Avian and Human Seasonal Influenza Hemagglutinin Proteins Elicit CD4 T Cell Responses That Are Comparable in Epitope Abundance and Diversity

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Abstract

Avian influenza viruses remain a significant concern due to their pandemic potential. Vaccine trials have suggested that humans respond poorly to avian influenza vaccines relative to seasonal vaccines. It is important to understand, first, if there is a general deficiency in the ability of avian hemagglutinin (HA) proteins to generate immune responses and, if so, what underlies this defect. This question is of particular interest because it has been suggested that in humans, the poor immunogenicity of H7 vaccines may be due to a paucity of CD4 T cell epitopes. Because of the generally high levels of cross-reactive CD4 T cells in humans, it is not possible to compare the inherent immunogenicities of avian and seasonal HA proteins in an unbiased manner. Here, we empirically examine the epitope diversity and abundance of CD4 T cells elicited by seasonal and avian HA proteins. HLA-DR1 and HLA-DR4 transgenic mice were vaccinated with purified HA proteins, and CD4 T cells to specific epitopes were identified and quantified. These studies revealed that the diversity and abundance of CD4 T cells specific for HA do not segregate on the basis of whether the HA was derived from human seasonal or avian influenza viruses. Therefore, we conclude that failure in responses to avian vaccines in humans is likely due to a lack of cross-reactive CD4 T cell memory perhaps coupled with competition with or suppression of naive, HA-specific CD4 T cells by memory CD4 T cells specific for more highly conserved proteins.

Keywords

CD4 T cells, influenza, HA, epitopes, HLA-DR, vaccine

Highly pathogenic avian influenza (HPAI) represents a significant threat to global health and security. Emerging in 1997, H5N1 has caused periodic outbreaks in humans, which have been marked by an alarmingly high case fatality rate of 33% to 85%, depending on the clade of the virus (1). More recently, in 2013, an outbreak of H7N9 avian influenza in China exhibited a similarly high case fatality rate of around 35% and showed some evidence of nonsustained, human-to-human transmission (2). Despite the fact that these outbreaks have been limited in their breadth and duration, both H5 and H7 HPAI viruses share certain characteristics that make them a viable threat. Of primary concern are the potential for increased transmissibility through point mutations or reassortment (3, 4), the threat of oseltamivir resistance (5, 6), and the lack of an effective vaccine for human use.

The threat from HPAI has prompted a number of human vaccine studies which, to date, have been largely ineffective in rapidly and efficiently generating protective immunity against H5 and H7 strains. H5N1 and H7N9 vaccines have proven to be poorly immunogenic in these trials, often requiring multiple doses of vaccine with high hemagglutinin (HA) content or the use of adjuvants to achieve the desired neutralizing antibody titers (7–11). A 2006 H5N1 vaccine trial showed that two doses, each con-
aining 90 μg of HA, were able to generate microneutralization (MN) titers of ≥1:40 in just half of the subjects (7). Similarly, a 2015 H7N9 vaccine trial demonstrated that two AS03- or MF59-adjuvanted doses were required to achieve desired MN titers in the majority of patients (9).

Although it appears that adjuvants, higher doses, and prime-boost strategies may be effective in overcoming the apparent low immunogenicity, it is still not clear why primary responses to avian influenza vaccines are so weak in humans. There have been several proposed explanations for this observation. It is possible that preferential boosting of low-frequency, stalk-reactive memory B cells in response to avian influenza HAs (12–15) may be responsible for the low magnitude of the neutralizing antibody response. Studies in mice have also suggested that H7 may be in general poorly immunogenic (16). Another possible explanation is that, while healthy humans do have circulating, cross-reactive CD4 T cells to H5 and H7 HA (17–20), prepandemic priming was required to generate a sufficiently robust H5-specific CD4 T cell response and subsequent antibody response (20). It was recently suggested, through the use of a predictive algorithm, that HA from H7N9 has poor “immunogenicity potential” as it predicted there to be a paucity of both major histocompatibility complex class I (MHC-I)- and MHC-II-restricted epitopes in humans (21, 22). If true, this could explain the poor antibody responses to H7 HA, as help from HA-specific CD4 T cells is critical for generation of high-affinity antibodies to HA (23).

