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Human Cytotoxic T Lymphocytes Directed to Seasonal Influenza A Viruses Cross-React with the Newly Emerging H7N9 Virus

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In February 2013, zoonotic transmission of a novel influenza A virus of the H7N9 subtype was reported in China. Although at present no sustained human-to-human transmission has been reported, a pandemic outbreak of this H7N9 virus is feared. Since neutralizing antibodies to the hemagglutinin (HA) globular head domain of the virus are virtually absent in the human population, there is interest in identifying other correlates of protection, such as cross-reactive CD8$^+$ T cells (cytotoxic T lymphocytes [CTLs]) elicited during seasonal influenza A virus infections. These virus-specific CD8$^+$ T cells are known to recognize conserved internal proteins of influenza A viruses predominantly, but it is unknown to what extent they cross-react with the newly emerging H7N9 virus. Here, we assessed the cross-reactivity of seasonal H3N2 and H1N1 and pandemic H1N1 influenza A virus-specific polyclonal CD8$^+$ T cells, obtained from HLA-typed study subjects, with the novel H7N9 virus. The cross-reactivity of CD8$^+$ T cells to H7N9 variants of known influenza A virus epitopes and H7N9 virus-infected cells was determined by their gamma interferon (IFN-γ) response and lytic activity. It was concluded that, apart from recognition of individual H7N9 variant epitopes, CD8$^+$ T cells to seasonal influenza viruses display considerable cross-reactivity with the novel H7N9 virus. The presence of these cross-reactive CD8$^+$ T cells may afford some protection against infection with the new virus.

Influenza viruses are an important cause of respiratory tract infections. Occasionally, animal influenza viruses cross the species barrier and infect humans after zoonotic transmission. In the past 2 decades, several avian influenza A viruses, like those of the H9N2 subtype (1), the H7N7 subtype (2, 3), and the H5N1 subtype (4–9), have infected humans. In 2009, H1N1 influenza A viruses of swine origin (H1N1pdm09) caused a pandemic outbreak, and these viruses continue to circulate in the human population (10).

In February 2013, the first human cases of infection with a novel avian influenza A virus of the H7N9 subtype were reported in China. As of September 2013, 135 laboratory-confirmed cases had been reported, 44 of which had a fatal outcome (11). Older male individuals especially seem to be at risk for developing severe disease upon infection (12–15). Most hospitalized patients developed severe viral pneumonia and acute respiratory distress syndrome (ARDS) (16–19).

Influenza A viruses with hemagglutinin (HA) and neuraminidase (NA) of subtypes H7 and N9, respectively, circulate in wild bird species (20, 21). The newly emerged H7N9 virus is most likely the result of multiple reassortment events of at least three avian viruses (17, 22, 23). Although the H7N9 virus has been classified as a low-pathogenic virus based on the intravenous pathogenicity index (IVPI) in chickens and the absence of a multibasic cleavage site in the HA, it is quite pathogenic in humans (17). The virus also replicates efficiently in the airways of other mammalian species, including mice, ferrets, and cynomolgus macaques (24, 25). It is more pathogenic than seasonal influenza A H3N2 (sH3N2) viruses or pandemic 2009 H1N1 (pH1N1) viruses and after intratracheal inoculation causes fatal disease in ferrets (26). The high pathogenicity in mammals correlates with the presence of known pathogenicity markers. Several human isolates of the H7N9 virus contain the E627K substitution in PB2, which allows avian influenza viruses to replicate at lower temperatures (27). A deletion of 5 amino acids in the NA of H7N9 virus is associated with enhanced virus replication (17). The presence of the Q226L substitution in the HA (17, 28) is associated with binding to alpha(2,6)-linked sialic acids found in the human upper respiratory tract (24) and has been associated with airborne transmission of avian H5N1 virus in ferrets (29). In the case of the novel H7N9 virus, only limited transmission between ferrets was observed (24, 25, 30, 31). Acquisition of gene segments from human influenza A viruses by the avian influenza H7N9 virus through genetic reassortment may lead to further adaptation to humans (10, 32–37). The detection of an H7N9 patient who was coinfected with an sH3N2 virus underscores this possible scenario (38). Although at present no sustained human-to-human transmission of the H7N9 virus has been reported (39), the pandemic potential of H7N9 virus should be considered seriously, especially since virus-neutralizing antibodies directed to the HA globular head domain of the virus are virtually absent in the human population (18), though low concentrations of stalk region-specific antibodies might be present (40, 41).

