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Comprehensive analysis of T cell epitope discovery strategies using 17DD yellow 2 fever virus structural proteins and BALB/c (H2^d) mice model

Milton Maciel Junior[1], **Srinivasan N. Kellathur**[1,3], **Pryia Chikhlikar**[1], **Rafael Dhalia**[5], **John Sidney**[2], **Alessandro Sette**[2], **Thomas J. August**[1], and **Ernesto T. A. Marques Jr**[1,4,5,*]

1*Johns Hopkins University, School of Medicine, Pharmacology Department, Baltimore*

2*La Jolla Institute of Immunology, CA*

3*Johns Hopkins Singapore, Singapore*

4*Johns Hopkins, School of Medicine, Department of Infectious Diseases, Baltimore*

5*Oswaldo Cruz Foundation (FIOCRUZ), Instituto Aggeu Magalhaes, Recife, Brazil*

Abstract

Immunomics research uses *in silico* epitope prediction, as well as *in vivo* and *in vitro* approaches. We inoculated BALB/c (H2^d) mice with 17DD yellow fever vaccine to investigate the correlations between approaches used for epitope discovery: ELISPOT assays, binding assays, and prediction software. Our results showed a good agreement between ELISPOT and binding assays, which seemed to correlate with the protein immunogenicity. PRED$^{BALB/c}$ prediction software partially agreed with the ELISPOT and binding assay results, but presented low specificity. The use of prediction software to exclude peptides containing no epitopes, followed by high throughput screening of the remaining peptides by ELISPOT, and the use of MHC-biding assays to characterize the MHC restrictions demonstrated to be an efficient strategy. The results allowed the characterization of 2 MHC class I and 17 class II epitopes in the envelope protein of the YF virus in BALB/c (H2^d) mice.

I. INTRODUCTION

In the last decade, many groups have devoted attention to the study of the immunomes of a variety of viruses with the goal of obtaining new insights that could support the development and improvement of vaccines, viral diagnosis, and further our understanding of the immune system (Peters et al., 2005; Sette and Fikes, 2003; Sette et al., 2005).

Immunome, as defined by Sette and co-workers (Sette et al., 2005), is the detailed map of immune reactions of a given host interacting with a foreign antigen, and immunomics can be defined as the study of immunomes. The identification of the T and B cell immune response targets allows the design of antigenic formulations focused on selected targets, for instance, promoting more robust responses against subdominant epitopes contained in conserved

*Corresponding author: email emarques@jhmi.edu; emarques@cpqam.fiocruz.br.

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sequences that do not easily tolerate mutants escape. In addition, it makes it possible to avoid the presence of inhibitory epitopes (Disis et al., 1996), allows the combination of many epitopes from different proteins or organisms (Sercarz et al., 1993) and the avoidance of immunopathogenic epitopes. Hence, immunomics offers a powerful approach to the rational design of vaccines (Sette and Fikes, 2003).

In addition to prediction software approaches (Moutaftsi et al., 2006; Yewdell, 2006), ELISPOT (Anthony and Lehmann, 2003), flow cytometry (Hoffmeister et al., 2003), binding assays (Sidney et al., 1998), and human leukocyte antigen (HLA)-transgenic mice (Sette and Fikes, 2003) are the most frequently used tools for epitope discovery. Among those, ELISPOT offers many positive advantages with regards to characterizing the immune response (e.g. looking at different cytokines and/or subpopulations of cells), by offering some degree of automation, broader options for readouts (e.g., IFN-γ, IL-2, and IL-4), the screening of large number of peptides, and rapid results (Anthony and Lehmann, 2003). Although the length of the peptides used in ELISPOT assays can vary from 9 to 20 amino acids, libraries of 15 aminoacids (15mers) with an offset of 11 amino acids seems to represent the best compromise between epitope coverage of a protein sequence and the ability to detect $CD4^+$ and $CD8^+$ responses (Kiecker et al., 2004).

Several factors are known to modulate the repertoire of T cells responses, such as the protein's three-dimensional structure, its processing, presentation, MHC-peptide affinity, T cell receptor (TCR) diversity, and avidity. These factors can be highly affected by how the antigen formulations are prepared and presented to the host organism. Therefore, a high-quality characterization of an immunome must include the testing of multiple immunization protocols using antigens inoculated via different routes and doses, with the immune responses tested at different points in time (Assarsson et al., 2007). Since it is nearly impossible to test all the potential antigen formulations and screen all the peptides that might induce responses, it is important to include in the immunome study reductionist approaches that are subjected to fewer variables. One of the most commonly used approaches is the biochemical MHC-peptide binding assay. The direct *in vitro* evaluation of peptides that can bind MHC molecules, as determined by competition assays, is likely to identify a larger pool of possible epitopes that would not actually bind *in vivo*, due to various processing and chaperone mechanisms, which are part of the immunodominant shaping mechanisms (Yewdell, 2006).

The use of prediction software has also become a very important tool for epitope discovery. Such software are intended to save time and money by allowing investigators to pre-screen the sequence of a protein or a list of peptides for the presence of possible targets for the immune response. Different mathematical models are used in the search for peptides that can bind to MHC molecules with high affinity. These models can be as simple as matrices of frequencies of individual amino acids in a given position in a peptide sequence or as complex as artificial neural network models; this topic has been reviewed elsewhere (Braga-Neto and Marques, 2006).

The YF virus is an interesting target for immunome studies, with a number of useful relevant tools available that facilitate these studies, such as an effective human live-attenuated virus vaccine and accepted animal challenge models. YF is a deadly arthropod-borne virus of the flavivirus family that is currently circulating in tropical and subtropical areas of the world (Figueiredo, 2007; Monath, 2001; World Health Organization, 2003). The 17D, 17D-213, and 17DD, YF strains used for human vaccination are products of multiple passages of the virulent parental Asibi virus (Galler et al., 1997). The molecular basis of the attenuation has been identified, and all the vaccine strains have been shown to differ from the wild-type virus in a few amino acids (dos Santos et al., 1995; Galler et al., 1997; Jennings et al., 1993; Post et al., 1992). The strain 17D has been successfully used worldwide for more than 65 years. It has an

extraordinary record of safety, conferring long-lasting immunity for up to 35 years in as many as 99% of those vaccinated (Poland et al., 1981; World Health Organization, 2003).

The humoral neutralizing response against the YF envelope protein, one of its three structural proteins, has been recognized as one of the major mechanisms to account for the protection elicited by the vaccine (Monath, 1986; Monath and Barrett, 2003), although data increasingly point to an important role for the cellular arm of the immune response in protection against infection (Co et al., 2002). A few MHC-restricted T cell epitopes have been described in mice (Regner et al., 2001; van der Most et al., 2002) and humans (Co et al., 2002), but their contribution to the immunity induced by the vaccine is still not understood. It was recently reported that the YF 17D vaccine can infect dendritic cells (DC) (Barba-Spaeth et al., 2005) and is able to activate innate immune responses through toll-like receptors (TLR) (Querec et al., 2006).

We used the 17DD YF virus as a surrogate model for our immunomics studies of the YF virus structural proteins. Murine T cell responses were evaluated *ex vivo* through ELISPOT IFN-γ assays with overlapping peptide libraries encompassing the entire length of each of the three YF structural proteins (envelope, capsid, and membrane). These results were then compared to a computational T cell epitope prediction method, $PRED^{BALB/c}$, which is based on binding matrix models for each of the five MHC alleles present in the BALB/c mouse, and to binding assay results for all five H2-D alleles. All the epitopes described in this study can be found in the Immune Epitope Database –IEDB (http://www.immuneepitope.org/home.do) (Peters et al., 2005).

III. RESULTS

Evaluation of the kinetics and dose-response of BALB/c mice to the immunization with the human 17DD YF vaccine

In order to define a suitable immunization protocol for immunome studies using the human 17DD YF virus, groups of BALB/c mice were immunized three times, at 2-week intervals, with either a high (10^5 PFUs) or low dose (10^4 PFUs) of the 17DD YF vaccine and were assayed *ex vivo* by IFN-γ ELISPOT assay using 16mer peptides from the YF envelope protein, (Supplemental Table I). A detectable T cell response was observed in both mice groups with the high and low doses 14 days after the first immunization (Supplemental Fig. 1A and B). The second immunization led to a more focused T cell response to fewer 16mer peptides (Supplemental Fig. 1C and D) and the third immunization did not change the pattern observed after the second immunization (Supplemental Fig. 1E and F). The evaluation of the humoral response after each immunization (data not shown) indicated that after two immunizations with either the high or low dose of the vaccine, both groups reached similar levels of IgG anti-YF (ELISA with virus lysate), and that the third immunization did not enhance the antibody levels. The results of these experiments (Supplemental Fig. 1) indicated that an immunization protocol with two low dosed of the 17DD YF vaccine given three weeks apart would be enough to induce an optimal T cell response, and hence, such protocol was applied in the subsequent experiments, except when indicated.

Analysis of $CD4^+$ and $CD8^+$ responses to the YF envelope protein peptides

Seven days after the second immunization, the splenocytes of the mice receiving two low doses (10^4 PFUs) of the human 17DD YF vaccine 3 weeks apart were assayed by ELISPOT with 15mer peptides (15×11) of the 17DD YF envelope protein (Supplemental table II). A high number of IFN-γ SFC was observed against several peptides of the envelope protein (Fig. 1A) and the main positive responses were directed to the same regions in the YF envelope protein previously observed with the 16mer peptides (16×10) (Supplemental Fig. 1).

We further characterized the cellular response using splenocytes depleted of $CD4^+$ or $CD8^+$ lymphocytes. The depletion approach consistently yielded >95% of depletion, as judged by flow cytometry (data not shown). CD4-depleted cells, which were used to address the $CD8^+$ response, showed significant numbers of IFN-γ SFC in response to three of the peptides (Fig. 1B), while the CD8-depleted cells, which correspond to the $CD4^+$ response, showed significant numbers of IFN-γ SFC in response to 17 peptides (Fig. 1C). These results were compatible with the findings obtained with total splenocytes (Fig. 1A) and characterized the immunogenic peptides either as MHC class I or class II antigens. As an additional control, experiments were performed with purified $CD4^+$ or $CD8^+$ T cells incubated with splenocytes from naïve mice as APC, and the ELISPOT results were comparable to those obtained with depleted populations (data not shown). Two of the peptides ($E_{57–71}$ and $E_{61–75}$) promoted IFN-γ secretion from both $CD4^+$ and $CD8^+$ T cells, suggesting that these 15mers contain epitope motifs for class I and class II molecules, whereas the remaining immunogenic peptides induced either $CD4^+$ or $CD8^+$ responses.