In this report, we evaluate the primary CD4 T cell response to recombinant H1, H3, H5, and H7 proteins in two transgenic mouse strains, each expressing a unique human MHC-II molecule (HLA-DR1 or HLA-DR4). This was done to assess whether avian HA proteins differ from seasonal HA proteins in their ability to be processed and presented on two common human MHC-II molecules. The magnitudes and diversities of the CD4 T cell responses to the four HAs were similar, suggesting that the poor immunogenicity observed for H5 and H7 is unlikely to be due to an intrinsic paucity of CD4 T cell epitopes and is, instead, due to other immunological or biochemical mechanisms.

RESULTS

Previous avian vaccine studies in humans conducted within our laboratory and others have shown that the initial response to avian influenza vaccine is very poor relative to what has been observed from seasonal influenza vaccination. However, our studies on H5N1 vaccines in humans revealed that previous H5N1 vaccination many years earlier primes human subjects for a high-titer neutralizing antibody response upon a second vaccination with a serologically distinct H5N1 vaccine (20). The improved B cell response was associated with the presence of enhanced H5-specific memory CD4 cells in the primed host prior to vaccination and expansion of these cells postvaccination. The H5-naive subjects had very few H5-reactive CD4 T cells. Based on these studies, as well as our survey of human CD4 T cell reactivity to H7 HA (24), we speculated that the explanation behind the generally poor immunogenicity of avian HA relative to isolates from seasonal H1 and H3 HA proteins lies with the composition of the CD4 T cell memory compartment. Most humans have abundant CD4 T cell memory to epitopes contained within seasonal HA proteins (25, 26), suggesting that it is this difference in the host that accounts for the overall ineffectiveness of avian vaccines. This contrasts with the speculation that avian HA proteins are inherently poorly immunogenic or have generalized deficits in T cell epitopes.

We therefore sought to empirically examine the inherent CD4 T cell immunogenicity of avian- and human-origin HA proteins in the primary response, essentially placing all HA proteins into identical host genetic backgrounds with no preexisting immunological memory. In order to do this, we utilized mouse models of vaccination, as virtually all human subjects have existing memory to seasonal H1N1 and H3N2 (25, 26). We designed experiments to identify the number and abundance of MHC-II epitopes contained within H1, H3, H5, and H7 HA proteins that were recognized by CD4 T cells in an unbiased and comprehensive way. Mice expressing the human class II molecule HLA-DR1 or HLA-DR4 were chosen for study because these class II molecules are commonly
expressed in humans (27) and have been useful in identifying epitopes that have been confirmed to be recognized in human subjects expressing these alleles of HLA class II molecules (28, 29). Use of two different mouse strains, each expressing a distinct class II molecule, allows a more broad-based assessment of CD4 T cell immunogenicity.

Full-length, purified recombinant HA proteins produced from the baculovirus-expressSF insect cell (derived from Spodoptera frugiperda) system were used as immunogens, introduced with alum as an adjuvant. Proteins generated by this system form trimers and generate structures containing heterogeneous mixtures of N-linked glycans, consisting mainly of oligomannose and paucimannose but also containing high-mannose, intermediate-mannose, and hybrid species (30–33). Mice were vaccinated subcutaneously and analyzed 10 to 11 days postvaccination. In order to identify peptide epitopes, a peptide pooling and cytokine enzyme-linked immunoabsorbent spot (ELISpot) strategy was employed as we (34) and others (35,36) have previously described.