On the other hand, virus-specific CD8$^+$ T cells (cytotoxic T lymphocytes [CTLs]), induced after infection with seasonal influenza A viruses, are mainly directed to the conserved internal proteins of influenza A viruses (33, 42–51). The presence of these cross-reactive CD8$^+$ T cells may afford a certain degree of heterosubtypic immunity against infection with novel H7N9 viruses. Using various combinations of influenza A virus subtypes for pri-

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TABLE 1 Variant amino acid sequences of known $CD8^+$ T cell epitopes in the influenza A H7N9 virus[a]

HLA restriction	Epitope	Amino acid sequence[b]					Reference
		Influenza A virus	sH3N2	sH1N1	pH1N1	H7N9	
HLA-A*3	M1 13-21	SIIPSGPLK	--V------	--V------	---------	---------	85
	M1 27-35	RLEDVFAGK	---------	---S-----	---------	---------	86
HLA-A*0201	M1 58-66	GILGFVFTL	---------	---------	---------	---------	44, 87
	M1 59-68	ILGFVFTLTV	----------	----------	----------	----------	87
HLA-B*35	M1 128-135	ASCMGLIY	--------	--------	--------	--------	88
HLA-B*44	M2 7-15	VETPIRNEW	---------	---------	----T-S--	----T-TG-	46
HLA-A*0201	NA 213-221	CVNGSCFTV	INGTCTVVM	--------I	---------	VCPVVFTDG	89
HLA-A*01	NP 44-52	CTELKLSDY	--------H	------N--	---------	--------N	90
HLA-A*6801	NP 91-99	KTGGPIYKR	-------R-	---------	-------R-	-------R-	91
HLA-B*1402	NP 146-154	TTYQRTRAL	A--------	---------	A--------	A--------	92
HLA-B*2705	NP 174-184	RRSGAAGAAVK	----------	----------	----------	----------	62
HLA-A*3	NP 188-198	TMVMELVRMIK	------I--V-	---L--I----	-IA---I----	------I----	85
HLA-A*03	NP 265-273	ILRGSVAHK	---------	---------	---------	---------	90
HLA-B*44	NP 338-346	FEDLRVLSF	-----L---	------S--	------S--	------S--	93
HLA-B*3701	NP 339-347	EDLRVLSFI	----L----	-----S---	-----S---	-----S---	94, 95
HLA-B*44	NP 379-387	LELRSRYWA	-----G---	---------	---------	---------	93
HLA-B*0801	NP 380-388	ELRSRYWAI	----G----	---------	---------	---------	96
HLA-B*2702	NP 381-388	LRSRYWAI	---G----	--------	--------	--------	97
HLA-B*2705	NP 383-391	SRYWAIRTR	-G-------	---------	---------	---------	98
HLA-B*35	NP418-426	LPFEKSTVM	-------I-	---D-A-I-	----RA---	----RA-I-	99
HLA-A*0201	NS1 122-130	AIMDKNIIL	---E---M-	---------	---E---V-	--V----T-	100
	NS1 123-132	IMDKNIILKA	--E---M---	----------	--E---V---	-V----T---	100
HLA-B*44	NS1 158-166	GEISPLPSL	--------F	--------F	---------	---------	93
HLA-A*01	PB1 591-599	VSDGGPNLY	---------	---------	---------	---------	90

[a] The A/Anhui/1/13 (H7N9) sequence was unavailable at the time of ordering the peptides. All epitopes, except LPFEKSTVM (H7N9 LPFERARIM), were conserved between the H7N9 viruses present in the database at April 22th and the A/Anhui/1/13 virus used in this study.