The T cell repertoire of the immunized mice was also investigated at 4.5 and 9 months after the initial immunization protocols, with or without a recall boost right before the experiments (Supplemental Fig. 2). In these long-term memory experiments, we observed the same set of immunogenic peptides that was found seven days after the second 17DD YF immunization. The peptides corresponding to the $CD8^+$ response appeared to be the most dominant immune responses present at all times tested.

Characterization of the $CD8^+$ responses to the YF envelope protein peptides

The $CD8^+$ T cells preferentially recognize peptides of 8–10 amino acids present in the groove of MHC class I molecules. Therefore, the pattern of $CD8^+$ response to 15mers does not define the minimal sequences that are being recognized. To address this issue, we examined how $CD8^+$ T cells from mice immunized with our standard 17DD YF virus protocol react *in vitro* with all the possible 9mers that are present within each of the three CD8-immunogenic 15mer peptides (Table I; Supplemental Table V). For the first two CD8-immunogenic 15mers, $E_{57–71}$ and $E_{61–75}$, we considered the amino acid sequence between position E_{57} and E_{75} of the envelope protein, which results in 11 peptides of 9 amino acids overlapped by 8 amino acids (9×8). For the third CD8-immunogenic 15mer ($E_{329–343}$), the sequence between E_{329} and E_{343} was considered, i.e., 7 peptides of 9 amino acids, overlapped by 8 amino acids.

The results of the IFN-γ ELISPOT assays using 9mer peptides to stimulate the *in vitro* responses are presented in Figures 2A and B. Within the first set of 9mers (E_{57} to E_{75}), the 9mer peptide $E_{60–68}$ induced the highest number of IFN-γ SFC, while in the second set of 9mers ($E_{329–343}$), the peptide $E_{330–338}$ stimulated the highest number of IFN-gamma SFC. However, in both 9mer sets, other 9mer peptides were also able to stimulate a significant number of IFN-gamma SFC. In order to determine how the concentration of peptide used in the ELISPOT assay could influence the results, we conducted a second round of experiments using decreasing *in vitro* concentrations of peptide (10, 1, and 0.1μg/mL) (Fig. 2C and D). In these experiments, peptide $E_{60–68}$ induced the highest number of IFN-γ SFC at all concentrations tested, showing a high *in vitro* stimulatory activity for $CD8^+$ T cells of mice immunized with the 17DD YF virus (Fig. 2C). Peptide $E_{64–73}$ showed an intermediate stimulatory level, while all the other peptides in the same set induced measurable IFN-γ secretion only at the highest concentration of 10μg/mL.

In the second set of 9mer peptides, $E_{332–340}$ showed the strongest $CD8^+$ stimulatory response with the peptide concentration of 10μg/mL (Fig. 2D). Contrarily to what was expected, peptide $E_{330–338}$, which consistently induced the highest number of IFN-γ SFC at 10μg/mL in the ELISPOT assays, showed only an intermediate stimulatory curve. Peptide $E_{335–343}$ also

showed an intermediate stimulatory activity, while the remaining peptides induced only a very low response, and only at 10μg/mL.

Taken together, the results pointed out that the 9mer $E_{332–340}$ was significantly less potent than $E_{330–338}$ to activate a large number of IFN-γ SFC after *in vitro* stimulation at 10μg/mL (Fig. 2B), whereas it could activate relatively higher number of IFN-γ SFC at lower peptide concentrations (Fig. 2D). It suggests that, in animals immunized with the 17DD YF vaccine, peptides $E_{332–340}$ and $E_{330–338}$ may be stimulating different populations of $CD8^+$ T cells. The $CD8^+$ T cell population activated by $E_{332–340}$ seems to be smaller in number, but with a higher avidity for its MHC-peptide complex then the $CD8^+$ T cell population stimulated by $E_{330–338}$. In these conditions, it is possible that two partially overlapping class I epitopes exist. However, we can not discard the hypothesis that the *in vivo* processing and availability of these two epitope are different after the immunization with the 17DD YF vaccine, leading to the priming and generation of different numbers of $CD8^+$ T cell clones with distinct MHC-peptide affinities.

In order to determine whether antigen peptide processing of the 17DD YF virus is affecting the availability of the peptides and therefore influencing the pattern of the *in vitro* recognition and activation of the cells, mice were immunized with each individual 9mer present in the two sets of immunogenic peptide sequences that produced the highest $CD8^+$ responses, namely E_{57} to E_{71} and E_{329} to E_{343}. Additional control groups were immunized with a mixture of all 9mers pooled together and with the 15mers from which the 9mers were generated. The cells of each group were then tested in ELISPOT assays with decreasing concentrations of all peptides, as means of evaluating the avidity of the CD8 response (Fig. 3).

Among the seven 9mers in the first set, peptides $E_{57–65}$, $E_{62–70}$ and $E_{63–71}$ failed to induce any response after individual immunization (Fig. 3A, F, G). Other four peptides, $E_{58–66}$, $E_{59–67}$, $E_{60–68}$, and $E_{61–69}$ were able to induce IFN-γ SFC (Fig. 3B–E). Peptide $E_{60–68}$, as previously observed, showed a very high avidity in the ELISPOT assays, inducing high numbers of IFN-γ SFC at all concentrations of the peptide used for the *in vitro* stimulation (Fig. 3D). The immunization with peptides that were closely related to peptide $E_{60–68}$ was also able to induce cells that preferably responded to peptide $E_{60–68}$, suggesting that a sharing of binding motifs may be the mechanism behind the indirect stimulations. As expected, direct immunization with the 15mer $E_{57–71}$ also induced a strong response to peptide $E_{60–68}$, further supporting the concept that this sequence contains the class I motif needed for the CD8 response (Fig. 3I).

The immunization with the 9mers within the E_{329} to E_{343} region produced results distinct of those observed after immunization with 17DD YF virus (Fig. 4). The peptides $E_{329–337}$, $E_{331–339}$, $E_{333–341}$, and $E_{334–342}$ were unable to elicit a significant response, as evidenced by the very low numbers or total lack of IFN-γ SFC after *in vitro* stimulation with the respective peptides (Fig. 4A, C, E, F). Peptide $E_{330–338}$, which induced a very strong stimulation after immunization with the 17DD YF vaccine, was less immunogenic when administered as a 9mer peptide emulsified in adjuvant (Fig. 4B). In contrast, the immunization with peptides $E_{332–340}$ and $E_{335–342}$ activated relatively higher numbers of IFN-γ SFC (Fig. 4D and G) then the immunization with $E_{330–338}$, to the *in vitro* stimulation with the respective peptides. Of note, peptides $E_{332–340}$ and $E_{335–343}$ were able to stimulate the secretion of IFN-γ in a cross-reactive fashion, in that both peptides could cross-stimulate the IFN-γ secretion in mice individually immunized with either one of them. Moreover, $E_{332–340}$ and $E_{335–343}$ were preferably recognized by splenocytes from mice immunized with the pool of 9mers, as well as those immunized with 15mer $E_{329–343}$ (Fig. 4H, I). The numbers of IFN-γ SFC obtained with $E_{332–340}$ were very high, even with the lowest *in vitro* concentration of 0.1μg/mL (Fig. 4D, G–I), paralleling the avidity curve previously obtained (Fig. 2D). These results further support the possibility that $E_{330–338}$ and $E_{332–340}$ are different epitopes and suggest that either

processing of the peptides and/or pos-translational modifications of the 17DD YF virus interfere with presentation of these epitopes.

Biochemical characterization of the class I epitopes of the YF envelope protein by binding assay

Subsequently, the affinity between the MHC class I molecules and the immunogenic 9mer peptides were evaluated in binding assays, by directly measuring the affinity of each peptide to all three MHC class I molecules present in the BALB/c mouse strain (Table I). All eighteen possible 9mers between residues E_{57} and E_{75} and between E_{329} and E_{340} were assayed to determine their binding affinities to H2-D^d, -K^d, and L^d molecules.

The binding assay results (Table I) demonstrated that peptide $E_{60–68}$ has the highest affinity of all the peptides tested, being able to inhibit the binding of the competitor peptide to the H2-K^d molecule at 1nmol, a result that was consistent with the findings from the ELISPOT assays. In addition, adjoining peptides $E_{59–67}$ and $E_{61–69}$, positioned right before and after $E_{60–68}$ and consequently sharing eight amino acids, also exhibited some degree of affinity for H2-K^d (211 and 93nmol, respectively); these results could explain why those peptides were also able to stimulate IFN-γ secretion, even without a high avidity, as observed in the ELISPOT assays (Fig. 2C), and also suggest that 8–10mer peptides covering that region can be immunogenic.

Among the 9mers present in the sequence E_{329} to E_{343}, peptide $E_{332–340}$ exhibited the highest affinity (IC_{50}=56nmol) for the H2-L^d molecule, while all the other peptides had an IC_{50} higher than the minimum cutoff value of 500nmol to be considered a good class I binder (Table I). Curiously, neither peptide $E_{330–338}$ nor $E_{335–340}$ performed well in terms of binding to any of the class I alleles, although they were able to stimulate IFN-γ SFC in the ELISPOT assays with splenocytes of mice immunized with 17DD virus or peptides, even at low peptide concentrations (Fig. 2 and 4).

Characterization of the $CD4^+$ peptides in mice immunized with the 17DD YF vaccine

The seventeen 15mer immunogenic peptides that had been considered positive in ELISPOT assays with splenocytes and with CD8-depleted cells (Fig. 1C) of mice immunized with the 17DD YF vaccine were further characterized. The peptides were tested in ELISPOT assays with decreasing *in vitro* concentrations (Supplemental Fig. 3). Peptides $E_{57–71}$, $E_{61–75}$, $E_{129–143}$, and $E_{133–147}$ were able to induce a high $CD4^+$ response even when stimulated *in vitro* 1 or 0.1μg/mL; the other peptides showed only an intermediate or low level of stimulatory activity.

Next, the affinity of the CD4-immunogenic peptides to the $H2^d$ class II molecules I-A^d and I-E^d was tested by binding assays (Table II). Peptides $E_{129–143}$, $E_{133–147}$, $E_{233–247}$, $E_{237–251}$, $E_{325–439}$, and $E_{465–479}$ showed affinities lower than 1000nmol (cutoff for MHC class II binders). Two of then, peptides E_{129-14} and $E_{133–147}$, were among the ones that also showed immunogenicity in ELISPOT assays at low peptide concentrations. All other immunogenic peptides tested showed a low affinity in the binding assays (IC_{50}>1000nmol). It is important to note that peptides $E_{129–143}$ and $E_{133–147}$, and $E_{233–247}$ and $E_{237–251}$ are adjacent and therefore share 11 amino acids, which may explain why both peptides in the two pairs showed similar IC_{50} values, as well as why they induced high numbers of IFN-γ SFC after the immunization with the 17DD YF vaccine. All six peptides below the cutoff showed a high affinity for I-A^d suggesting that these peptides are being presented by the MHC class II I-A^d molecule. In addition, peptide $E_{233–247}$, also showed a high affinity for I-E^d (Table II) suggesting that this peptide may be presented by both, I-A^d and I-E^d molecules.