The general procedure is illustrated in Fig. 1A, where an iterative strategy is used to first eliminate many candidate peptides from consideration and then to finally identify the CD4 T cell peptide epitopes that are recognized by the host CD4 T cells. Postvaccination, the draining lymph nodes were excised and CD4 T cells enriched by negative paramagnetic bead isolation. Peptide pools consisting of individual 15- to 17-mer peptides, representing the entire translated sequence of each unique HA protein, were arrayed in a “matrix” illustrated in Fig. 1 and detailed in Materials and Methods and the legend to Fig. 1, with each pool consisting of 9 to 11 peptides of 15 to 17 amino acids (aa), used at a final concentration of 2 μM. In this way, all potential HA epitopes recognized by the host CD4 T cells would be presented and available to recall the primed CD4 T cells. The peptide pools were coincubated for 16 h with the primed CD4 T cells, and syngeneic splenocytes from naive mice or MHC-II-expressing fibroblasts were used as a source of antigen-presenting cells. Activation of CD4 T cells was measured by interleukin-2 (IL-2) cytokine ELISpots. This process allowed identification of pools of peptides that contained CD4 T cell epitopes recognized by the host.
and those pools lacking any epitopes. Pools tested as negative (<30 cytokine-producing cells per million) in two independent assays were eliminated from further analyses. Candidate peptides at the intersection of “rows” and “columns” were then tested as single peptides. An example of the results is illustrated in Fig. 1B and C, which involves HLA-DR1 mice and the H3 protein. Through this sequential, iterative process, the single-peptide epitopes derived from the 4 different HA proteins in both strains of mice were identified.

Shown in Fig. 2 is a comparison of the H1-, H3-, H5-, and H7-derived CD4 single-
peptide epitopes that were recognized postvaccination as detected and quantified by cytokine ELISpots. Each 15- to 17-mer peptide is indicated on the y axis by the first amino acid in its sequence. The range between two independent experiments is indicated for each epitope, allowing us to confidently identify the epitopes recognized in the response to vaccination as well as eliminate other peptides from further consideration. From this direct ex vivo quantification, it is evident that the total number of HA-specific CD4 T cells varies for each HA-HLA combination, as does the abundance of CD4 T cells elicited for each peptide. For example, in the HLA-DR4 mice, shown in the right panels, 4 major epitopes are recognized within H7, but in the HLA-DR1 mice (left panel), at least 8 to 10 epitopes are detected (depending on whether overlapping peptides include one overlapping or two separate epitopes). For pH1, there are 3 to 5 peptide epitopes recognized in HLA-DR1 mice, whereas HLA-DR4 mice respond to one major epitope. However, comparing among different HA proteins and between the two different strains of HLA-DR transgenic mice, it is clear that the diversity of epitopes recognized does not vary consistently with the species source of the HA protein. The peptide epitopes recognized in each combination of HA and mouse strain are presented in Table 1, where only peptides that elicit at least 50 spots are considered reliable and major epitopes are indicated in boldface. Generally, the HLA-DR1 mice mounted a more diverse and robust CD4 T cell response than the HLA-DR4 strain, but differences in the CD4 T cell responses did not segregate between the human- and avian-derived HA strains.

The overall abundance of CD4 T cells elicited for H1, H3, H5, and H7 is shown in Fig. 4, where the average of the response to all of the peptide epitopes shown in Fig. 2 was summed, with overlapping peptides considered a single peptide. The data for HLA-DR1 mice are shown on the left, and those for the HLA-DR4 mice are shown on the right. Interestingly, in both strains, H7 and H3 recruit the most CD4 T cells and H5 and H1 the least. The HLA-DR1 strain exhibited a greater overall abundance of CD4 T cells specific for HA than does the HLA-DR4 strain. (Note the differences in the scales used in Fig. 2 and 4.) This correlates with their lower cell surface density of HLA-DR4 relative to HLA-DR1 on dendritic cells in these two transgenic mice (not shown).

DISCUSSION

Here, in naive hosts, we found that there were no generalizable differences in the location, distribution, or overall number of CD4 T cell epitopes within HA from seasonal human viruses compared to avian viruses. This leads us to conclude that avian HA proteins do not have a paucity of CD4 T cell epitopes that can be presented by human class II molecules. There were variations in the fine specificity of the CD4 T cells elicited by the different HA proteins, consistent with the consensus that the particular MHC-II molecule in the host “selects” the peptides that can be presented to the circulating CD4 T cells (37, 38). In addition to addressing a question that has existed in the literature for some time, the experiments completed in this study provide an unbiased source of information for derivation of HLA-DR tetramers that can be used to track responses in humans pre- and postvaccination with either seasonal or avian-derived vaccines.