[b] Peptides used in the present study are shaded and were selected based on variation in the H7N9 sequence and correspondence to the HLA alleles of the study subjects. Synthetic immunograde peptides were ordered with >85% purity. The dashes indicate identity with the amino acids in the influenza A virus sequence.

mary and secondary infection, this type of immunity and the contribution of virus-specific $CD8^+$ T cells were demonstrated in various animal models (52–57). Evidence for heterosubtypic immunity and the role of $CD8^+$ T cells in humans is limited (58–61), though the presence of $CD8^+$ T cells cross-reactive with avian H5N1 and swine origin triple-reassortant A H3N2 (vH3N2) viruses has been demonstrated (49–51, 62). It is unknown to what extent $CD8^+$ T cells elicited by a seasonal or 2009 pH1N1 influenza A virus infection cross-react with the novel H7N9 virus. Here, we show that polyclonal $CD8^+$ T cell populations specific for seasonal H1N1 (sH1N1), sH3N2, or pH1N1 virus cross-react with the H7N9 virus by determining their gamma interferon (IFN-γ) response upon *in vitro* stimulation with the novel H7N9 virus and their lytic activity toward H7N9 virus-infected human leukocyte antigen (HLA)-matched target cells. The preexisting cross-reactive $CD8^+$ T cells may afford some level of protection and may reduce morbidity and mortality caused by infections with the novel H7N9 virus.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells (PBMCs) were obtained from 6 HLA-typed healthy blood donors (35 to 50 years of age) between 2008 and 2013 (Sanquin Bloodbank, Rotterdam, The Netherlands). Lymphoprep (Axis-Shield PoC, Oslo, Norway) gradient centrifugation was used to isolate PBMCs, which were subsequently cryopreserved at −135°C. Donors were selected based on their HLA class I alleles for which functionally confirmed influenza A virus HLA class I epitopes have been identified and had the following HLA haplotypes: subjects 1 and 2, HLA-A*0101, -A*0201, -B*0801, and -B*3501; subjects 3 and 4, HLA-A*0101, -A*0201, -B*0801, -B*2705; and subjects 5 and 6, HLA-A*0101, -A*0301, -B*0801, and -B*3501. The use of PBMCs for scientific research was approved by the Sanquin Bloodbank after informed consent was obtained from the blood donors.

Peptides. The amino acid sequences of confirmed influenza A virus HLA class I epitopes were aligned with their H7N9 analogues from human isolates between February 2013 and 22 April 2013 (Table 1). Sequences were obtained from the influenza virus resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database). In addition, conservation of these epitope sequences in the prototype viruses used in the present study, sH3N2 (A/Netherlands/348/07), sH1N1 (A/Netherlands/26/07), and pH1N1 (A/Netherlands/602/09), was determined (Table 1). The H7N9 variant epitopes for which the HLA restriction was compatible with the HLA type of the study subjects were ordered as synthetic immunograde peptides (>85% purity) (Eurogentec, Seraing, Belgium).

Viruses. Influenza virus A/Anhui/1/2013 (H7N9) was isolated from a fatal human case (Anhui Province, People's Republic of China) and was kindly provided through the WHO Pandemic Influenza Preparedness (PIP) framework and subsequently passaged once in Madin Darby Canine Kidney (MDCK) cells. Prototypic seasonal influenza A viruses A/Netherlands/348/07 (sH3N2), A/Netherlands/26/07 (sH1N1), and A/Netherlands/602/09 (pH1N1) were propagated in MDCK cells. Culture supernatants were clarified by low-speed centrifugation and subsequently purified by ultracentrifugation through a sucrose gradient. Their infectious-virus titers were determined as described previously (63).

Amino acid sequence identity. The amino acid sequence identity of the viral proteins of influenza viruses A/Anhui/1/2013 (H7N9) and the prototype sH3N2, sH1N1, and pH1N1 was determined using BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 2). The consensus sequence of A/Anhui/1/2013 was obtained from the GISAID database (http://platform.gisaid.org), and the consensus sequence of the influenza

TABLE 2 Percent amino acid sequence identity with A/Anhui/1/2013 (H7N9)

Gene segment	% Identity		
	sH3N2	sH1N1	pH1N1
PB2	94	94	97
PB1	97	95	96
PA	94	95	96
HA	47	41	41
NP	91	92	93
NA	45	43	45
M1	91	91	92
M2	82	78	89
NS1	76	80	78
NS2	93	90	88

virus A/Anhui/1/2013 preparation used in the present study was confirmed by sequence analysis (30).

In vitro expansion of influenza A virus-specific CD8$^+$ T cells. PBMCs obtained from HLA-typed study subjects were stimulated with sH3N2, sH1N1, and pH1N1 viruses at a multiplicity of infection (MOI) of 3, as described previously (64). Eight days after stimulation, polyclonal CD8$^+$ T cells were isolated from the expanded PBMC cultures by means of CD8$^+$ magnetically activated cell sorting (MACS) bead sorting according to the manufacturer's recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany) and subsequently used as effector cells in IFN-γ enzyme-linked immunosorbent spot (ELISpot) and lytic assays (see below).

