera and PBMCs from 90 healthy vaccinia-naïve humans before (day 0) and 45 d after (day 45) vaccinia virus vaccination were prepared at Saint Louis University Center for vaccine development during a study of smallpox vaccines generated by Acambis (Cambridge, MA) after approval by the Saint Louis University Institutional Review Board (58). Thirty donors each were vaccinated with Dryxav, ACAM1000, and ACAM2000, respectively. The ACAM1000 and ACAM2000 (Acambis, Cambridge, MA) vaccines are derived from Dryxav (Wyeth Laboratories, Marietta, PA) by plaque purification cloning in Vero cells and purified from disrupted infected cells by ultrafiltration and diafiltration, and lyophilized (58–60). Sera and PBMCs from seven long-term vaccinia-immune donors (vaccinated with Dryxav >4 y before this study) and four nonimmunized donors were collected under a protocol approved by the Medical School Institutional Review Board of University of Massachusetts.

Recombinant proteins

Recombinant vaccinia proteins A27L (BEI resources ID: NR-2622), A33R (NR-2623), B5R (NR-2624), and L1R (NR-2625) from the WR strain were obtained from the Biodefense Repository (http://www.beiresources.org/).

IFN-γ–ELISPOT for CD4+ T cell responses

We measured CD4+ T cell responses against A27L, A33R, B5R, and L1R by IFN-γ ELISPOT assay. In brief, 5 × 105 PBMCs from each donor were stimulated with 5 μg/ml of each recombinant protein in 200 μl cRPMI medium (RPMI 1640 supplemented with 10% human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 μM of 2-ME and 1 mM nonessential amino acids from Life Technologies) in Immobilon-P 96-well MultiScreen plates (Millipore Corporation, Billerica, MA) for 48 h. A 1:800 dilution of vaccinia virus (MVA strain)–infected monkey kidney CV-1 cell lysate originally containing 1.7 × 104 PFU/ml or 1:800 dilution of noninfected CV-1 cell lysate or medium only was used as control to stimulate the PBMCs. Number of IFN-γ–secreting cells (spots per well [SPW]) was determined using ELISPOT analyzer equipped with ImmunoSpot 5.0.3 software (CTL, Shaker Heights, OH).

IFN-γ and IL-2 ELISA for CD4+ T cell responses

We also measured the CD4+ T cell responses against A27L, A33R, B5R, and L1R in donors 09, 22, 34, and 39 by IFN-γ and IL-2 ELISA. In brief, 5 × 105 PBMCs from each donor were stimulated with 5 μg/ml of each recombinant protein or medium only, or VV-infected CV-1 cell lysate in 200 μl cRPMI medium. A total of 100 μl supernatant from each well was collected after 48 h. The production of IFN-γ and IL-2 were measured using the Human IFN-γ ELISA set and Human IL-2 ELISA set, respectively (BD Biosciences, San Diego, CA).

Human cytokine/chemokine 96-well plate multiplex assay

The human cytokine/chemokine production post vaccinia virus infection was measured by MILLIPLEX MAP Kit containing different sizes of anti-human GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, MCP-1, and TNF-α Ab-immobilized beads (EMD Millipore Corporation, Darmstadt, Germany). In brief, 5 × 105 PBMCs from each donor were stimulated with heat-inactivated vaccinia virus–infected CV-1 cell lysate or control noninfected lysate in 200 μl cRPMI medium for 48 h. A total of 100 μl supernatant from each well was collected and 25 μl of each sample was added to a Microtiter Filter Plate. Subsequently, 25 μl anti-human cytokines/chemokines Ab-coated pre-mixed beads was added to each well and incubated at room temperature for 1 h. Then 25 μl streptavidin-PE was added and the mixture was incubated at room temperature for 30 min. The plate was washed, 150 μl PBS was added to all wells, and the beads were resuspended on a plate shaker for 5 min. The
amount of each cytokine/chemokine was read out on a Luminex 200 analyzer (Luminex, Austin, TX).