The results of this study suggest that the defects in human neutralizing antibody responses to avian vaccination are not secondary to inherent deficits of CD4 T cell epitopes within these avian H5 and H7 proteins. We suspect that the major mechanism that underlies poor responses of humans to avian vaccines is simply a low abundance of memory helper CD4 T cells that are recruited into the response. In our studies on CD4 T cell reactivity to H7 HA-derived epitopes in humans that have never encountered H7
avian viruses or vaccines, we have found that cross-reactivity is detectable in many but is of relatively low abundance for most individuals (24). In contrast, memory CD4 T cells specific for seasonal H1N1- and H3N2-derived HA proteins are readily detectable in humans (25, 26). Even when the novel swine-origin pandemic H1N1 virus (A/California/04/09) emerged in 2009, responses to vaccines were robust, requiring only a single dose (39). Our laboratory (40) has shown that the rapid and protective antibody responses to the serologically distinct, swine-origin HA proteins were likely a consequence of HA-specific CD4 T cells that were cross-reactive between the previous "seasonal" HA [i.e., A/Brisbane/59/2007 (H1N1)] and the novel HA. These cross-reactive CD4 T cells were likely drawn into the response to infection with the 2009 pH1N1 virus and may have potentiated early and protective antibody responses during the infection.

### TABLE 1 CD4 T cell peptide epitopes recognized by HLA-DR1 and HLA-DR4 transgenic mice

| MHC-II restriction | HA protein | Peptide name | Amino acid sequence | Avg no. of spots/10⁶ T cells |
|--------------------|------------|--------------|---------------------|-----------------------------|
| HLA-DR1 pH1        | aa221      | 221 SRYSKKFKEIAIRP 235 | 405                     |
|                    | aa225      | 225 KKFKEIAIRPKVDR 239 | 476                     |
|                    | aa305      | 305 TSLPQNIHPITIGK 319 | 578                     |
|                    | aa437      | 437 TYNALVLENERT 451   | 188                     |
|                    | aa441      | 441 ELLVLLENERTLDYH 455 | 318                     |
| H3                 | aa113      | 113 CYPYDVPDYASLRLVA 129 | 732                     |
|                    | aa119      | 119 PDYASLRLVASSGTE 135 | 965                     |
|                    | aa315      | 315 RITYGACPYVKQNTLK 331 | 127                     |
|                    | aa321      | 321 CPRYVKQNLKLTGMR 337 | 1,697                   |
|                    | aa386      | 386 TQAANIQINGKLNRLIG 402 | 139                     |
|                    | aa391      | 391 NQINGKLNRLIGKTNE 407 | 166                     |
| H5                 | aa157      | 157 TSFFRNVWVWLIKKNST 173 | 807                     |
|                    | aa259      | 259 SNGNFIAPFAYKIKKKV 275 | 1,484                   |
|                    | aa301      | 301 INSSMPFHNIHPLTIGE 317 | 172                     |
|                    | aa307      | 307 FHNHPHTIGECPKY 323   | 311                     |
|                    | aa433      | 433 VWTNYANELLVLENERT 449 | 184                     |
|                    | aa439      | 439 ELLVLLENERTLDHFS 455 | 474                     |
| H7                 | aa81       | 81 PPQCDQFLEFADLIE 97   | 223                     |
|                    | aa106      | 106 YPGKVFNEALRQILRE 122 | 803                     |
|                    | aa181      | 181 RKSPALIVWHHHSVST 197 | 909                     |
|                    | aa241      | 241 FHWLMLNPNTDYTFVS 257 | 275                     |
|                    | aa266      | 266 ASFLRQGSMQGQV 282   | 473                     |
|                    | aa271      | 271 GKSMLQGQGQVQV 287   | 114                     |
|                    | aa336      | 336 PKRGFLGAAAFHH 352   | 200                     |
|                    | aa341      | 341 LFAGAAGSGVEGGL 357   | 119                     |
|                    | aa381      | 381 QSAIDQITGKLRLIE 397   | 339                     |
|                    | aa386      | 386 QITGKLRLJKTNNQF 402   | 339                     |
|                    | aa406      | 406 DNEFNEKQIQNINW 422   | 111                     |
|                    | aa431      | 431 WSYNELLVAMENQHTI 447 | 506                     |
|                    | aa436      | 436 ELLVAMENQHTIDLADS 452 | 476                     |
| HLA-DR4 pH1        | aa269      | 269 SYRIMARYMAGSII 283   | 240                     |
| H3                 | aa37       | 37 PNGTVKKTITNDQIVET 53   | 265                     |
|                    | aa315      | 315 RITYGACPYVKQNTLK 331 | 155                     |
|                    | aa321      | 321 CPRYVKQNLKLTGMR 337 | 685                     |
| H5                 | aa61       | 61 DGVKPLIRDCSVAGW 77    | 559                     |
|                    | aa439      | 439 ELLVLLENERTLDHFS 455 | 73                      |
| H7                 | aa106      | 106 YPGKVFNEALRQILRE 122 | 174                     |
|                    | aa156      | 156 AEMKMWLNSNTDAAFPQ 172 | 304                     |
|                    | aa181      | 181 RKSPALIVWHHHSVST 197 | 269                     |
|                    | aa511      | 511 QIDPVKLSGYKDVILW 527 | 141                     |