Target cells. HLA-matched B lymphoblastoid cell lines (BLCLs) were prepared as described previously (65). The cells (10^6) were incubated with or without 100 μM peptide for 16 h at 37°C and subsequently washed and resuspended in RPMI 1640 medium (Lonza, Basel, Switzerland) containing antibiotics and 10% fetal bovine serum (Sigma-Aldrich, Zwijndrecht, The Netherlands) (R10F medium). Virus-infected target cells were prepared by inoculating BLCLs at an MOI of 3 with sH3N2, sH1N1, pH1N1, or H7N9 virus. After 1 h, the cells were washed and resuspended in R10F medium and cultured for 16 to 18 h at 37°C before being used for the stimulation of T cells or as target cells.

IFN-γ ELISpot assay. The IFN-γ responses of *in vitro*-expanded polyclonal CD8$^+$ T cells were determined by ELISpot assays, which were performed according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden). In brief, 10,000 or 5,000 *in vitro*-expanded polyclonal CD8$^+$ T cells were used as effector cells and incubated for 16 to 18 h with 30,000 peptide-loaded, virus-infected, or untreated HLA class I-matched target cells, in triplicate. The average number of spots was determined using an ELISpot reader and image analysis software (Aelvis, Sanquin Reagents, Amsterdam, The Netherlands).

CTL assay. The lytic capacity of the *in vitro*-expanded polyclonal CD8$^+$ T cells was determined using a CTL assay with carboxyfluorescein succinimidyl ester (CFSE)-labeled target cells. In brief, 5×10^6 cells of HLA class I-matched BLCLs were incubated with 50 μM CFSE (Sigma-Aldrich, Zwijndrecht, The Netherlands) for 5 min at 37°C. Subsequently, these cells were inoculated with sH3N2, sH1N1, pH1N1, or H7N9 virus at an MOI of 3 for 16 to 18 h. The infected and CFSE-labeled BLCL target cells were cocultured with the *in vitro*-expanded polyclonal CD8$^+$ effector T cells in effector-to-target cell (E:T) ratios of 5, 2.5, and 1.25. After a 3-h incubation period, the cells were fixed using Cytofix/Cytoperm (BD Biosciences, Breda, The Netherlands), and lysis in the target cell population was determined by flow cytometry using BD FACSDiva software (Becton, Dickinson B.V., Breda, The Netherlands). Experiments were performed in triplicate.

Statistical analysis. The data were analyzed using an independent *t* test, and differences were considered significant at a *P* value of <0.05.

RESULTS

Comparison of amino acid sequences of CD8$^+$ T cell epitopes. The amino acid sequences of 24 confirmed influenza A virus HLA class I epitopes were compared with their influenza A H7N9 virus analogues. As shown in Table 1, most epitopes (>50%) were fully conserved in H7N9 viruses. Based on these results, four variant H7N9 epitopes that were conserved in our prototypic sH3N2, sH1N1, and/or pH1N1 viruses (Table 1) and were compatible with the HLA type of the study subjects under investigation were further tested for cross-recognition in the ELISpot assay. All epitopes except $NP_{418-426}$ were conserved among H7N9 viruses available in the influenza virus resource database (22 April 2013) and the A/Anhui/1/13 (H7N9) virus used in this study.

Cross-recognition of influenza A (H7N9) analogues of known influenza A HLA class I epitopes. *In vitro*-expanded CD8$^+$ T cell preparations specific for sH3N2, sH1N1, and pH1N1 influenza viruses were tested for their cross-reactivity with the selected H7N9 variant epitopes listed in Table 1 using peptide-loaded HLA-matched BLCLs.

Virus-specific CD8$^+$ T cells obtained from study subjects 1 and 2 (HLA-A*0101, -A*0201, -B*0801, and -B*3501) displayed strong reactivity with the homologous epitopes, except for epitope $NS_{123-132}$ (IMDKNIILKA) (Fig. 1A and B). The H7N9 variant of the $NP_{418-426}$ (LPFERATIM) epitope was recognized by sH3N2-specific CD8$^+$ T cells derived from subject 1, although the IFN-γ response was lower than the response to the homologous epitope (LPFEKSTIM) (Fig. 1A). None of the other H7N9 variant epitopes were recognized by virus-specific CD8$^+$ T cells of these HLA-A*0101, -A*0201, -B*0801, and -B*3501 study subjects.