Comparison between the ELISPOT results and PRED$^{BALB/c}$ prediction software results for the YF envelope protein

Prediction software is generally used before *in vitro* testing in order to identify possible MHC binder peptides in a protein or to test the likelihood of a specific peptide inducing an immune response when tested by methods such as ELISPOT. Here, we used PRED$^{BALB/c}$ after our ELISPOT and binding experiments in order to evaluate how well the software would perform in anticipating the positive peptides, as well as in avoiding testing of peptides that did not induce a positive response. Thus, we evaluated the performance of PRED$^{BALB/c}$ to predict possible binders to all the H2^{d} alleles (D^{d}, K^{d}, L^{d}, I-A^{d}, I-E^{d}). We subjected the list of 120 15mer peptides from the envelope protein of the 17DD YF virus (Supplemental table II) to PRED$^{BALB/c}$ prediction analysis for class I and class II alleles, independently. It is important to note that in our simulation, the use of either1 the list of 15mers or the whole sequence of the envelope protein rendered comparable results, possibly due to the high degree of coverage offered by the scheme of 15 amino acids overlapping 11 (Kiecker et al., 2004).

The software analyzed all possible 9mers within each peptide, ranking each peptide according to the highest score found (scores range from 1 to 10) (Zhang et al., 2005). For each one of the alleles, an output result list with 120 scored peptides was generated. Once these lists of peptides were sorted from high to low score, we compared each list to the ELISPOT results (Fig. 1), attempting to visualize the PRED$^{BALB/c}$ score threshold that eliminated the largest number of negative peptides while keeping the positive peptides.

In the case of the class I prediction results for the 120 15mer peptides from the envelope protein, a threshold score of 9 eliminated all positive peptides, while a threshold of 8 included all three positive 15mers observed in the CD8 response ($E_{57–71}$, $E_{61–75}$, and $E_{329–434}$; Fig. 1B). After removing all the repeated peptides in each allele's list, the PRED$^{BALB/c}$ indicated 91 peptides as highly probable binders regardless of the class I allele. If the 91 peptides had been assayed instead of 120 15mer peptides, this approach would have reduced by 24% the number of peptides to test by ELISPOT.

We further evaluated the discrimination of the possible binders found in the PRED$^{BALB/c}$ results by comparing the results produced by the software to all 9mer peptides that were eventually used to characterize the CD8^{+} response by ELISPOT (Fig. 2), as shown in Table I. The software correctly predicted $E_{60–68}$ (score 8.66 for H2-K^{d}), which was positive by ELISPOT and also presented a high affinity for H2-K^{d} in binding assays. However, it failed by positively predicting peptide $E_{62–70}$ (score 8.42 for H2-D^{d}), which was not positive by ELISPOT or by binding assay. Peptide $E_{331–339}$ was predicted (score 8.50 for H2-D^{d}) although it induced only a secondary ELISPOT response and showed no affinity in the binding assay. On the other hand, peptide $E_{330–338}$, which induced a very strong ELISPOT response, was not predicted by the software (Table I).

The overall comparison of the ELISPOT results, binding assay results, and PRED$^{BALB/c}$ predictions can be seen in the Fig. 7. The diagram illustrates the differences in immunogenicity as defined by ELISPOT responses, epitope prediction, and binding assays.

The analysis of the immunogenic peptide sequences revealed that some of them contained known anchor residues, which in the case of the MHC class I molecules, were consistent with the MHC restrictions found in the binding assays (Supplemental table VI). However, the MHC class II restriction of 15mer peptides was less evident since anchor motifs for both I-A^{d} and I-E^{d} could be found in a same peptide (Supplemental Table VII).

Analysis of the T cell response to the YF virus capsid and membrane protein peptides

As described before, BALB/c mice were immunized twice with the 17DD YF vaccine, 3 weeks apart. The ELISPOT experiments were performed with a set of 15mers, overlapping by 11 amino acids, which comprise the entire length of the capsid (Supplemental Table III) and membrane proteins (Supplemental Table IV).

Figures 5A and B show the profiles of the responses of CD4- and CD8- depleted cells to the capsid peptides in IFN-γ ELISPOT assays. Peptide $C_{33–47}$ corresponded to the $CD8^+$ response while three other peptides, $C_{61–75}$, $C_{77–91}$, and $C_{81–95}$, were positive for the $CD4^+$ response. Among those, $C_{77–91}$ and $C_{81–95}$ showed a high affinity for the MHC class II I-A^d molecule in binding assays (Table III). In addition, other peptides showed a high affinity for MHC class II I-A^d and I-E^d alleles, as seen on Table III. Unfortunately, the binding properties of the 9mers within the CD8 positive region C_{33} to C_{47} were not evaluated because these peptides were not available.

The analysis of the capsid 15mer peptides using the $PRED^{BALB/c}$ software yielded 18 peptides above the $PRED^{BALB/c}$ software threshold of 8 (18/28) with probable class I binding motifs and 27 peptides above the threshold of 9 (27/28) with probable class II binding motifs (data not shown). Although the prediction algorithm produced a high number of false-positives for the capsid protein, all the peptides that were defined as immunogenic by ELISPOT were found in the software prediction list. (Fig. 6B).

The number of IFN-γ SFC for the 15mers from the YF virus membrane protein were overall relatively very low in the BALB/c mice immunized with the 17DD YF vaccine as compared to the responses elicited against the envelope protein, and no peptide was considered positive by ELISPOT (Fig. 5C and D). However, the binding analysis of the membrane 15mer peptides for class II molecules indicated that several peptides bound to I-A^d (results on Table IV). The $PRED^{BALB/c}$ software predicted 10 peptides for class I epitopes above a threshold of 8 (10/16) and 12 peptides for class II epitopes with a threshold above 9 (data not shown). Among the 12 peptides predicted to have class II epitopes, 6 showed high affinity in the binding assays (Table IV; Fig. 6C).

IV. DISCUSSION

Others studies have previously analyzed the immune response to the YF vaccine using different immunization protocols with higher doses of the YF virus (van der Most et al., 2002). In those investigations, only a limited number of epitopes were reported; thus, it appears that the immunization protocol or infection model may be an important variable determining the epitope repertoire, and protocol variations can lead to differences in the results obtained. However, it is also possible that differences in the sensitivity of the readout assays used (e.g. flow cytometry versus ELISPOT) as well as the strain of the virus and mouse strain used, could account for the observed results.

The immunogenic $CD8^+$ peptides found among the YF envelope protein elicited higher numbers of IFN-γ SFC than the immunogenic $CD4^+$ peptides did. This pattern was also observed in the memory experiments, in which the peptides inducing responses in $CD8^+$ T cell led to the highest numbers of IFN-γ SFC. The two most immunodominant peptides were able to stimulate both $CD8^+$ and $CD4^+$ T cells to secrete IFN-γ ($E_{57–71}$ and $E_{61–75}$) in 17DD YF vaccine immunized mice, likely boosting the immune response by allowing a closer level of collaboration between the cytotoxic and helper arms of the immune response.

The sequence $E_{60–68}$ was shown to promote the highest number of IFN-γ SFC in 17DD YF vaccine immunized mice. However, other related 9mer adjacent peptides were also

immunogenic. It has previously been demonstrated that peptides of 8 to 10 amino acids can bind $H2^b$ class I alleles ($H2\text{-}K^b$ and $\text{-}D^b$) (Moutaftsi et al., 2006), and likely this is also true for $H2^d$ class I alleles. The ELISPOT assays using decreasing *in vitro* concentrations of peptides may provide a measure of the avidity between the MHC-peptide complex and the TCR (Hesse et al., 2001). At the same time, this approach can indirectly reflect the affinity of the TCR-peptide interaction (Hesse et al., 2001), which can lead to distinct cell responses, as well as to different degrees of activation (Wilson et al., 2004). Our results showed that 9mer $E_{60–68}$ not only induced the highest number of IFN-γ SFC but also had the highest dose-response curve, indicating a very high avidity for the MHC-peptide[$E_{60–68}$]:TCR complex in 17DD YF vaccine immunized mice. Peptide $E_{64–72}$ showed an intermediate level of avidity, while all the other 9mers had a very low avidity, supporting the conclusion that the results can be dependent on the *in vitro* peptide concentration used in the ELISPOT assays. However, a biological role for the responses that were observed only at high *in vitro* concentrations cannot be disregarded. The additional binding data showed that peptide $E_{60–68}$ indeed bound the class I allele $H2\text{-}K^d$ with very high affinity and that the adjoining peptides, $E_{59–67}$ and $E_{61–69}$, also had a high affinity for $H2\text{-}K^d$, despite the fact that they showed a low dose-response curve in the ELISPOT assays.

Peptide $E_{330–338}$, which induced the high number of IFN-γ SFC in mice immunized with the 17DD YF vaccine, showed only an intermediate dose-response curve with decreasing peptide concentrations in the ELISPOT, suggesting that the MHC-peptide[$E_{330–338}$]:TCR avidity was low. This peptide also failed to induce a strong immune response when used directly in immunization protocols, and its recognition was inhibited when the immunization was performed in the presence of other peptides covering the adjacent amino acid sequences (Fig. 5H). Moreover, $E_{330–338}$ also had a low affinity in the binding assay. On the other hand, peptide $E_{332–340}$, which activated about half of the number of IFN-γ SFC induced by $E_{330–338}$, showed a very high ELISPOT avidity curve in 17DD YF vaccine immunized mice and had very high affinity for $H2\text{-}L^d$, as determined in the binding assays. Furthermore, peptide $E_{332–340}$ was able to induce a very strong and dominant immune response when used directly in the immunization protocol with adjuvant (Fig. 4D). These results point out the benefits of performing ELISPOT assays with different concentrations of peptides, after a first round of screening. This approach can identify peptides that only induce responses at high concentration, as well as evaluate the peptide avidity.

The identification of a class I epitope in E_{329}–E_{343} of the YF virus envelope protein is partially supported by previous work of Rothman and colleagues, who mapped the $H2^d$ class I epitope ^{331}SPCKIPFEI^{339} in the envelope protein of the dengue-2 virus, another member of the flavivirus family (Rothman et al., 1996). Although YF and the dengue virus do not share complete amino acid identity in the 331–339 region of the envelope protein, the presence of a class I epitope at the same position in the envelope protein in both viruses suggests a selective evolution of the immune system to target the same region.