**ELISA for Ab responses**

Ab responses against A27L, A33R, B5R, and L1R were measured by ELISA. A 96-well high-binding polystyrene microtiter plate (Thermo Scientific, Rochester, NY) was coated with pretitrated optimal concentration of each recombinant protein at 0.5 μg/ml in 100 μl overnight at 4°C. Control wells were coated with 0.5 μg/ml BSA. The plates were washed with PBST (1× PBS with 0.1% Tween 20) and blocked with 5% BSA at 37°C for 2 h. Subsequently, 100 μl human serum diluted in PBST + 2.5% BSA from each donor was added and the plates were incubated at 37°C for 1 h. Binding of human Abs was revealed by using 100 μl 1:4000 dilution of peroxidase-labeled goat anti-human IgG (KPL, Gaithersburg, MD) after the washing steps and incubation at 37°C for 1 h. Finally the plates were developed with ABTS solution (Roche Applied Science, Mannheim, Germany) and read at 405 nm for absorbance using Victor plate reader (PerkinElmer, Shelton, CT).

**Calculation of expected number of donors positive for each combination of four proteins assuming random independent association**

The experimental number of positive donors against each protein was used to calculate the expected number of donors positive for each combination of A27L, A33R, B5R, and L1R based on probability theory of independent events. For example, expected number of donor with A27L and A33R was calculated as: (number of A27L/total number)*(number of A33R/total number)*(number of B5R/total number)*(number of L1R/total number) (total number); and expected number of A27L+A33R+B5R+L1R donor is calculated as: (number of A27L/total number)*(number of A33R/total number)*(number of B5R/total number)*(number of L1R/total number) (total number — number of L1R)/(total number)* (total number). The total number is number of donors analyzed, 57 for CD4+ T cell responses and 88 for Ab responses.

**Correlation coefficient analysis**

Correlation coefficient analyses relating CD4+ T cell responses (shown as SPW) and Ab responses (shown as absorbance at 405 nm) were done using GraphPad Prism 5 (GraphPad Software, San Diego, CA). Pearson’s correlation coefficient and two-tailed p value were calculated.

**Results**

**Vaccinia virus vaccination induces robust cellular and humoral responses against A27L, A33R, B5R, and L1R**

To study the linkage between CD4+ T cell responses and Ab responses, we obtained PBMCs and sera from 90 healthy vaccinia-naive donors before (day 0) and 45 d after inoculation with smallpox vaccines developed by Acambis (ACAM1000 and ACAM2000). Thirty donors each were vaccinated with ACAM1000, ACAM2000, or Dryvax (the only previously licensed smallpox vaccine). ACAM1000 and ACAM2000 are identical at the genome level, both derived from Dryvax by plaque purification cloning in Vero cells (59, 60). All these three smallpox vaccines showed similar protective immunity in mice and nonhuman primates (59, 60), and in humans, with a detailed characterization of safety and efficacy (58) (study number: Acambis H-400-002). ACAM2000 was approved by the U.S. Food and Drug Administration on August 31, 2007, to replace Dryvax for smallpox vaccine.

To identify which functional responses would be most useful in following the response to smallpox vaccination, we measured the production of GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, MCP-1, and TNF-α in PBMC samples obtained 45 d after vaccination (Fig. 1). Samples were stimulated with heat-inactivated vaccinia virus–infected CV-1 cell lysate or control noninfected CV-1 cell lysate, and cytokine production was measured by multiplex bead assay. Among the 14 cytokines/chemokines measured, IL-8 and MCP-1 were nonspecifically produced in all 5 donors, and IL-6 was nonspecifically produced in donor 42. IL-4, IL-5, IL-7, IL-12, and IL-13 were secreted specifically upon vaccinia stimulation but at relatively low levels. The other cytokines, GM-CSF, IFN-γ, IL-1β, IL-2, IL-10, and TNF-α, were specifically produced at high levels in responses to vaccinia infection. The relative pattern of response did not vary greatly from donor to donor. Of the cytokines specifically produced at a high level, IFN-γ had the greatest signal-to-background ratio (~2000-fold over background, as compared with ~700-fold for IL-2 and 10- to 300-fold for the others) and the lowest relative SD (9%, as compared with 17% for IL-2 and 19–27% for the others). Thus, we selected IFN-γ for analysis of a larger set of vaccinated and nonvaccinated donors. IFN-γ detection is widely used to characterize the CD4+ T cell responses against vaccinia virus and to identify CD4+ T cell epitopes in both mice (20) and humans (47–49, 61).