Virus-specific CD8$^+$ T cells obtained from study subjects 3 and 4 (HLA-A*0101, -A*0201, -B*0801, and -B*2705) displayed a minor response to homologous epitopes $NS_{122-130}$ and $NS_{123-132}$ (Fig. 1C and D). This is in agreement with the subdominant nature of the responses to these epitopes in these subjects (data not shown). CD8$^+$ T cells from both subjects did not display any response to the H7N9 variant of the $NS_{122-130}$ and $NS_{123-132}$ epitopes (Fig. 1C and D). Although CD8$^+$ T cells of the two subjects displayed reactivity with the homologous NP_{44-52} epitope, they did not respond to the H7N9 variant of the epitope (CTELK LSDN).

Virus-specific CD8$^+$ T cells from study subjects 5 and 6 (HLA-A*0101, -A*0301, -B*0801, and -B*3501) displayed a strong response to the homologous sH3N2 variant of the $NP_{418-426}$ epitope (Fig. 1E and F). Some minor cross-reactivity with the H7N9 variant (LPFERATIM) was observed with CD8$^+$ T cells derived from subject 5 (Fig. 1E). As for the other subjects, no cross-reactivity was observed with the H7N9 variant of the NP_{44-52} (CTELKL SDN) epitope with CD8$^+$ T cells obtained from subject 5.

Overall, the extent of cross-reactivity of influenza virus-specific CD8$^+$ T cells against individual H7N9 variant epitopes was low and dependent on the study subjects and peptides tested.

CD8$^+$ T cells cross-react with influenza A H7N9 virus-infected cells. Since more than 50% of previously identified influenza virus HLA class I epitopes were present in the H7N9 virus, we wished to compare the overall amino acid sequence identities between the H7N9 virus and the prototypic sH3N2, sH1N1, and pH1N1 viruses used in the present study. BLAST analysis revealed that the sequence identity of most viral proteins was high (>76%), except for hemagglutinin and neuraminidase (Table 2).

