Cutting Edge: CD4 T Cells Generated from Encounter with Seasonal Influenza Viruses and Vaccines Have Broad Protein Specificity and Can Directly Recognize Naturally Generated Epitopes Derived from the Live Pandemic H1N1 Virus

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Cutting Edge: CD4 T Cells Generated from Encounter with Seasonal Influenza Viruses and Vaccines Have Broad Protein Specificity and Can Directly Recognize Naturally Generated Epitopes Derived from the Live Pandemic H1N1 Virus

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The unexpected emergence of pandemic H1N1 influenza has generated significant interest in understanding immunological memory to influenza and how previous encounters with seasonal strains influence our ability to respond to novel strains. In this study, we evaluate the memory T cell repertoire in healthy adults to determine the abundance and protein specificity of influenza-reactive CD4 T cells, using an unbiased and empirical approach, and assess the ability of CD4 T cells to recognize epitopes naturally generated by infection with pandemic H1N1 virus. Our studies revealed that most individuals have abundant circulating CD4 T cells that recognize influenza-encoded proteins and that a strikingly large number of CD4 T cells can recognize autologous cells infected with live H1N1 virus. Collectively, our results indicate that a significant fraction of CD4 T cells generated from priming with seasonal virus and vaccines can be immediately mobilized upon infection with pandemic influenza strains derived from antigenic shift. *The Journal of Immunology*, 2010, 185: 4998–5002.

The online version of this article contains supplemental material.

Abbreviations used in this paper: AF, allantoic fluid; CA, A/California/04/09 E3; HA, hemagglutinin; HAI, hemagglutinin inhibition; MOI, multiplicity of infection; NA, neuraminidase; NC, A/New Caledonia/20/1999; NP, nucleoprotein; NS, nonstructural protein.

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Materials and Methods
Isolation of CD4 T cells from human PBMCs and ELISPOT assays

Blood samples from healthy adults (ages 18–50 y) were obtained with informed consent and Biosafety Committee approval. PBMCs were isolated using Histopaque-1077 centrifugation, washed, and frozen at a controlled rate, −80°C, in FCS containing 10% DMSO. Frozen PBMCs were thawed and plated overnight, and CD4 T cells were purified by negative selection using MACS no-touch CD4 purification (Miltenyi Biotec, Auburn, CA). As a source of APCs, PBMCs from the same donor were T depleted using CD4 and CD8 microbeads (Miltenyi Biotec). ELISPOT assays were performed as described (8, 9), with some modifications for human samples. Briefly, 96-well filter plates (Millipore, Billerica, MA) were coated with 15 μg/ml purified anti-human IFN-γ (clone 1-D1K, MabTech, Cincinnati, OH), washed, and incubated with RPMI 1640 containing 10% FCS to block nonspecific binding. A total of 200,000 T cells were cocultured with 100,000 APCs and peptide pools or virus-infected APCs for 24–36 h at 37°C in 5% CO2, in RPMI containing 10% FBS (Life Technologies, Rockville, MD). Postincubation, cells were removed from the plates by washing, and biotinylated anti-human IFN-γ (clone 7-B6-1, MabTech) was added at 2 μg/ml and incubated at room temperature for 2 h. The plates were processed for spot counting as previously described using an Immunospot reader series 2A using Immunospot software, version 3.2 (Cellular Technology, Shaker Heights, OH).

Synthetic peptide libraries
The 17-mer peptides overlapping by 11 aa encompassing the entire sequences of HA, neuraminidase (NA), NP, M1, NS1, and PB1 were obtained from the National Institutes of Health Biodefense and Emerging Infections Research Resources Repository, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD) as previously described (9, 10) and reconstituted in PBS, with or without added DMSO, to increase solubility of hydrophobic peptides, and 1 mM DTT, for peptides that contained cysteine. Peptides were pooled and used at 2 μM (final concentration) for each peptide.

Infection of APCs
Influenza A/New Caledonia/20/99 and A/California/04/2009 E3 viruses were prepared as previously described (8) and used to infect APCs. APCs purified as described above were washed in serum-free DMEM media and infected with virus [multiplicity of infection (MOI) of 1 or 10 as indicated in the figure legends] for 1 h in serum-free media at 37°C, followed by dilution and washing with complete culture media, described above.