We chose the vaccinia proteins A27L, A33R, B5R, and L1R for detailed analysis. These are the proteins targeted most strongly by both humoral and cellular responses during vaccinia virus infection (50, 51, 56, 57). Vaccination with genes encoding these four proteins and corresponding recombinant proteins provided protective immunity in mice and nonhuman primates (52–55), and these have been a focus of subunit vaccine development efforts. Moreover, the proteins are representative of both forms of the virus: A27L and L1R from the intracellular IMV form, and A33R and B5R from the extracellular secreted EEV form. CD4+ T cell responses against A27L, A33R, B5R, and L1R were measured by IFN-γ–ELISPOT in PBMCs stimulated by recombinant proteins. Donors showing no responses to vaccinia virus–infected CV-1 cell lysate at day 45 (1 donor) or nonspecific responses at day 0 (8 donors), or without enough PBMCs to repeat at least 2 times (24 donors) were excluded from the analysis and we ended up analyzing data from 57 donors (Fig. 2A, Table I). We found that vaccinia virus vaccination induces significant and diverse CD4+ T cell responses against these four proteins (Fig. 2A). More than half of the donors show positive responses, and there is no preference for CD4+ T cells targeting IMV proteins (A27L and L1R, 27/57 and 29/57, respectively; Table I) or EEV proteins (A33R and B5R, 25/57 and 26/57, respectively; Table I).

We verified the T cell responses against the four proteins in donors 09, 22, 34, and 39 using two additional assays: IFN-γ–ELISA (Supplemental Fig. 1A–D) and IL-2–ELISA (Supplemental Fig. 1E–H), and compared them with IFN-γ–ELISPOT measurement (Supplemental Table I). Significantly, in donors 09, 22, and 34, IFN-γ–ELISPOT and IFN-γ–ELISA resulted in the same response profile against A27L, A33R, B5R, and L1R. In the other donor (donor 39), IFN-γ–ELISPOT identified positive responses against B5R and L1R, whereas IFN-γ–ELISA was negative for those (Supplemental Fig. 1D). The positive responses against B5R and L1R in donor 39 were verified by the IL-2–ELISA (Supplemental Fig. 1H). Consistently, IFN-γ–ELISA and IL-2–ELISA resulted in similar response profile against these four proteins as IFN-γ–ELISPOT (Supplemental Table I).

One caveat of this assay is that we may detect IFN-γ secreted by CD8+ as well as CD4+ T cells. However, it has been shown that the potent stimulator for CD8+ T cells in ex vivo stimulating assay is peptides (62, 63), instead of whole recombinant proteins, which can be processed and presented to activate CD4+ T cells (64, 65). Also, multiple CD4+ T cell epitopes from A27L, A33R, B5R, and L1R have been identified in humans (36, 48, 57, 61), whereas CD8+ T cell epitopes were identified only for B5R and A27L (61). Moreover, the same set of donors in this study has been tested for CD8+ T cell responses against all previously identified CD8+ T cell epitopes, and only one donor (donor 44) showed positive CD8+ T cell responses against B5R epitope (63). Importantly, in our measurement, that donor did not show any responses when stimulating with recombinant B5R (Fig. 4C, arrow indicates donor).
FIGURE 1. Human cytokine/chemokine production profile followed by vaccinia virus vaccination. A total of $5 \times 10^5$ PBMCs from donors 45 d after vaccination were stimulated with heat-inactivated vaccinia virus–infected CV-1 cell lysate (solid black bar) or control noninfected CV-1 cell lysate (open bar) for 48 h. The supernatants were collected and the production of 14 cytokines/chemokines as listed in the figure was quantified by fluorescence intensity of Ab-immobilized beads as described in Materials and Methods for (A) donors 09, (B) 22, (C) 34, (D) 39, and (E) 42. Each cytokine/chemokine was distinguished by the different sizes of the corresponding Ab-immobilized beads, and the concentration was converted from fluorescence intensity using five-parameter logistic curve model (Bio-Rad, Hercules, CA). The concentrations for IFNg were out of the range of the standard curve in the Multiplex assay, which were then determined by ELISA. Each sample had three replicates.
These considerations suggest that the responses after recombinant protein stimulation measured by IFN-γ–ELISPOT were predominantly CD4+ T cell responses. For Ab responses, we also excluded the donor showing no response at day 45 to vaccinia virus Ag (1 donor) or showing a nonspecific response to BSA (1 donor). Finally, we analyzed data from 88 donors (Fig. 2B, Table I). Robust and diverse Ab responses against A27L, A33R, and B5R were also found post vaccinia virus vaccination in the majority of the donors (75, 75, and 80, respectively, of 88 donors), whereas Ab responses to L1R were