<Characteristics of major epitopes are indicated in boldface.>
An additional potential factor limiting the response to avian vaccines relative to seasonal vaccines in humans is competition at the level of antigen presentation between naive CD4 T cells specific for new epitopes in the avian vaccines and more abundant and more easily triggered memory cells specific for conserved peptide epitopes. Most of the avian vaccines tested in humans have been derived from infected embryonated chicken eggs. We have shown that although HA protein is the only viral protein quantified in the vaccine and that biochemical procedures are implemented to enrich these vaccines for HA, these split and subunit vaccines also contain internal virion proteins such as nucleoprotein (NP) and matrix 1 (M1) protein. These are readily detectable by biochemical methods and at sufficient levels to recruit CD4 T cells (41, 42). Because of the high degree of genetic conservation of these internal virion proteins and use of standard “backbone” genes from A/PR/8/34 (H1N1) virus that are used for high efficiency vaccine production (43), CD4 T cells specific for these epitopes are repeatedly boosted and expanded in humans. CD4 T cells specific for these internal virion proteins may outcompete or suppress CD4 T cells specific for the divergent, avian-derived HA (and neuraminidase) epitopes. In several recent studies (20, 23), we have shown that although CD4 T cells specific for M1 and NP are expanded postvaccination, CD4 T cells specific for HA are the most effective at promoting anti-HA antibody responses to influenza vaccines and infection. Therefore, the specificity, rather than the absolute abundance of the CD4 T cells drawn into the response to vaccination, is likely to be critical in facilitating neutralizing antibody responses to vaccination, and here, avian vaccines will be at a disadvantage.

One additional factor that may contribute to immunogenicity differences between avian and human HA that was not addressed here relates to generalized features in glycosylation (44, 45). Differences in the number and location of glycosylation sites could alter the efficiency of carbohydrate receptor recognition by innate immune cells.
that can promote uptake of antigens, such as dendritic cells (45–49). It is known that HA from viruses that are adapted to circulate in humans contains more glycosylation sites in the globular head domain compared to avian HA (44). However, because unique features in alternative vaccine platforms (egg, mammalian, and insect cells) lead to distinct patterns of glycosylation, the role of glycosylation in protein vaccine immunogenicity is still an open question that will require further detailed study (31). In this regard, however, it is interesting to note that three epitopes restricted by HLA-DR1 that are shared within avian and human HA proteins elicit comparable CD4 T cell responses in the DR1 transgenic mice (Table 1; pH1, aa 437; H5, aa 433; H7, aa 431). This result argues that in this vaccination strategy, carbohydrate differences between avian and human HA do not substantially alter processing and presentation, which was described for different viral model antigens (50).