Peptide $E_{335–343}$, which induced a low number of IFN- γ SFC and presented an intermediate ELISPOT avidity curve after immunization with 17DD YF vaccine (Fig. 2D), was able to induce a vigorous immune response when used to immunize mice directly (Fig. 4G). Interestingly, in the direct immunization with 9mer peptides plus adjuvant, $E_{332–340}$ and $E_{335–343}$ induced IFN- γ SFC in a cross-reactive fashion (Fig. 4D and G), even though $E_{335–343}$ apparently did not seem to be a good MHC binder in the *in vitro* binding assay. Other studies have demonstrated that the binding affinity in biochemical assays does not always correlate with cytotoxic potential and that, for some epitopes, a lower affinity favors a higher level of cytotoxicity by $CD8^+$ T lymphocytes (Ochoa-Garay et al., 1997). In this sense, the identification of low affinity epitopes can be useful in vaccine development.

Recently, two groups described protective MHC class I restricted-epitopes in the envelope of the West Nile flavivirus in the context of $H2^b$ alleles (C57Bl/6 mouse), (Brien et al., 2007; Purtha et al., 2007). There is no data supporting the protective role of the citotoxic response against YF and it would be interesting to test if the MHC class I epitopes described here can confer some degree of protection to the YF infection. A better characterization of the cellular response against the YF could lead to new vaccine formulations and new parameters of protection besides the humoral response used today.

It is possible that the broader repertoire of the CD4-positive peptides observed here is partially responsible for the strong humoral response observed against the YF envelope protein (Monath, 1986; Monath and Barrett, 2003), which can provide help to B cells that recognize neutralizing epitopes. In fact, Zhao and colleagues have demonstrated that class II epitopes of the SARS coronavirus nucleoprotein are able to support the production of neutralizing antibodies against the virus (Zhao et al., 2007). It is possible that other cytokines such as IL-4 and IL-5 are also secreted in response to other peptides of the envelope protein. Furthermore, the $CD4^+$ T cells activated by peptides of the envelope protein can help support $CD8^+$ T cell cytotoxic clones that are important to the defence against the virus as well as contributing to the memory response (Supplemental Fig. 2) (Johansen et al., 2004; Sun et al., 2004).

The fact that each of the CD4-immunogenic peptides produced different dose-response curves in the ELISPOT experiments suggests that the MHC-epitope complex can exhibit distinct avidities for the $CD4^+$ T cells TCRs (Hesse et al., 2001), a situation that can have important implications in the choice of epitopes for vaccine development. It is important to note that the precise definition of class II epitopes is somewhat less clear than class I epitopes. In fact, mouse MHC class II alleles have been reported to be able to accommodate a variety of motifs (Kurata and Berzofsky, 1990).

It has been previously reported that the envelope protein and non-structural protein 3 (NS3) are the main targets for the immune response against the YF virus (van der Most et al., 2002). Here we have extended these observations by evaluating the cellular response against the three structural proteins of the virus. In fact, the number of immunogenic peptides in the YF envelope protein found in 17DD YF virus inoculated mice was by far larger than the numbers found in the capsid and membrane proteins. These results also underscore the secondary nature of the immune response against the capsid and membrane proteins of the YF virus when compared to the envelope protein.

$PRED^{BALB/c}$ is an *in silico* peptide identification tool that uses quantitative matrices based on binder and non-binder peptide training datasets to compose equations based on the frequency of amino acids at specific positions within the training set of 9mer peptides (Zhang et al., 2005). In our hands, the software showed to be very sensitive, predicting almost all positive peptides. However, the ability of the software to eliminate negative peptides was low and, if used before the ELISPOT assays, it would have avoided the testing of only about one-quarter of the 120 15mers of the YF envelope protein. In another system, given a list of 41 peptides from the nucleoprotein of the SARS coronavirus, $PRED^{BALB/c}$ predicted 17 peptides with possible class II epitopes, and two peptides were found to be positive by *in vitro* epitope mapping (Zhao et al., 2007). Taken together, these results suggest that the $PRED^{BALB/c}$ prediction algorithm is indeed very sensitive, but its specificity still needs to be improved.

In conclusion, the results of our epitope mapping of the three structural proteins of the YF virus in BALB/c ($H2^d$) mice suggest that initial computational screening with high thresholds, followed by peptide screening of ELISPOT responses in immunized hosts and by binding assays, may prove to be the most efficient strategy for epitope discovery and analysis. In

addition, the results presented here have the potential to identify novel correlates for YF protection in a mouse strain that is commonly used for the validation of YF vaccines.

II. MATERIAL AND METHODS

YF vaccine and peptides

The human YF vaccine, composed of attenuated 17DD YF virus, was a generous gift from Dr. Ricardo Galler (Oswaldo Cruz Foundation, Rio de Janeiro, Brazil). The vaccine was reconstituted with chilled PBS, kept in an ice bath, and used for immunization of mice within 4 h of reconstitution.

A set of 81 peptides of 16 amino acids (16mers), overlapping by 10 amino acids (16×10) and comprising the entire length of the envelope protein (E) of the YF virus (sequence NCBI entry U17066), was synthesized by Synpep (California, USA) (Supplemental Table I). The purity of this peptide set was stated by the manufacturer to be 70% or lower, it was not HPLC-purified, and the amount of peptide per vial was not provided.

Additional sets of peptides of 15 amino acids (15mers), overlapping by 11 amino acids (15×11) and comprising the entire length of the envelope (n=120) (Supplemental Table II), capsid (C; n=28) (Supplemental Table III), or membrane (M; n=16) (Supplemental Table IV) proteins of the 17DD YF virus (sequence NCBI entry U17066), were synthesized by Schafer-N (Copenhagen, Denmark). Peptides of nine amino acids (9mers), overlapping by 8 amino acids, were synthesized for regions E57 to E75 ($E_{57–75}$) and E329 to E343 ($E_{329–343}$) of the YF envelope protein (Supplemental Table V). These peptides were HPLC-purified to 80% purity or greater, with the exception of a few peptides that could not be purified and were used as crude products. The identity of each peptide was confirmed by mass spectrometry, and the amount of purified peptide was precisely measured. Stock solutions of all peptides were prepared by dilution in water when possible, or in a solution of 10 to 100% of DMSO, to a final concentration of 20mg/mL, and stored at −20°C. For the ELISPOT assays, the peptides were used at 10, 1, or 0.1μg/mL as indicated. The maximum DMSO concentration in the ELISPOT assay was 0.05%.

Animals and immunization protocols

Female BALB/c ($H2^d$) mice, 6 to 8 weeks old (Charles River, Kingston, NY), were used in the immunization protocols. They were housed in micro-isolator cages under specific pathogen-free conditions and manipulated according to Johns Hopkins Institutional Animal Care and Use Committee (IACUC) protocol number MO05M336 Three different immunization protocols were used: 1) Mice were immunized three times subcutaneously (s.c.) at the base of the tail with 10^5 or 10^4 PFUs of the human YF vaccine (strain 17DD, Oswaldo Cruz Fondation, Rio de Janeiro, Brazil) in 50μL on days 0, 14 and 28. 2) Mice were immunized s.c. with 10^4 PFUs of the vaccine on days 0 and 21. Memory experiments were performed with animals immunized s.c. with 10^4 PFUs of the vaccine on day 0, with or without a boost on day 21, and boosted or not after 4.5 and 9 months. 3) For the evaluation of epitope avidity/specificity by ELISPOT assay, BALB/c mice were immunized once at the base of the tail with 100μL of an emulsion v/v of TiterMax® adjuvant (CytRx Corporation, Norcross, GA) containing 50μg of an individual 9mer, a pool of 9mers (50μg of each), or the 15mer $E_{57–71}$, $E_{63–75}$ or $E_{329–343}$ (50μg) from the YF envelope protein, or PBS alone. Mice immunized with PBS emulsified in TiterMax® served as a negative control. The experiments were performed 15 and 30 days after the immunization.

ELISPOT assays for enumeration of IFN-γ spot-forming cells (SFC)

Seven to 10 days after the last immunization, the mice were killed and their spleens were removed. Splenocytes were isolated by standard methods, and single-cell suspensions, depleted of red blood cells, were prepared from freshly isolated splenocytes in culture medium (RPMI 1640 medium supplemented with 10% v/v fetal bovine serum, 100 units/ml penicillin/streptomycin, 2mM L-glutamine, 50μM 2-mercaptoethanol and 1M HEPES buffer). Experiments for evaluation of CD4 and CD8 responses were performed with CD8- or CD4-depleted cells, following the procedures from Miltenyi Microbeads kits (Miltenyi, Auburn, California, USA), using LD columns. IFN-γ ELISPOT assays were performed by using an ELISPOT set from BD-Biosciences Pharmingen (San Diego, CA), according to the manufacturer's protocol. Initially, the ELISPOT plates were coated with anti-IFN-γ at 5μg/ml and incubated at 4°C/overnight. The plates were blocked with RPMI 1640 containing 10% FCS for 2 h at room temperature, and either total splenocytes (1×10^6 cells/well) or CD8- or CD4-depleted cells ($0.5–1.0\times10^6$ cells/well) from immunized mice were then added. The cells were cultured at 37°C in 5% CO_2 with culture medium only (RPMI 1640 medium supplemented with 5% v/v of fetal calf serum, 100 units/ml penicillin/streptomycin, 2 mM L-glutamine), or in the presence of concanavalin A (2.5 μg/ml; Sigma), 10^9 PFUs/mL of inactivated YF virus as a positive control (strain 17DD), or individual 15mers or 9mers from the envelope, capsid, or membrane proteins of the 17DD YF virus at 10μg/ml, 1μg/mL, or 0.1μg/mL, according to the experiment. After 16 h of culture, the plates were washed and incubated with biotinylated anti-IFN-γ for 2 h at room temperature, followed by HRP-conjugated avidin for 1 h at room temperature. Reactions were developed with AEC substrate (Calbiochem-Novabiochem Corporation, San Diego, CA). Final enumeration of IFN-γ. SFC was performed using the Immunospot Series 3B Analyzer ELISPOT reader (Cellular Technologies Ltd, Shaker Heights, OH) with aid of the Immunospot software version 3.0 (Cellular Technologies Ltd). The data indicate the number of spot-forming cells (SFC)/10^6 cells. The results were considered positive if the number of SFC was above 20 and higher than the background (culture with medium alone) plus three standard deviations. The results are presented after subtraction of the background, which was consistently found to be 10 to 35 spots/10^6 cells throughout the experiments.