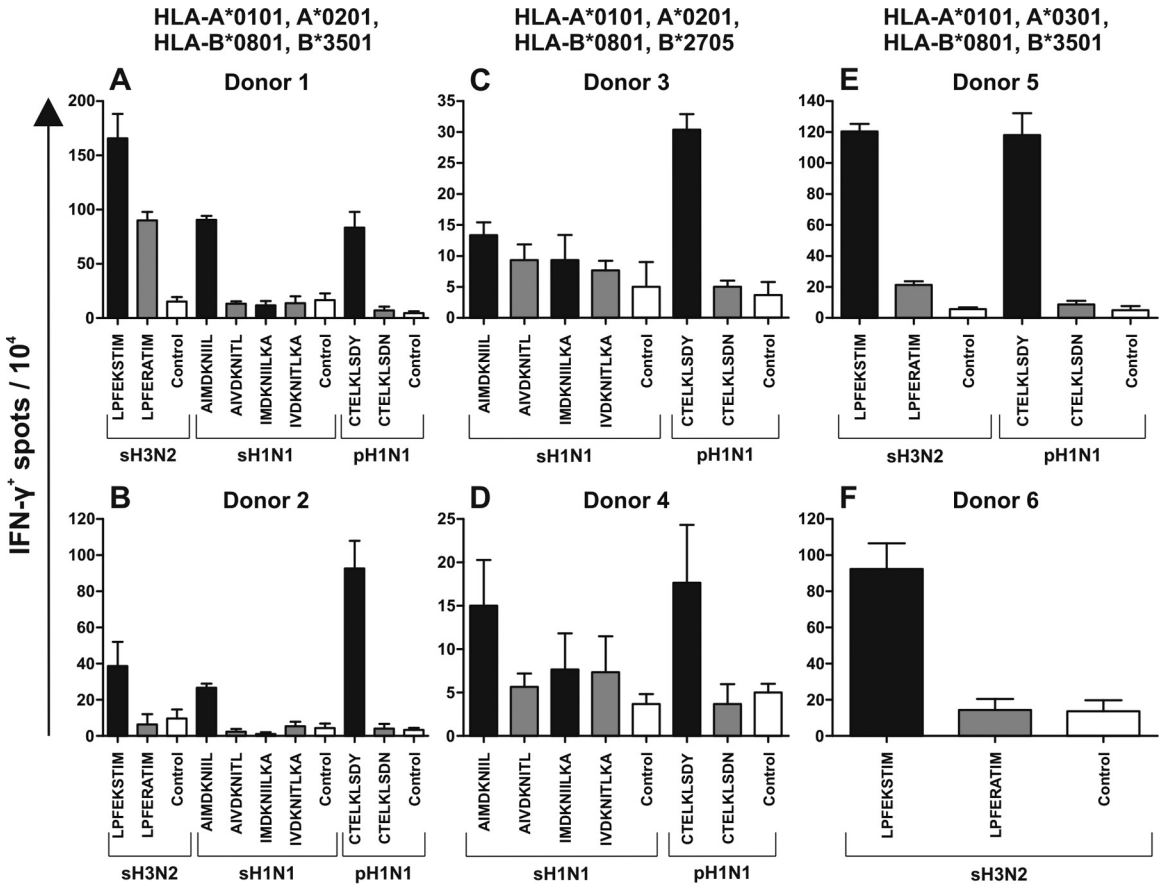


FIG 1 Epitope-specific IFN-γ production by seasonal influenza virus-specific CD8$^+$ T cells after stimulation with peptide-loaded BLCLs. Polyclonal CD8$^+$ T cells were isolated from PBMCs *in vitro* stimulated with sH3N2, sH1N1, or pH1N1, as indicated. No pH1N1 *in vitro* stimulation was performed for subject 6, since those PBMCs were isolated in 2008, prior to the 2009 pandemic outbreak. The polyclonal CD8$^+$ T cells were subsequently stimulated with peptide-loaded and untreated HLA class I-matched BLCLs. Stimulation with homologous peptides is indicated by black bars, stimulation with H7N9 variant peptides is indicated by gray bars, and control cells without peptide are indicated by white bars. The number of IFN-γ-producing cells per 10,000 polyclonal CD8$^+$ T cells was determined by ELISpot assay. The results represent the averages of triplicate wells. Peptides were selected based on the variation in the H7N9 sequence and their compatibility with the HLA haplotypes of our study subjects. The error bars indicate standard deviations of results from the triplicate wells.