Finally, it is also formally possible that there are limitations in the primary human T cell receptor repertoire that diminish T cell responses to avian HA that were not revealed in this study of murine responses. This is an extremely difficult question to address experimentally because in the United States, infants are vaccinated several times in the first few years of life, making comprehensive analysis of the primary response to even seasonal HA extremely challenging with current technology. Also, thus far, experimental vaccines such as those that contain avian HA have only been evaluated for efficacy in adults. Also important in consideration of the CD4 T cell repertoire is the extensive degree of polymorphism in human HLA class II proteins. Humans express multiple isotypes (HLA-DR, HLA-DQ, and HLA-DP) and are most commonly heterozygous in the HLA complex (reviewed in reference 51). This will lead to a typical display of as many as 8 to 10 different MHC-II proteins that can each present pathogen-derived peptides and recruit CD4 T cells. This complexity in HLA expression makes it difficult to envision systematic defects in the CD4 T cell repertoire to the subset of peptides that are derived from avian HA proteins. In this regard, it is noteworthy that some of the HLA-DR-restricted peptides identified in this study were also found in humans using MHC-II tetramers (18, 52), suggesting that antigen handling and CD4 T cell epitope repertoires within humans and mice share some relevant features important for antigen presentation and CD4 T cell recruitment.

Based on our results suggesting that there are no inherent deficits in CD4 T cell recognition of avian HA-derived proteins, it is likely that strategies to enhance representation of these CD4 T cells in humans would be a good prepandemic strategy. Prepandemic priming of humans with avian-derived HA proteins devoid of other internal virion proteins would likely poise humans for more protective antibody responses to avian viruses and vaccines. Because the H5 and H7 sequences among different serotypes have many regions of sequence identity (53), it is likely that even vaccination with serologically distinct HA would prime many cross-reactive, avian HA-specific CD4 T cells that could be recalled during infection or vaccination with newly emerging avian viruses. Our recent studies with H5N1 vaccination in human subjects support this view. We found that memory CD4 T cells specific for H5 HA persisted in subjects vaccinated many years earlier and that these could be recruited into a subsequent immune response to vaccination, enabling a greatly enhanced antibody response to a serologically distinct H5N1-derived virus (20). Should avian influenza viruses continue to pose a threat to humans (54–64), such a prepandemic strategy could enhance vaccine responsiveness in “at risk” subjects and enhance early and potentially protective antibody responses in infected individuals.

MATERIALS AND METHODS

MICE. The HLA-DR1 (B10.M/J-TgN-DR1) and HLA-DR4 (C57BL/6Tac-Abb<tm->TgNDR4) transgenic mice were obtained from D. Zaller (Merck) through Taconic Laboratories and were maintained in the specific-pathogen-free facility at the University of Rochester according to institutional guidelines. Mice were used at 3 to 8 months of age.

ETHICS STATEMENT. All mice were maintained in a specific-pathogen free facility at the University of Rochester Medical Center according to institutional guidelines. All animal protocols used in this study adhere to the AAALAC International, the Animal Welfare Act, and the PHS guide, and were approved by the University of Rochester Committee on Animal Resources, Animal Welfare Assurance no. A3291-01.
The protocol under which these studies were conducted was originally approved 4 March 2006 (protocol 2006-030) and has been reviewed and reapproved every 36 months, with the most recent review and approval on 6 February 2015.