Binding assays

Binding of peptides to H-2 allomorphs was determined using quantitative assays based on the inhibition of binding of a radiolabeled standard peptide to purified MHC molecules, essentially as described previously (Sidney et al., 1998). Briefly, 0.1 to 1 nM of a known MHC-binding radiolabeled peptide was co-incubated at room temperature with varying amounts of unlabeled test peptides, 1μM to 1nM H-2 molecules purified by affinity chromatography, a cocktail of protease inhibitors and, for class I assays, 1μM human β2-microglubulin (Scripps Laboratories, San Diego, CA). After 2-day incubation, MHC-peptide complexes were captured on Lumitrac 600 microplates (Greiner Bio-one, Longwood, FL) coated with monoclonal antibody 28-14-8S, SF1-1.1.1, 34-5-8S, MKD6 or 14.4.4 for L^d, K^d, D^d, I-A^d and I-E^d, respectively. Bound radioactivity was measured using a TopCount microscintillation counter (Packard Instrument Co., Meriden, CT). The concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC_{50}) was then calculated. Peptides were typically tested at six different concentrations covering a 100,000-fold dose range, and in three or more independent assays. Under the conditions utilized, where [label]<[MHC] and $IC_{50} \geq$ [MHC], the measured IC_{50} values are reasonable approximations of the K_D values. The peptides used for binding assays were typically ≥80% pure (Supplemental Tables II, III, IV, and V).

PRED$^{BALB/c}$ epitope prediction software

PRED$^{BALB/c}$ is a freely available, website-based *in silico* epitope prediction program (http://antigen.i2r.a-star.edu.sg/predBalbc/) that scans protein and peptide sequences to look for epitopes that are capable of being presented in the context of $H2^d$ alleles of the BALB/c mouse strain. It utilizes quantitative matrices that have been validated using experimentally determined binders and non-binders and also by *in vivo* studies using viral proteins to score and rank proteic sequences of nine amino acids according to their predicted peptide binding to BALB/c major histocompatibility complex ($H2^d$) class I (H2-K^d, -D^d and -L^d) and class II (I-E^d and I-A^d) molecules (41). This software analyzes the sequence of each peptide and scores each one according to the known binding motifs identified for each allele throughout a core sequence of nine amino acids within the peptide sequence. The software supplies a list of all the peptides, ranked from 10 to 0, for each one of the five alleles of $H2^d$. An additional software feature allows the use of a threshold, which can be defined by the user.

For the identification of possible epitopes, PRED$^{BALB/c}$ was given the list of all 15mer peptides used in the ELISPOT experiments: 120 peptides from the envelope, 28 peptides from the capsid, and 16 peptides from the membrane protein, according to the published sequence of the YF virus 17DD (NCBI entry U17066).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ELISPOT, Enzyme-linked immunosorbent spot assay; IC_{50}, 50% inhibitory concentration; YF, yellow fever; DC, dendritic cells; SFC, spot forming cells.

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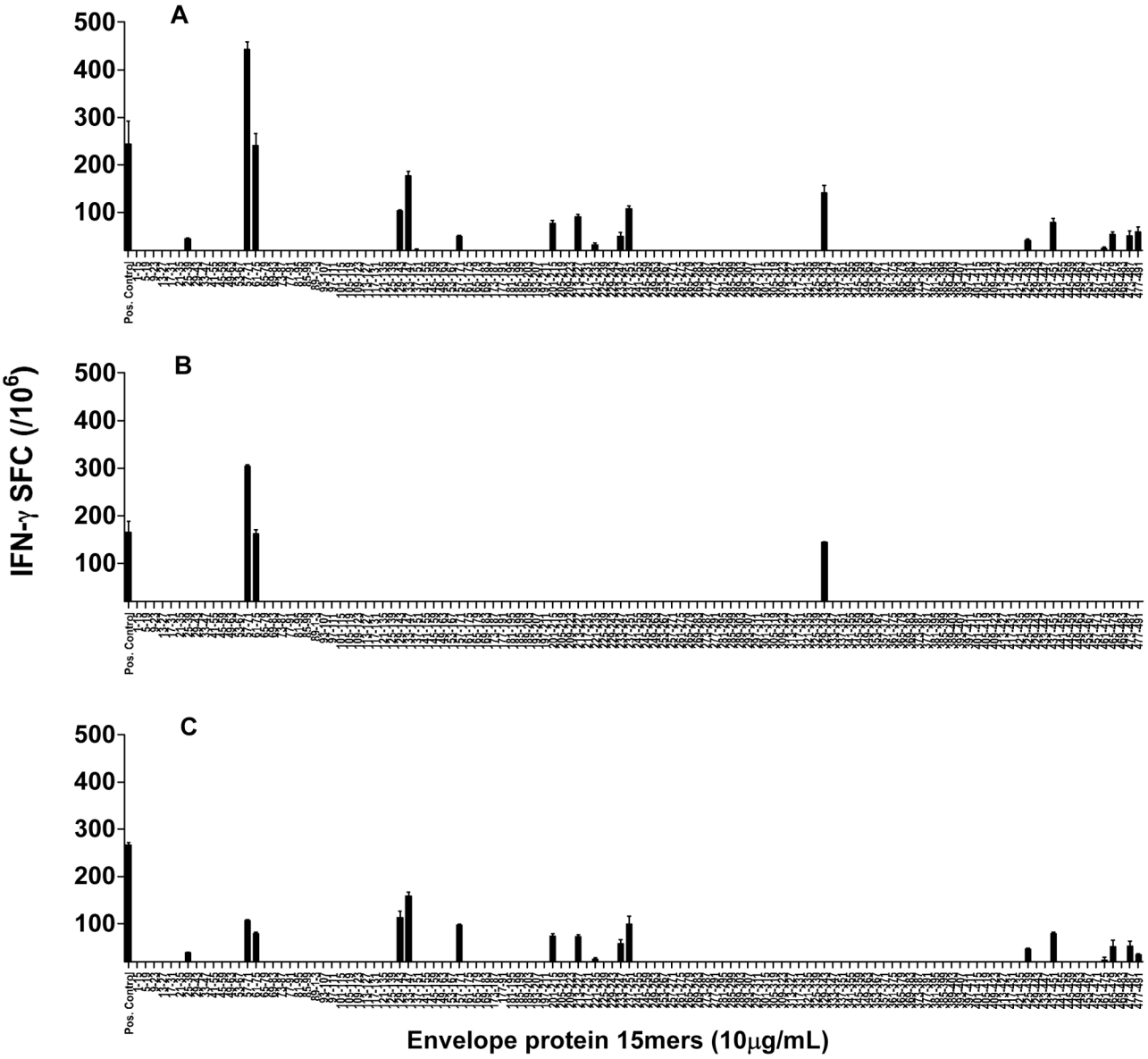

Figure 1.

Response of total splenocytes and $CD4^+$ and $CD8^+$ T cells to the peptides of the YF envelope protein. BALB/c mice were immunized on day 0 and boosted on day 21 with 10^4 PFUs of the human 17DD YF vaccine, and the splenocytes were tested in IFN-γ ELISPOT assays 7–10 days after the boost. The peptides used for *in vitro* stimulation were 15mers, overlapping by 11 amino acids and comprising the length of the envelope protein (10 μg/mL). (A) splenocytes; (B) CD4-depleted splenocytes; (C) CD8-depleted splenocytes. Figures represent the average of two to four experiments performed with a pool of three to five mice each. Bars indicate the mean ± SD.

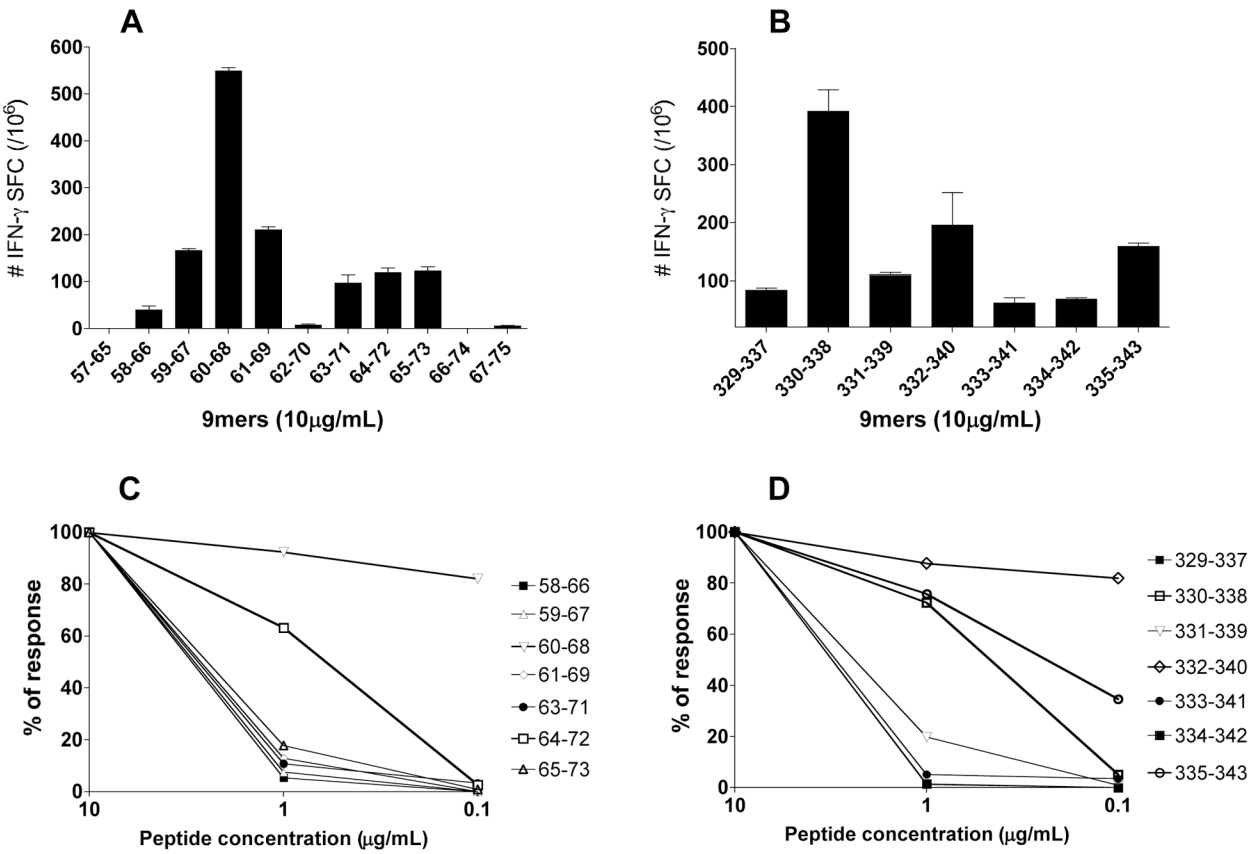

Figure 2.