Table I. Immune responses summary

<table>
<thead>
<tr>
<th>Protein</th>
<th>Recently Vaccinated Donors</th>
<th>Nonimmunized and Long-Term Immunized Donors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+ T Cell Response</td>
<td>Ab Response</td>
</tr>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 45</td>
</tr>
<tr>
<td>A27L</td>
<td>3/57 1.3</td>
<td>27/57 17.9</td>
</tr>
<tr>
<td>A33R</td>
<td>6/57 1.4</td>
<td>25/57 19.8</td>
</tr>
<tr>
<td>B5R</td>
<td>3/57 0.3</td>
<td>26/57 17.8</td>
</tr>
<tr>
<td>L1R</td>
<td>4/57 0.9</td>
<td>29/57 16.4</td>
</tr>
<tr>
<td>Any</td>
<td>12/57 3.9</td>
<td>43/57 71.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonimmunized</td>
<td>Ab Response</td>
</tr>
<tr>
<td>A27L</td>
<td>0/4</td>
<td>0.6</td>
</tr>
<tr>
<td>A33R</td>
<td>0/4</td>
<td>1.7</td>
</tr>
<tr>
<td>B5R</td>
<td>0/4</td>
<td>1.1</td>
</tr>
<tr>
<td>L1R</td>
<td>0/4 (day 45)</td>
<td>1.4</td>
</tr>
<tr>
<td>Any</td>
<td>0/4</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Sera and PBMCs from healthy vaccinia-naive humans before (day 0) and 45 d after (day 45) vaccinia virus vaccination were prepared during a study of smallpox vaccines generated by Acambis (Cambridge, MA).

The CD4+ T cell response against each protein was considered positive if SPW stimulated with protein >2*SPW of medium and SPW (protein) – SPW (medium) >5.

The average SPW in ELISPOT assay.

The donor was considered positive for any if the donor showed positive responses against any of the proteins.

Sera and PBMCs from four nonimmunized donors and seven long-term vaccinia-immune donors were collected during a study at University of Massachusetts Medical School.
weaker, less variable, and observed at a lower frequency (41/88 donors) (Fig. 2B, Table I). Previous studies on Ab responses post vaccinia virus vaccination in humans using protein array or multiple Ags from EEV and IMV also identified A33R and B5R as the most potent targets, with A27L in the middle and least response against L1R (51, 56). The magnitude and diversity of Ab and CD4+ T cell responses that we observed are consistent with previous reports on the efficacy of the smallpox vaccines (48, 49, 51, 58), and protective immunity elicited by A27L, A33R, B5R, and L1R immunization (52–55).

No correlation between CD4+ T cell and Ab responses against each individual protein is observed

The linkage between CD4+ T cell responses and Ab responses in humans for a large and complex pathogen such as vaccinia virus is complicated by its large size and diverse responses elicited during

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**FIGURE 3.** CD4+ T cells provide intermolecular help to generate Ab responses. Ab responses (B, solid black unit bar) and CD4+ T cell responses (T, open unit bar) against (A) A27L, (B) A33R, (C) B5R, and (D) L1R in each donor were shown for the 57 donors measured for both responses. A unit bar was shown if the donor had positive responses against the corresponding protein. The number of donors showing positive responses for each protein, and matched Ab and CD4+ T cell responses against the same protein are summarized in parentheses on the top of each plot. (E–H) Ab responses against each protein (B, solid black unit bar) and CD4+ T cell responses against any of the four proteins (any-T, open unit bar) are shown for (E) A27L, (F) A33R, (G) B5R, and (H) L1R. The number of donors showing positive responses, and matched Ab and any CD4+ T cell responses in the same donor are summarized in parentheses on the top of each plot.