**Peptides and peptide array matrix strategy.** Peptide arrays H1 HA A/California/04/2009 (NR-15433), H3 HA A/New York/384/2005 (NR-2603), HS HA A/Vietnam/1203/2004 (NR-18974), and H7 HA A/Anhui/1/2013 (NR-44011) were obtained through BEI Resources, NIAID, NIH. The H1 array contained 139 15-mer peptides overlapping by 11 amino acids. The H3 array contained 94 17-mer peptides overlapping by 11 amino acids, and the H5 array contained 93 17-mer peptides overlapping by 11 amino acids. The 111 peptides in the H7 array were 17-mers overlapping by 12 amino acids. The peptides were reconstituted at 10 mM in phosphate-buffered saline (PBS), with or without added dimethyl sulfoxide to increase solubility of hydrophobic peptides and 1 mM dithiothreitol for cysteine-containing peptides. Working stocks (1 mM) were prepared in complete Dulbecco's modified Eagle's medium (DMEM), filter sterilized, and stored at 20°C, as were concentrated stocks. Peptides were grouped into pools containing 8 to 10 peptides, where no overlapping peptides are included within any given pool. These pools are arrayed into rows (labeled “R”) and columns (labeled “C”), as described previously (34, 35) and illustrated in Fig. 1. This strategy allows efficient identification of immunodominant peptides elicited in response to infection or vaccination. Peptide pools were considered positive if they were at least 2-fold over background in two experiments (typically greater than 30 spots/million). Peptides in nonstimulatory pools were eliminated from further study. The sequences of the peptides used were generally greater than 95% conserved with the immunizing recombinant HA proteins.

**Immunizations.** The recombinant HA proteins used were produced and purified from a baculovirus-insect cell (expressSF+) expression system and included A/California/04/2009 (NR-13691), A/Brisbane/10/07 (NR-19238), A/Indonesia/05/05 (NR-10511), and A/Netherlands/219/03 (NR-42022), obtained through BEI Resources (30, 31). HLA-DR transgenic mice were immunized subcutaneously in the rear footpad with 1.0 μg recombinant HA in the presence of alum, at a 1:1 ratio by volume. Popliteal lymph nodes (pLNs) were excised 10 to 11 days postimmunization, pooled, and used as the source of CD4 T cells for ELSpot analyses. Cells were depleted of red blood cells (RBCs) using ACK lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Na2-EDTA in H2O [pH 7.2]), followed by depletion of B cells, CD8 cells, and class II− cells by negative selection using MACS (magnetically activated cell sorting) technology (Miltenyi Biotec, San Diego, CA), according to the manufacturer’s instructions.

**ELSpot assays.** ELSpot assays were performed as previously described (34). Briefly, 96-well filter plates (Millipore, Billerica, MA) were coated with 2 μg/ml purified rat anti-mouse interleukin-2 (IL-2) (clone JES6-1A12; BD Biosciences, San Jose, CA) in PBS overnight at 4°C, washed with medium to remove any unbound antibody, and incubated with 100 μl medium per well for 1 h to block nonspecific binding. CD4 T cells (200,000) were isolated from the pLNs and cocultured with 500,000 syngeneic spleen cells (HLA-DR4) or DAP.3 fibroblast cells transfected with the genes encoding HLA-DR1, generously provided by E. Long, NIAID, NIH (35,000), and with either a pool of peptides for matrices or a single peptide at a final concentration of 2 or 5 μM, respectively, in a total volume of 200 μl for 18 to 20 h at 37°C and 5% CO2. The cells were removed, and the plates were washed with wash buffer (1× PBS, 0.1% Tween 20). Biotinylated rat anti-mouse IL-2 (clone JES6-5H; BD Biosciences) was added at a concentration of 2 μg/ml at 50 μl/well in wash buffer with 10% fetal bovine serum (FBS) and incubated at room temperature for 30 min. The plates were washed again, and streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA) was added at a dilution of 1:1,000 in wash buffer with 10% FBS at 50 μl/well and incubated for 30 min at room temperature. The plates were washed with wash buffer and developed using Vector Blue substrate kit III (Vector Laboratories, CA) prepared in 100 mM Tris (pH 8.2). After drying, quantification of spot was performed with an Immunospot reader series 5.2, using Immunospot software, version 5.1.

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