CD8 responses to 9mer peptides of the YF envelope protein. Splenocytes of BALB/c mice immunized twice with the human 17DD YF vaccine were used for IFN-γ ELISPOT assays. The percentage of responses for each peptide was calculated according to the formula: number of spots at 1μg/mL (or 0.1μg/mL) × 100/number of spots at 10μg/mL. (A) Number of IFN-γ SFC for peptides of 9 amino acids within the $E_{57–75}$ sequence of the YF envelope protein; (B) number of IFN-γ SFC for the 9mers within the $E_{329–343}$ sequence of the YF envelope protein; (C) IFN-γ ELISPOT evaluation of the affinity of CD8 responses to decreasing *in vitro* concentrations of 9mers within the $E_{57–75}$ sequence of the YF envelope protein; (D) IFN-γ ELISPOT evaluation of the affinity of CD8 responses to decreasing *in vitro* concentration of 9mers within the $E_{329–343}$ sequence of the YF envelope protein. Figures A and B represent the average of three experiments with a pool of two to three mice. Figures C and D illustrate one representative experiment out of three.

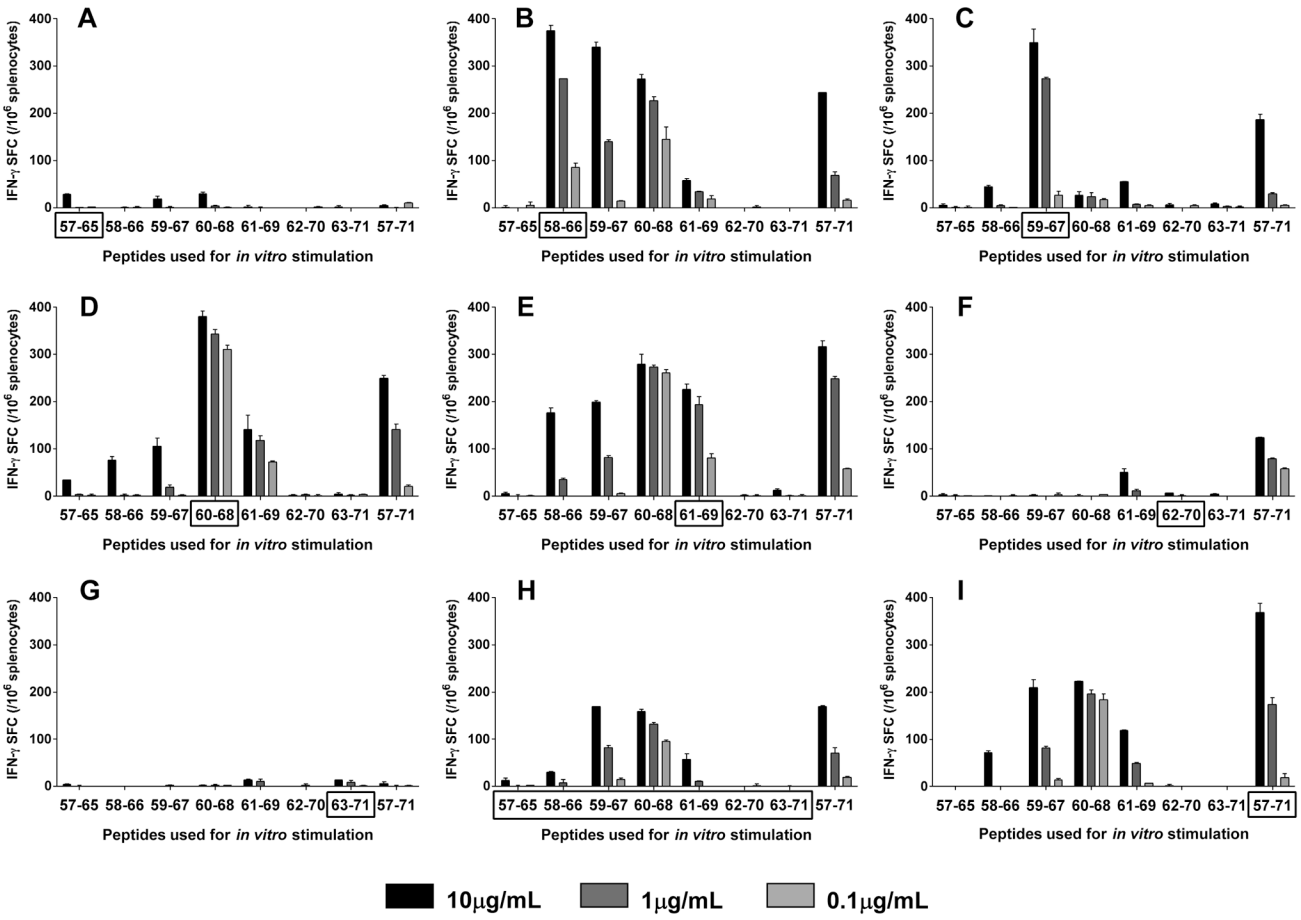

Figure 3.

Cellular responses to immunization with 9mers from the sequence E_{57} to E_{71} of the YF envelope protein. BALB/c mice were immunized s.c. once with 50μg/mL of individual 9mers, a pool of 9mers, or a 15mer, emulsified v/v in adjuvant (TiterMax®). The splenocytes were used in IFN-γ ELISPOT assays 15–30 days after the immunization. The cells were stimulated *in vitro* with individual peptides in decreasing concentrations. The box on the *x*–axis indicates the peptide(s) used in the immunization: Mice were immunized with (A) peptide $E_{57–65}$; (B) peptide $E_{58–66}$; (C) peptide $E_{59–67}$; (D) peptide $E_{60–68}$; (E) peptide $E_{61–69}$; (F) peptide $E_{62–70}$; (G) peptide $E_{63–71}$; (H) a pool of 9mers; (I) 15mer peptide $E_{57–71}$. Each plot represents one of three experiments using a single mouse; bars represent the average ± SD of the ELISPOT duplicate.

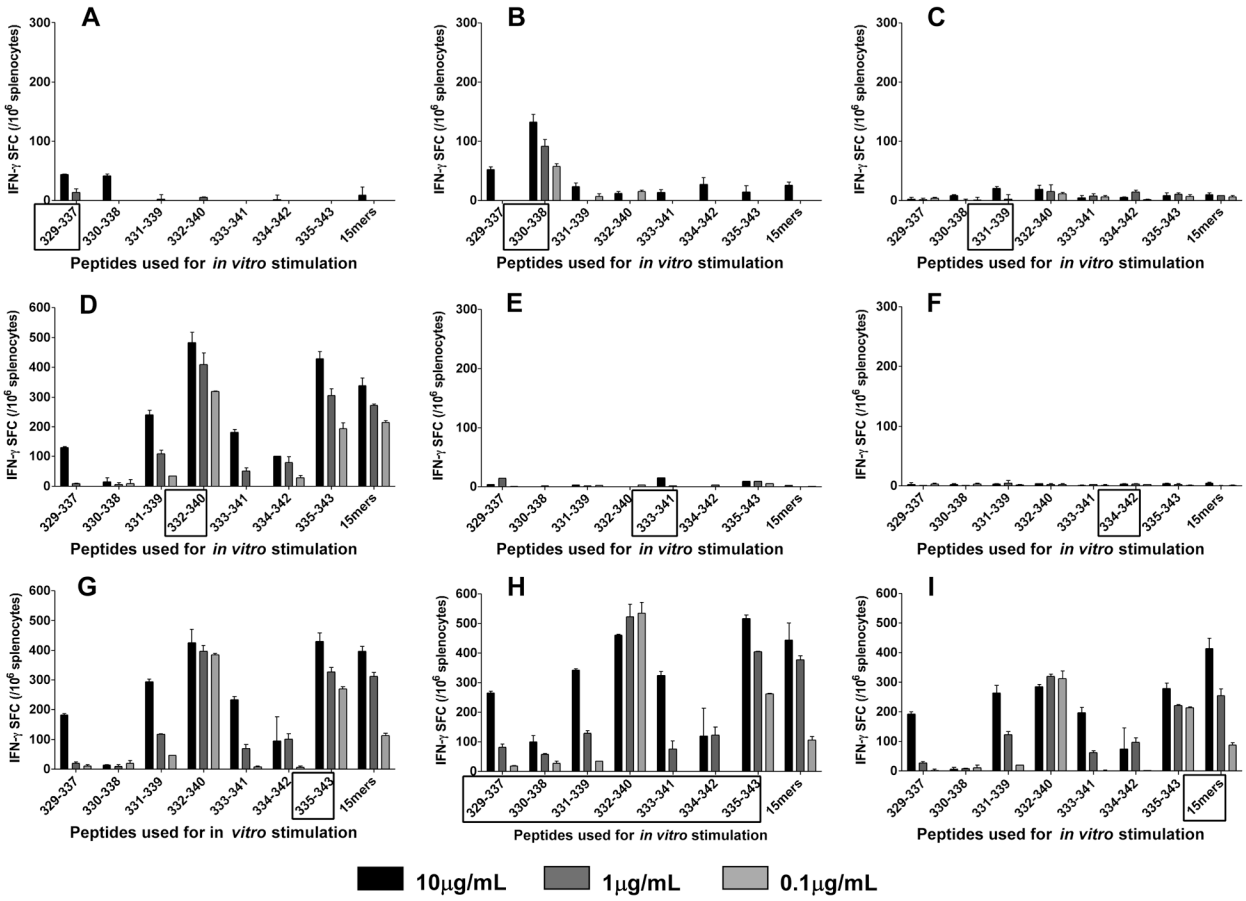

Figure 4.

Cellular responses to immunization with peptides from $E_{329–343}$ of the YF envelope protein. BALB/c mice were immunized s.c. once with 50μg/mL of individual 9mers, a pool of 9mers, or a 15mer, emulsified v/v in adjuvant (TiterMax®). Splenocytes were used in IFN-γ ELISPOT assays 15–30 days after the immunization. The cells were stimulated *in vitro* with individual peptides in decreasing concentrations. The box on the *x*-axis indicates the peptide(s) used in the immunization: Mice were immunized with (A) peptide $E_{329–337}$; (B) peptide $E_{330–338}$; (C) peptide $E_{331–339}$; (D) peptide $E_{332–340}$; (E) peptide $E_{333–341}$; (F) peptide $E_{334–342}$; (G) peptide $E_{335–343}$; (H) a pool of 9mers; (I) 15mer peptide $E_{329–343}$. Each plot represents one of three experiments using a single mouse; bars represent the average ± SD of the ELISPOT duplicate.