Results and Discussion
To enumerate the abundance and protein specificity of circulating human CD4 T cells to influenza A, several factors were considered. Due to the MHC heterogeneity in humans and the limited information on binding motifs for many class II alleles, even with sophisticated binding algorithms (11, 12), we did not bias our studies by preselecting peptides based on predictions of affinity or genetic conservation among influenza segments, as other studies have done (13, 14). Accordingly, overlapping peptides representing the translated sequence of influenza proteins were used. A subset of virus Ags was chosen because these represent the classes of Ags that humans encounter during vaccination or infection: highly conserved internal proteins (NP, M, and PB1), frequently encountered through natural infection, genetically variable, membrane-expressed proteins (HA and NA), for which sequences will vary but that are enriched in subunit vaccines (15, 16), and the NS1 protein, expressed in infected cells but not in virions from which subunit vaccines are made. Because of the limited numbers of PBMCs obtainable from normal healthy
The results of the experiments evaluating the magnitude and specificity of CD4 T cells from nine healthy human donors are shown in Fig. 1, in which purified CD4 T cells were tested with pools of peptides representing (from left to right in each panel) HA from H1N1 (A/New Caledonia/20/99) or H3N2 (A/New York/384/2005), NA from H1N1 or H3N2, NP, NS1, M1, or PB1 from H1N1, using autologous cells depleted of T cells as APCs. CD4 T cells reactive with the influenza peptides were enumerated using IFN-γ ELISPOT assays. Several important observations were made from these analyses. First, each donor had circulating CD4 T cells reactive with influenza-derived peptides, although the degree of total influenza reactivity varied somewhat. We found that those individuals with very low reactivity to influenza frequently had low reactivity to the positive control tetanus toxoid protein tested (summarized in Supplemental Fig. 1).

With regard to the Ag specificity of the influenza-reactive CD4 T cells, peptides from most proteins could recruit CD4 T cells from each donor, and the distribution of reactivity differed among the individuals tested. The overall reactivity pattern of CD4-enriched populations from all of the donors is shown in Fig. 1B, which illustrates that within this population, both genetically conserved and highly variable proteins were able to recruit significant numbers of cells from the peripheral blood pool, typically on an average of 200–300 cells per million CD4 T cells. This result indicates that repeat encounters with influenza viruses and vaccines does not lead to dominance of reactivity toward conserved internal viral proteins over variable HA and NA. Based on the number of CD4 T cells circulating in peripheral blood, we can estimate that the number of CD4 T cells specific for the influenza in a typical human is in the range of 5 million to 6 million. This number is a lower estimate because we have only measured IFN-γ-producing cells and have tested for only two types of HA and NA.

We expect that in each individual, CD4 T cells that recognize the conserved M1, PB1, and NP proteins and the variable HA and NA proteins will be diverse in peptide specificity due to heterogeneity in the human MHC class II molecules that can present the peptides in the different hosts and because each individual will have encountered different viruses and vaccines. However, we predict each individual might have some influenza-specific CD4 T cells that recognize epitopes shared among divergent influenza viruses because of the genetic conservation of many of the viral protein segments. Of particular interest to evaluate was reactivity to the recently emerging pandemic strain A/California/04/09 (1–3). There has been considerable uncertainty regarding whether confrontation with seasonal viruses and vaccines in any way facilitates the immune response toward vaccines containing the novel H1N1 virus proteins or the H1N1 virus itself.

Priming of CD4 T cells to influenza-derived peptides via virus infection and vaccination can occur by many mechanisms of Ag uptake and processing, and it is possible that the influenza-specific CD4 T cells identified in this study through use of synthetic peptides may not be able to recognize the epitopes generated through natural infection of APCs. To explicitly examine the potential of the human influenza-specific CD4 T cell repertoire to be rapidly mobilized upon infection with live A/California/04/09 virus, CD4 T cells were evaluated for their ability to be activated by autologous cells infected with this novel virus. Seasonal H1N1 A/New Caledonia/20/99 was used as a control. CD4 T cells were purified, and a separate group of cells, depleted of CD4 and CD8 T cells, were used as APCs. APCs were incubated briefly with live virus, prepared as described (8), then washed, and added to autologous CD4 T cells for a 24 h coculture. Alloantibody (AF) was used as a negative control, and this was a set of APCs alone, infected with each virus, without CD4 T cells.

**FIGURE 1.** Human CD4 T cells from individuals not previously exposed to the virus can recognize autologous cells infected with A/California/04/09. In A, CD4 T cells were tested for reactivity to virus-infected APCs, where T-depleted PBMCs were incubated with either A/New Caledonia/20/1999 (NC, black) or A/California/04/09 E3 (CA, white) virus at an MOI of 1 or 1. CD4 T cells were tested for reactivity to AF, and APCs were tested for cytokine responses from virus infection, both of which were below background for all donors. Serum was tested by HA inhibition (HAI) for exposure to novel H1N1 (at least 3 mo after the collection of PBMCs). In B, CD4 T cell reactivity to influenza-infected APCs from donors collected in the autumn of 2008 (prior to the novel H1N1 outbreak) is shown using either A/New Caledonia/20/99 (NC, black bars) or A/California/04/09 E3 (CA, white bars) virus at an MOI of 10. Reactivity to AF was below background for all donors. *, HAI titer was not determined; −, nondetectable HAI response; +, HAI titer >40.

**FIGURE 2.** Cross reactivity of anti-HA–specific CD4 T cells between seasonal H1N1 (A/New Caledonia/20/99) and novel H1N1 (A/California/04/09) viruses. CD4 T cells were cocultured with APCs and a pool of HA peptides (shown in Supplemental Table I) from seasonal H1N1 or pandemic H1N1 as indicated in the figure at a final concentration of 2 μM in IFN-γ ELISPOT assays. Serum or plasma Ab from each donor was tested in HAI assays for reactivity to A/California/04/09 and was grouped as either having some evidence of contact with the pandemic H1N1 strain (HAI > 40, filled symbols) compared with those with an HAI ≤ 40 (open symbols) comparable to donor serum collected before 2009.
The number of CD4 T cells recognizing the viral Ags was quantified by IFN-γ ELISPOT assays.