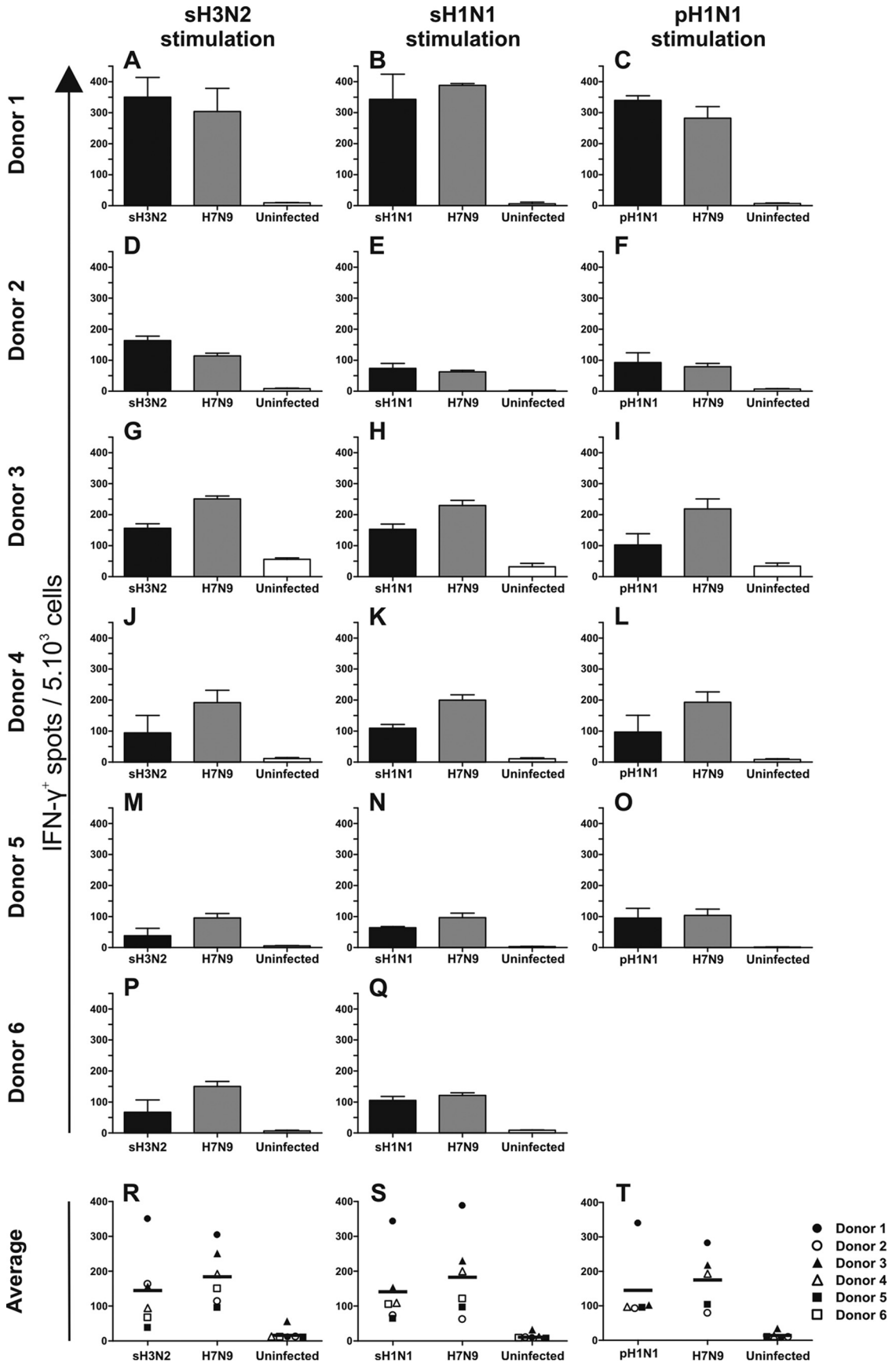
Since the sequence identity between seasonal influenza viruses used in this study and H7N9 virus is high, we wished to determine the cross-reactivity of polyclonal CD8$^+$ T cells specific for sH3N2, sH1N1, or pH1N1 viruses with H7N9 virus. To this end, *in vitro*-expanded seasonal influenza virus-specific polyclonal CD8$^+$ T cells were stimulated with HLA class I-matched BLCLs infected with the homologous seasonal influenza A virus or H7N9 virus (A/Anhui/1/2013). The number of IFN-γ-producing cells per 5,000 CD8$^+$ T cells was determined in an IFN-γ ELISpot assay (Fig. 2).

Study subject 1 showed a high response to homologous seasonal influenza viruses (sH3N2, sH1N1, and pH1N1), but also after stimulation with H7N9 virus-infected cells (Fig. 2A, B, and C). Although the frequency of seasonal influenza virus-specific CD8$^+$ T cells derived from subject 2 was lower than that of cells derived from subject 1, these T cells also cross-reacted with H7N9 virus-infected cells (Fig. 2D, E, and F). Subjects 3 and 4 responded to both the homologous viruses and H7N9 virus (Fig. 2G, H, I, J, K, and L). Subjects 5 and 6 (who lack the HLA-A*0201 allele) showed the lowest response to stimulation with homologous viruses. However, the virus-specific CD8$^+$ T cells of these two subjects also displayed cross-reactivity with H7N9 virus (Fig. 2M, N, O, P, and Q).

Thus, although the frequency of virus-specific IFN-γ-producing T cells varied between the study subjects, the cells cross-reacted with the H7N9 virus. This was independent of the sH3N2, sH1N1, or pH1N1 virus used for the *in vitro* expansion of the polyclonal CD8$^+$ T cells (Fig. 2). The average number of spots tended to be higher after restimulation with H7N9 virus than after restimulation with the homologous viruses, although the difference was not statistically significant (Fig. 2R, S, and T).

Cross-recognition of CD8$^+$ T cells with influenza A H7N9 virus assessed by lytic activity. Based on the IFN-γ ELISpot results, we selected high-responding study subjects from each HLA group to test the lytic capacity of the CD8$^+$ T cells against HLA class I-matched BLCLs infected with the homologous or H7N9 virus. To this end, polyclonal CD8$^+$ T cells derived from sH3N2, sH1N1, or pH1N1 virus-stimulated PBMC cultures from study subjects 1, 3, and 5 were incubated with CFSE-labeled BLCLs infected with the sH3N2, sH1N1, pH1N1, or H7N9 virus.