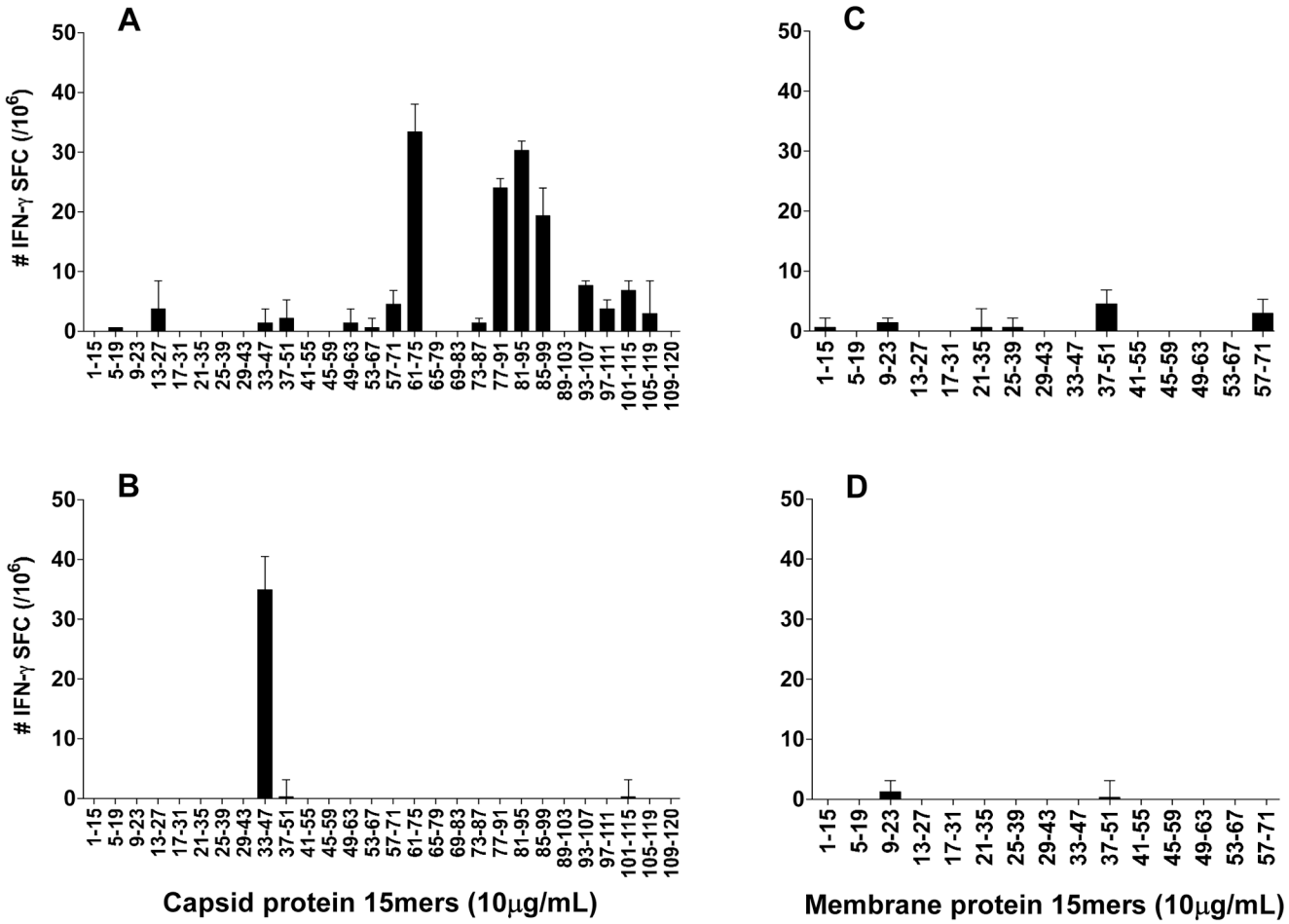

Figure 5.

Cellular responses of BALB/c mice to the peptides of the YF capsid and membrane protein. BALB/c mice were immunized s.c. with the human 17DD YF vaccine and assessed by ELISPOT assays for the (A) number of IFN-γ SFC for CD8-depleted splenocytes and (B) number of IFN-γ SFC for CD4-depleted splenocytes, using 15mer peptides, overlapping by 11 amino acids from the YF capsid protein (10 μg/mL). (C) number of IFN-γ SFC in CD8-depleted splenocytes and (D) number of IFN-γ SFC in CD4-depleted splenocytes, using 15mers overlapping by 11 amino acids from the YF membrane protein (10 μg/mL). Results represent the mean ± SD from three experiments.

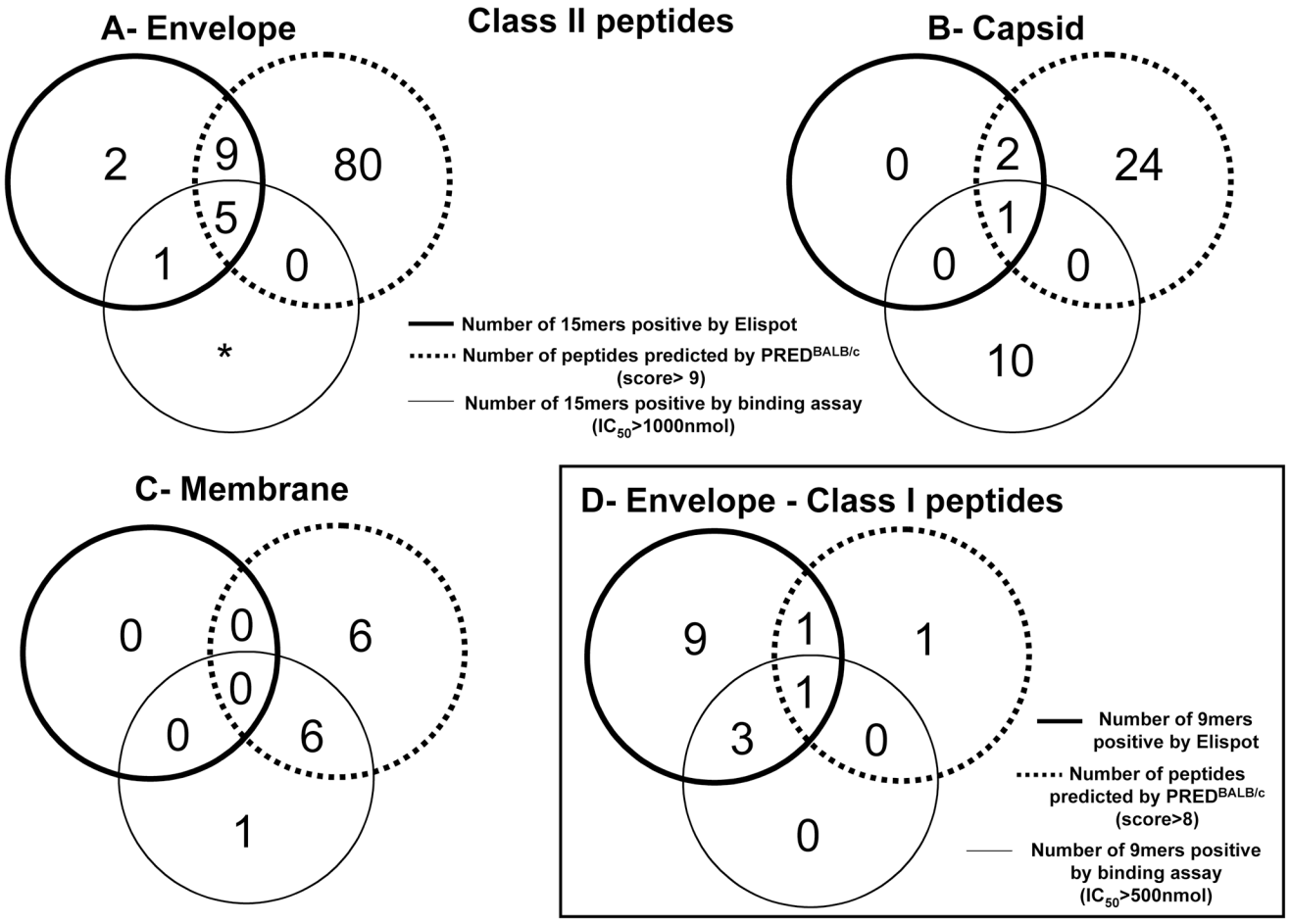

Figure 6.

Venn diagram representation of results obtained from ELISPOT assay binding assay, and $PRED^{BALB/c}$ prediction. (A) ELISPOT results with 120 15mer peptides from the envelope of the YF virus with CD8-depleted splenocytes; $PRED^{BALB/c}$ was used to analyze the 120 15mer peptides from the envelope for the presence of class II (I-A^d and I-E^d) binders; *only the seventeen peptides positive by ELISPOT were analyzed by binding assays for class II molecules, and those showing an IC_{50} <1000nM were considered possible binders. (B) ELISPOT results for the 3 out of 28 15mer peptides from the capsid of the YF virus that were considered positive with CD8-depleted splenocytes; $PRED^{BALB/c}$ was used to analyze the 28 15mer peptides from the capsid for the presence of class II (I-A^d and I-E^d) binders; all 28 15mers from the capsid were tested in binding assays for class II molecules, and those showing an IC_{50} <1000nM were considered possible binders. (C) ELISPOT results with the 16 15mer peptides from the membrane of the YF virus with CD8-depleted splenocytes; $PRED^{BALB/c}$ was used to analyze the 16 15mer peptides from the membrane for the presence of class II (I-A^d and I-E^d) binders; all 16 were tested in binding assays for class II molecules, and those showing an IC_{50} <1000nM were considered possible binders. (D) ELISPOT results for the 18 9mers from the envelope of the YF virus tested with CD4-depleted splenocytes; $PRED^{BALB/c}$ was used to analyze the 18 9mer for the presence of class II (I-A^d and I-E^d) binders; all 9mers were tested in binding assays for class I molecules, and those showing an IC_{50} <500nM were considered possible binders. The intersection represents the number of peptides that were positive in two or three of the approaches.