Fig. 2A shows the results of these experiments. Strikingly, many CD4 T cells from healthy donors produced IFN-γ when stimulated with autologous APCs infected with the pandemic H1N1 A/California/04/09 virus. The recall of circulating CD4 T cells by the novel H1N1 strain was equivalent to that obtained by the seasonal virus. No IFN-γ was produced when AF was used as an Ag, and no IFN-γ was produced by cultures containing only infected APC with no T cells added (data not shown). These data show that the typical human infected with the novel H1N1 virus has circulating CD4 T cells that can be rapidly mobilized upon infection and that many of the CD4 T cells measured using the peptide arrays can recognize naturally processed peptides displayed by infected cells.

Two pieces of data argue that the reactivity of circulating CD4 T cells to the pandemic strain of influenza that we have detected is not dependent on unreported encounter of these normal donors with the novel H1N1 virus or vaccines. First, most of the individuals tested in our study had no detectable circulating Ab reactive with the novel H1N1 virus, as indicated by the − symbol in the appropriate panels of Fig. 2A. Second, we have evaluated several donors whose PBMCs were collected in the autumn of 2008, well before any reports of infection with the novel H1N1 virus. Fig. 2B shows the reactivity of the CD4 T cells from these donors with autologous APCs infected with either the seasonal H1N1 virus (A/New Caledonia, left bar) or the novel H1N1 virus (A/California/04/09, middle bar), where it is clear that each virus strain is able to infect autologous APCs and stimulate many CD4 T cells to produce cytokines. These data show that the normal composition of memory CD4 T cells can be recalled by epitopes displayed by autologous cells infected by novel influenza virus strains that diverge from seasonal influenza.

For peptide epitopes within NP, M1, and PB1, there should be significant cross-reactivity because of the high homology of most regions of these proteins. The current pandemic H1N1 strain is thought to be composed of influenza genes from multiple species not previously identified in humans or swine in the United States (1, 3, 17). Sequence comparisons show a broad range in amino acid identity between pandemic H1N1 and seasonal viruses. The differences are unevenly distributed, with PB1 displaying the least (5%) amino acid diversity and HA the most (80%). CD4 T cell reactivity to HA is particularly important to quantify because of the recent evidence that CD4 help for B cell responses might occur optimally if the epitopes are derived from the same Ag (18). Accordingly, HA-specific CD4 T cells might be most potent for production of neutralizing HA-specific Abs.

In our previous studies on the primary response to infection with A/New Caledonia 20/99, we found that the HA-specific CD4 T cell repertoire was broad and included >30 epitopes (8). Comparison of HA sequences expressed by the pandemic H1N1 A/California/04/09 virus and other seasonal H1N1 strains of virus (not shown) indicates that there are segments of high sequence diversity where little T cell cross-reactivity is expected and regions of complete conservation where we assume full cross-recognition. Our studies using a DR1-transgenic mouse model of infection (9) and those of Roti et al. (19) using tetramer staining of human PBL have shown that even within HA, there are epitopes that are shared between seasonal virus and pandemic H5N1. Less predictable are the regions of HA that have a limited degree of sequence divergence. We searched for sequences that had limited sequence divergence between seasonal and the pandemic influenza. We anticipated that these might elicit CD4 T cells that recognize pandemic H1N1 influenza independently of previous exposure, with reactivity depending on whether the substituted residues were TCR or MHC class II anchor substitutions. The peptide sequences chosen are shown in Supplemental Table I. CD4 T cells from healthy donors were tested for their ability to secrete cytokine in response to either the peptides derived from the seasonal virus or those corresponding to the pandemic H1N1 strain. As shown in Fig. 3, there was significant cross-reactivity between peptides derived from regions of HA that had some sequence divergence from the seasonal strain and the pandemic strain. We also found that HA-specific CD4 T cell reactivity to pandemic H1N1 was not higher in those individuals who had serum reactivity to the H1N1 virus (Fig. 3, filled symbols) compared with those that did not (Fig. 3, open symbols). Therefore, there are HA-specific CD4 T cells circulating in normal individuals that can be mobilized upon infection with novel strains of H1N1 viruses.

In future experiments, it will be of interest to examine how the specificity and magnitude of pre-existing CD4 memory toward influenza in humans influences the quality and magnitude of the response to vaccination or infection with pandemic H1N1 or other pandemic strains and if neutralizing Ab responses to novel influenza viruses and resistance to virus-induced pathology can be predicted by the specificity or magnitude of the pre-existing repertoire of CD4 T cells. If such linkages are shown, it should be possible to enhance vaccine effectiveness by selectively arming the CD4 T cell compartment with those T cells specific for the most useful subset of influenza-derived proteins.

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Disclosures

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