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**FIGURE 4.** No direct correlation between CD4+ T cell responses and Ab responses. Correlation between CD4+ T cell responses (shown as SPW) and Ab responses (shown as Ab405, absorbance at 405 nm) was analyzed for (A) A27L, (B) A33R, (C) B5R, and (D) L1R for the 57 donors measured for both responses. Pearson’s correlation coefficient and two-tailed p value were indicated in the upper right of each plot. In (C), the only donor (donor 44) that was shown to have positive responses against B5R-derived CD8+ T cell epitopes in Terajima et al. (63) is highlighted by a black arrow.
infection (48, 51, 66). This linkage in humans has yet to be investigated in detail mainly because of the lack of availability of human donors tested for both responses. In this study, we studied this linkage for the 57 donors for whom we measured both CD4+ T cell and Ab responses (Fig. 3). Our data suggest that the Ab responses against A27L, A33R, B5R, and L1R are not accompanied with matched CD4+ T cell responses against the same protein (Fig. 3A–D). For A27L, A33R, and B5R, only about half of the donors showing positive Ab responses had matched positive CD4+ T cell responses against the same protein (Fig. 3A–C, Table I). Although L1R had similar number of donors positive for Ab responses or CD4+ T cell responses, only 15 donors showed the matched pattern positive for both (Fig. 3D, Table I). Clearly, there are donors showing positive Ab responses but no CD4+ T cell response, and vice versa. One possibility of observing this nonmatched pattern would be that some donors with overall lower responses skew the linkage. Instead, the responses were extremely diverse and randomly distributed among donors (data not shown). Another possibility is that the matched CD4+ T cell help can only be observed in donors with strong Ab responses. However, when we considered only the strong Ab responses as positive, again only about half of the donors showed matched CD4+ T cell and Ab responses (Supplemental Fig. 2).

To test the hypothesis that CD4+ T cells provide intermolecular help to generate Ab responses, we plotted the Ab responses against each protein and CD4+ T cell responses against any of the four proteins (Fig. 3E–H, any-T). Additional matches were observed, but 13 donors still exhibited Ab responses in the absence of measurable CD4+ T cell responses against A27L, A33R, B5R, or L1R, suggesting that CD4+ T cells specific for other vaccinia proteins might provide help for these responses. This is consistent with the finding that diverse CD4+ T cell epitopes from vaccinia virus in humans have been found (47, 48, 61). Notably, all 43 donors showing positive CD4+ T cell responses against at least 1 of the 4 proteins also were positive for an Ab response (Supplemental Fig. 3).

It is possible that there might be a quantitative correlation between the strength of responses even if no qualitative linkage between presence or absence of responses was observed. We performed correlation coefficient analysis between CD4+ T cell and Ab responses (Fig. 4). Quantitatively, there was no direct correlation between CD4+ T cell and Ab responses against A27L, A33R, B5R, and L1R (Fig. 4A–D). The total CD4+ T cell responses also did not correlate with total Ab responses against these four proteins (data not shown). Previous studies also have demonstrated lack of correlation between CD4+ T cell memory and long-term Ab response (67, 68).

Taken together, in contrast with the observed deterministic linkage reported in mice (19), our data indicate that in humans, there is no direct linkage of CD4+ T cell and Ab targets. Instead, CD4+ T cells provide intermolecular help to generate robust and diverse Ab responses.

Responses to A27L, A33R, B5R, and L1R all contribute to neutralizing Ab titers

Neutralizing Abs are of great importance in the protection from smallpox (7). EEV surface glycoproteins A33R and B5R are targets for protective Abs in animal models (52, 53, 55, 69–71),
although B5R has been shown to be the major target for EEV-neutralizing Abs in humans (56). A27L and L1R are major targets of IMV-neutralizing Abs in both animal models (52, 53, 55) and humans (56). The overall neutralizing Ab titers for the set of human donors tested in this study were reported previously (58). By comparison, we found that all of the 54 donors with positive neutralizing Ab responses were accompanied with Ab responses against A27L, A33R, B5R, or L1R (Fig. 5A), although only 41 donors had positive CD4+ T cell responses against these 4 proteins (Fig. 5B). It is likely that CD4+ T cell responses against other proteins also can help to generate neutralizing Abs or that some posttranslation modifications in native proteins are not represented by the recombinant proteins used in this study. Consistent with previous studies, Ab responses against B5R contributed most to neutralizing Ab titers, as indicated by the 49 matched donors and 3 donors showing neutralizing Abs with only Ab responses against B5R (donors 53, 55, and 72; Fig. 5A). Also, most of the donors showing CD4+ T cell or Ab responses against A27L, A33R, and L1R were positive for neutralizing Abs (Fig. 5). Collectively, our data indicate that responses against A27L, A33R, B5R, and L1R all contribute to neutralizing Abs in humans.