Table I

Results of ELISPOT, binding assay and PRED$^{BALB/c}$ prediction for the 9mer peptides from the CD8-positive sequences of the yellow fever envelope protein

9mer position (Yellow Fever Envelope)	Sequence	# IFN-γ SFC (CD4-depleted splenocytes/10^6) *(2)*	Binding assay (IC_{50} in nmol) (PRED$^{BALB/c}$ score)		
E_{57} to E_{75}	**RKVCYNAVLTHVKINDKCP***(1)*		D^d*(3)*	K^d*(3)*	L^d*(3)*
57–65	RKVCYNAVL*(4)*	1	>70000*(5)* (7.98)	>70000 (5.72)	39442 (4.70)
58–66	KVCYNAVLT	93	>70000 (2.32)	>70000 (1.84)	>70000 (0.90)
59–67	VCYNAVLTH	265	>70000 (7.98)	211* (1.60)	55368 (4.62)
60–68	CYNAVLTHV	403	>70000 (6.48)	1.0* (8.66**)	>70000 (5.18)
61–69	YNAVLTHVK	143	>70000 (3.46)	93* (1.92)	>70000 (0.90)
62–70	NAVLTHVKI	7.0	>70000 (8.42**)	1542 (5.56)	>70000 (0.30)
63–71	AVLTHVKIN	60	>70000 (4.10)	54670 (1.10)	20708 (0.88)
64–72	VLTHVKIND	60	>70000 (2.94)	>70000 (5.22)	>70000 (2.22)
65–73	LTHVKINDK	51	>70000 (4.02)	>70000 (1.60)	>70000 (1.50)
66–74	THVKINDKC	1	>70000 (0.80)	>70000 (2.26)	>70000 (0.40)
67–75	HVKINDKCP	6.0	>70000 (1.64)	>70000 (0.58)	48666 (2.5)
E_{329} to E_{343}	**PCRIPVIVADDLTAA***(6)*				
329–337	PCRIPVIVA	158	>70000 (4.78)	>70000 (0.50)	>70000 (0.44)
330–338	CRIPVIVAD	371	>70000 (2.36)	915 (2.40)	54239 (2.12)
331–339	RIPVIVADD	140	>70000 (8.50**)	>70000 (4.18)	70000 (1.42)
332–340	IPVIVADDL	63	>70000 (6.72)	33005 (6.00)	56* (7.50)
333–341	PVIVADDLT	26	>70000 (3.40)	>70000 (1.80)	3719 (0.00)
334–342	VIVADDLTA	86	>70000 (2.70)	>70000 (3.10)	19242 (1.50)
335–343	IVADDLTAA	46	>70000 (3.46)	>70000 (1.96)	38032 (0.90)

(1) Sequence of 19 amino acids that correspond to 15mer peptides $E_{57–71}$ and $E_{61–75}$ that were positive for CD8 response by ELISPOT (Fig. 2B)

(2) peptides were considered ELISPOT positives when >20 SFC/10^6 cells

(3) molecules of $H2^d$ class I

(4) sequence of each 9mer in relation to the sequence from where it is found in the envelope protein

(5) IC50 for the binding assay for class I molecule (nmol)

(6) sequence of 15 amino acids from the 15mer peptide $E_{329–343}$ (Fig. 2B)

* values of IC_{50} below the cutoff of 500nmol, suggestive of a possible binder peptide

** score above 8 from $PRED^{BALB/c}$ software prediction.

Table II

Results of ELISPOT, binding assay and PRED$^{BALB/c}$ prediction for the CD4-positive ELISPOT 15mer peptides of the yellow fever envelope protein

Peptide position/allele	Peptide sequence	# IFN-γ SFC CD8$^+$-depleted	Binding Assay (IC_{50} in nmol)		PRED$^{BALB/c}$ score *(2)*
			I-A^d*(1)*	I-E^d	
$E_{25–39}$	LEQDKCVTVMAPDKP	39	2412*(4)*	>45000	
I-A^d	EQDKCVTVM*(3)*				9.40
	DKCVTVMAP				9.60
	CVTVMAPDK				9.50
$E_{57–71}$	RKVCYNAVLTHVKIN	106	2848	5408	
I-A^d	YNAVLTHVK				9.20
E^d	YNAVLTHVK				9.14
$E_{61–75}$	YNAVLTHVKINDKCP	78	11582	>45000	
I-A^d	YNAVLTHVK				9.20
I-E^d	YNAVLTHVK				9.14
	LTHVKINDK				9.80
	THVKINDKC				9.50
$E_{129–143}$	EVDQTKIQYVIRAQL	113	53*	>45000	
I-A^d	QTKIQYVIR				9.30
	TKIQYVIRA				9.50
	IQYVIRAQL				9.10
I-E^d	QTKIQYVIR				9.42
	KIQYVIRAQ				9.70
$E_{133–147}$	TKIQYVIRAQLHVGA	158	14*	9073	
I-A^d	TKIQYVIRA				9.50
	IQYVIRAQL				9.10
	QYVIRAQLH				9.60
I-E^d	KIQYVIRAQ				9.70
	VIRAQLHVG				9.42
$E_{157–171}$	KTLKFDALSGSQEVE	97	1348	>45000	
I-E^d	KTLKFDALS				9.44
$E_{201–215}$	ESWIVDRQWAQDLTL	73	>45000	6358	
I-A^d	SWIVDRQWA				9.34
$E_{213–227}$	LTLPWQSGSGGVWRE	72	>45000	38812	
$E_{221–235}$	SGGVWREMHHLVEFE	26	2519	23454	
I-A^d	SGGVWREMH				9.20
$E_{233–247}$	EFEPPHAATIRVLAL	58	89*	111*	
I-A^d	AATIRVLAL				9.70
$E_{237–251}$	PHAATIRVLALGNQE	98	89*	7053	
I-A^d	AATIRVLAL				9.70
	TIRVLALGN				9.68
I-E^d	ATIRVLALG				9.38

Peptide position/allele	Peptide sequence	# IFN-γ SFC CD8$^+$-depleted	Binding Assay (IC_{50} in nmol)		PRED$^{BALB/c}$ score *(2)*
			I-A^d*(1)*	I-E^d	
$E_{425–439}$	GFFTSVGKGIHTVFG	46	60*	6170	
$E_{437–451}$	VFGSAFQGLFGGLNW	79	3511	14977	
$E_{461–475}$	LIWVGINTRNMTMSM	24	2393	31407	
I-A^d	INTRNMTMS				9.10
I-E^d	LIWVGINTR				9.80
$E_{465–479}$	GINTRNMTMSMSMIL	52	964*	3186	
I-A^d	INTRNMTMS				9.10
	TRNMTMSMS				9.60
$E_{473–487}$	MSMSMILVGVIMMFL	52	4588	>45000	
I-A^d	MSMILVGVI				9.70
	MILVGVIMM				9.60
$E_{477–491}$	MILVGVIMMFLSLGV	35	1368	>45000	
I-A^d	MILVGVIMM				9.60
	VGVIMMFLS				9.20

A list of 94 peptides selected by PRED$^{BALB/c}$ with score above 9 was compared to the CD4 ELISPOT results. The 9mers selected by the prediction software are shown below each 15mer peptide.

(1) Molecules of H2^d class II

(2) PRED$^{BALB/c}$ software scores

(3) options of 9mer peptides within each 15mers analyzed and scored above 9 by PRED$^{BALB/c}$

(4) IC_{50} (nmol) results for the binding assay of H2^d class II molecules

* values of IC_{50} below the cutoff of 1000nmol, suggestive of a possible binder peptide; Shading – peptides positive for CD4 response by ELISPOT (Fig. 2C) did not predict by PRED$^{BALB/c}$ above a threshold of 9.

Table III

Results of class II binding assay for the 15mer peptides from the yellow fever capsid protein

Position[(1)]	Sequence	Binding Assay (IC_{50} in nmol)	
		I-A^d[(2)]	I-E^d
$C_{1–15}$	SGRKAQGKTLGVNMV	3810[(3)]	>45000
$C_{5–19}$	AQGKTLGVNMVRRGV	1547	16452
$C_{9–23}$	TLGVNMVRRGVRSLS	56*	9379
$C_{13–27}$	NMVRRGVRSLSNKIK	801*	11260
$C_{17–31}$	RGVRSLSNKIKQKTK	38215	5697
$C_{21–35}$	SLSNKIKQKTKQIGN	>45000	18106
$C_{25–39}$	KIKQKTKQIGNRPGP	25182	24418
$C_{29–43}$	KTKQIGNRPGPSRGV	323*	33245
$C_{33–47}$	IGNRPGPSRGVQGFI	9683	>45000
$C_{37–51}$	PGPSRGVQGFIFFFL	11783	>45000
$C_{41–55}$	RGVQGFIFFFLFNIL	9301	>45000
$C_{45–59}$	GFIFFFLFNILTGKK	3535	>45000
$C_{49–63}$	FFLFNILTGKKITAH	562*	992*
$C_{53–67}$	NILTGKKITAHLKRL	378*	8234
$C_{57–71}$	GKKITAHLKRLWKML	5867	389*
$C_{61–75}$	TAHLKRLWKMLDPRQ	>45000	3788
$C_{65–79}$	KRLWKMLDPRQGLAV	539*	6193
$C_{69–83}$	KMLDPRQGLAVLRKV	2523	>45000
$C_{73–87}$	PRQGLAVLRKVKRVV	22243	1844
$C_{77–91}$	LAVLRKVKRVVASLM	93*	1081
$C_{81–95}$	RKVKRVVASLMRGLS	60*	6687
$C_{85–99}$	RVVASLMRGLSSRKR	41*	522*
$C_{89–103}$	SLMRGLSSRKRRSHD	1326	545*
$C_{93–107}$	GLSSRKRRSHDVLTV	17646	13392
$C_{97–111}$	RKRRSHDVLTVQFLI	1631	14460
$C_{101–115}$	SHDVLTVQFLILGML	29907	>45000
$C_{105–119}$	LTVQFLILGMLLMTG	37394	>45000
$C_{109–120}$	FLILGMLLMTGG	>45000	>45000

(1) Position of each 15mer peptide in the capsid protein (C) of the yellow fever virus

(2) molecules of $H2^d$ class II

(3) IC_{50} result for the binding assay for $H2^d$ class II molecules (nmol);

* values of IC_{50} below the cutoff of 1000nmol, suggestive of a possible binder peptide; Shading – peptides positive for CD4 response by ELISPOT (Fig. 6A)

Table IV

Results of class II binding assay for the 15mer peptides from the yellow fever membrane protein

Position[(1)]	Sequence	Binding Assay (IC_{50} in nmol)	
		I-A[d]	I-E[d]
$M_{1–15}$	AIDLPTHENHGLKTR	>45000	36639
$M_{5–19}$	PTHENHGLKTRQEKW	13183	>45000
$M_{9–23}$	NHGLKTRQEKWMTGR	40852	>45000
$M_{13–27}$	KTRQEKWMTGRMGER	1903	>45000
$M_{17–31}$	EKWMTGRMGERQLQK	20419	20683
$M_{21–35}$	TGRMGERQLQKIERW	42531	>45000
$M_{25–39}$	GERQLQKIERWFVRN	>45000	13242
$M_{29–43}$	LQKIERWFVRNPFFA	593*	1559
$M_{33–47}$	ERWFVRNPFFAVTAL	7.3*	2278
$M_{37–51}$	VRNPFFAVTALTIAY	40*	1917
$M_{41–55}$	FFAVTALTIAYLVGS	6213	>45000
$M_{45–59}$	TALTIAYLVGSNMTQ	102*	>45000
$M_{49–63}$	IAYLVGSNMTQRVVI	383*	29666
$M_{53–67}$	VGSNMTQRVVIALLV	30*	32305
$M_{57–71}$	MTQRVVIALLVLAVG	6484	>45000
$M_{61–75}$	VVIALLVLAVGPAYS	380*	>45000

[(1)] Position of each 15mer peptide in the membrane protein of the yellow fever virus

[(2)] molecules of $H2^d$ class II

[(3)] IC_{50} result for the binding assay for $H2^d$ class II molecules (nmol)

* values of IC_{50} below the cutoff of 1000nmol, suggestive of a possible binder peptide.