The presence of responses against A27L, A33R, B5R, and L1R is linked together within donors

We next wanted to find whether the presence of responses against A27L, A33R, B5R, and L1R was linked together within donors, which would be the case if vaccinia virions rather than individual proteins were the primary recognition unit. For CD4+ T cell responses, using the experimental number of positive donors against each protein, we calculated the expected number of donors positive for each combination of these four proteins under the assumption that the presence of responses to each protein is independent. By comparing with experimental values, we found that the observed numbers of donors positive for all four proteins (10/57) and negative for all proteins (14/57) are significantly higher than that of independently expected values (2.7/57 and 4.5/57, respectively; Fig. 6A). This suggests that the presence of CD4+ T cell responses against A27L, A33R, B5R, and L1R is linked together within donors.

For Ab responses, we found that 39 of 41 donors positive for L1R are also positive for A27L, A33R, and B5R, which is significantly higher than the noncorrelated expected values (27.1; Fig. 6B). The experimental numbers of donors positive for two- or three-protein combination matched with the noncorrelated expected numbers (Fig. 6A, 6B). Consistent with CD4+ T cell responses, the presence of Ab responses against A27L, A33R, B5R, and L1R is also linked together within donors.

No linkage between CD4+ T cell and Ab responses against A27L, A33R, B5R, and L1R in long-term vaccinated donors

Finally, we looked at this linkage in long-term memory stage. A previous study on multiple Ags from EEV and IMV, including A27L, A33R, B5R, and L1R, showed that human Ab responses against these proteins decreased between 21 d and 6 mo after smallpox vaccination (56). In this study, we measured the Ab responses and CD4+ T cells responses against A27L, A33R, B5R, and L1R in four unvaccinated and seven long-term vaccinia virus–vaccinated healthy donors (vaccinated with Dryvax >4 y ago; Fig. 7). The average Ab responses in the seven long-term vaccinated donors against each protein were slightly lower than those measured 45 d after vaccination, whereas CD4+ T cell responses were ~2- to 3-fold lower (Table I), which confirmed that immunization with vaccinia virus can induce long-term immune responses to these four proteins (52–55) and the magnitude of responses decreases with time (56). None of the nonvaccinated donors showed any positive responses against any recombinant protein (Fig. 7A–D). Diverse responses against A27L, A33R, B5R, and L1R were elicited in the seven long-term vaccinated donors, and consistent with previous short-term vaccinated subjects, no deterministic linkage between Ab responses and CD4+ T cell responses was observed for each protein (Fig. 7A–D). Quantitatively, there was no correlation between CD4+ T cell responses and Ab responses against the four recombinant proteins (Fig. 7E–H).

Discussion

Despite the eradication of smallpox by widespread vaccination with vaccinia virus, the potential use of smallpox as a bioweapon (72) and the importance of using vaccinia virus as an expression vector for immunization against other infectious diseases (73–75) and cancer (76, 77) make the understanding of immune responses to vaccinia virus extremely important. In this study, we have evaluated IFN-γ–CD4+ T cell responses and Ab responses against the vaccinia proteins A27L, A33R, B5R, and L1R in a large set of vaccinia virus–vaccinated donors. Within this data set, no deterministic linkage between CD4+ T cell and Ab responses against each individual protein was observed, although the presence of responses against the four tested proteins seemed to be linked within donors.
The lack of direct linkage between CD4+ T cell and Ab responses would imply that in vaccinia-immunized donors, B cells recognize vaccinia virion rather than individual proteins to generate MHC class II epitopes for presentation to cognate CD4+ T cells. One potential argument against this model is that the vaccinia virion (∼360 nm in diameter) is much larger than typical endocytic vesicles (50–150 nm in diameter), which would result in size exclusion at the level of cellular uptake for large and complex pathogens. However, recent studies have shown that vaccinia IMV enters by fusion with plasma membrane (78), whereas EEV enters cells by macropinocytosis and nonfusogenic acid–activated membrane rupture (79–81), both consistent with the model of entire virus uptake, although the mechanisms for IMV and EEV entry are different and still in debate. Our experiments support the standard model wherein the relevant particle taken up by B cells is larger than a single protein, and our observation that responses against A27L, A33R, B5R, and L1R are linked together within donors also adds evidence to this model. A recent study of lymph nodes of mice injected with viruses revealed that subcapsular macrophages capture virus particles for transfer to B cells, and that this transfer occurs without virus internalization or degradation. This process provides a mechanism by which B cells could encounter vaccinia viruses for uptake and processing (82).

In contrast with the results presented in this study, a previous study in a mouse model demonstrated a deterministic linkage, showing that each Ab response was accompanied by a matched CD4+ T cell response targeting the same protein, as if individual protein is the recognition unit for B cells (19). We consider that there are at least three plausible reasons for why the strong linkage was not observed in our human study. The first plausible reason is the difference between mouse and human immune responses to large and complex vaccinia virus. Compared with genetically homogeneous laboratory mice housed in relatively germ-free conditions, outbred humans are genetically heterogeneous and have also great variability in their environmental exposure to other pathogens. The complexity of responses to large viruses, such as vaccinia virus, CMV, HSV, and EBV, in humans compared with mice has been extensively reviewed (83). These studies suggest that many factors, including route of infection, genetic differences, and experience encountering multiple Ags, all contribute to the greater variability of antiviral responses in humans compared with mice. The second factor is that the mouse study highlighted the
linkage of the CD4+ T cell and Ab responses in mice immunized with peptide and then challenged with vaccinia virus. However, the linkage of the responses reported in that publication is considerably less striking in mice immunized with just virus (19), which was also the case in our human study. It is possible that the deterministic linkage observed in the mouse study might reflect an alternate mechanism for B cell–T cell interaction under conditions in which a high frequency of CD4+ T cells is present or Ag presentation is dominated by fluid-phase uptake. A final plausible reason is that the mouse and human experiments evaluated different vaccinia proteins. The four proteins characterized for linkage in the mouse study, III, H3L, D8L, and L4R, are all IMV proteins. Instead, of the four proteins tested in our human study, A33R and B5R are EEV proteins, whereas A27L and L1R come from IMV, although we also did not see the linkage in either case. The hierarchy of help model specifies that Ab responses against viral surface proteins can use intermolecular T cell help from any Ag in the virion, whereas Ab responses against internal proteins are limited to intramolecular help involving Ags from that same protein (16). In the somewhat complicated case of vaccinia virus, IMV surface proteins (A27L and L1R) can be considered external proteins in the form of IMV and internal proteins in the form of EEV. The vaccines used in our study were likely to have both IMV and EEV forms, but the Western Reserve strain used in the mouse study was prepared from the supernatant of infected HeLa cells and is likely to contain predominantly EEV forms (19). Thus, at least for A27L and L1R, the lack of linkage in our human study might derive from the differences in the forms of vaccinia virus used for immunization and assay. Because of the limitations of manipulation in human donors, a study directly addressing the linkage between CD4+ T cell and Ab responses against A27L, A33R, B5R, and L1R in EEV and IMV immunized mice might distinguish the different possibilities proposed earlier. In addition, testing the linkage for a larger set of proteins from vaccinia virus in humans would help to validate our observations.

The potential for deterministic linkage of Ab and CD4+ T cell responses in the same protein has received attention for its academic and practical implications in vaccine development and mapping of T cell epitopes (36, 37). Our results show that in some demic and practical implications in vaccine development and responses in the same protein has received attention for its academic implications in vaccine development and responses against the same protein, and vice versa. Thus, CD4+ T cell and Ab responses against the same protein, and vice versa. Thus, CD4+ T cell epitope-mapping efforts directed only at Ags eliciting Ab responses might miss important immunodominant epitopes derived from Ags against which no Ab responses are made.

In summary, to our knowledge, we have for the first time in humans studied the linkage between CD4+ T cell and Ab responses to a large and complicated virus. We observed minimal linkage between CD4+ T cell and Ab responses against A27L, A33R, B5R, and L1R in vaccinated donors. However, we did observe that the presence of responses against these proteins is linked together within individual donors. These results imply that in human, CD4+ T cells provide intermolecular help to generate robust Ab responses against these four abundant and immunodominant vaccinia virus proteins.

Acknowledgments
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Disclosures
The authors have no financial conflicts of interest.

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