CD8$^+$ T cells from subject 1 obtained after sH3N2, sH1N1, and pH1N1 virus stimulation not only displayed lytic activity to the respective homologous viruses, but also displayed similar or even stronger lytic activity to H7N9 virus-infected cells, as was observed for sH1N1 virus-specific CD8$^+$ T cells (Fig. 3A, B, and C).


sH3N2 stimulation
sH1N1 stimulation
pH1N1 stimulation
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor 6
Average
IFN-γ+ spots / 5.10^3 cells
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
sH3N2
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Donor 3
Donor 4
Donor 5
Donor 6

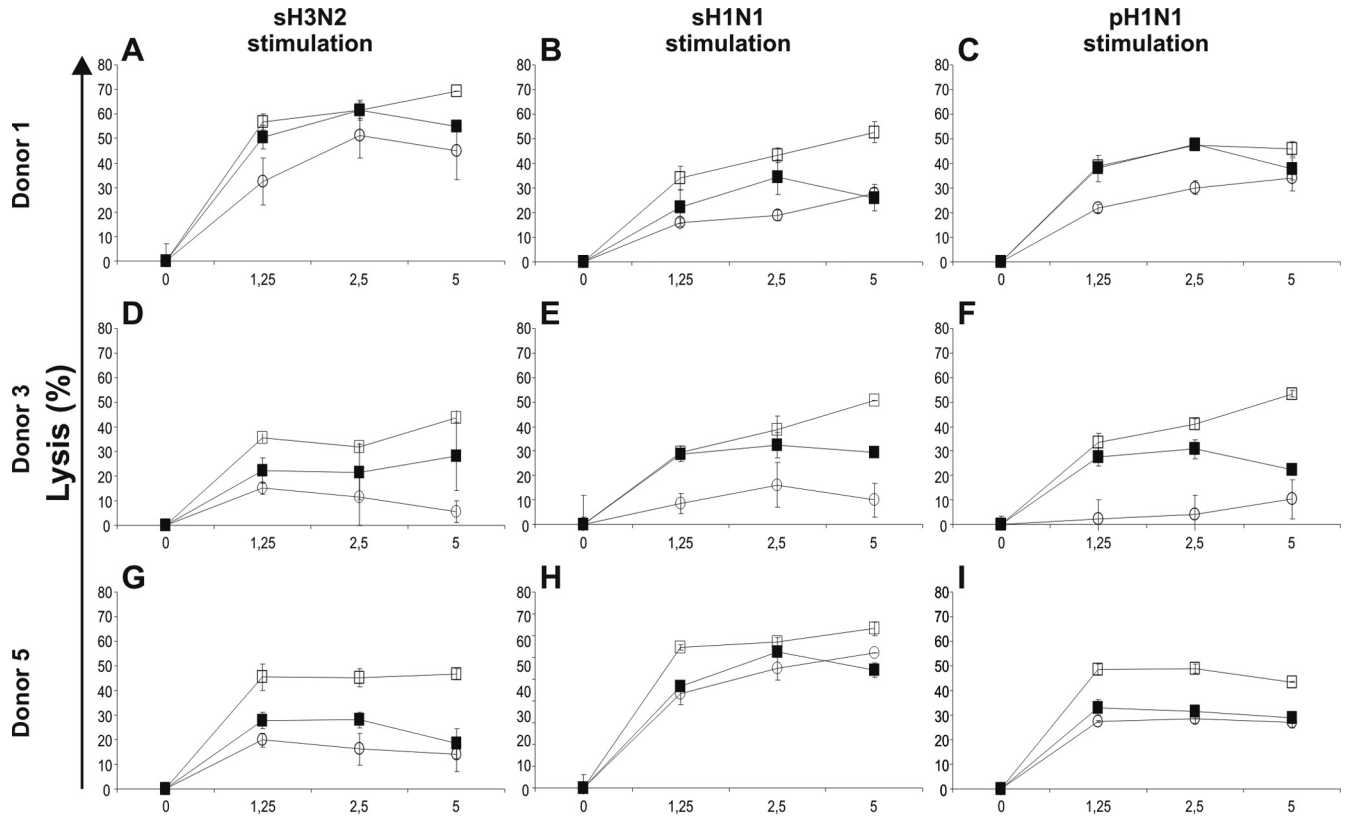

FIG 3 Lytic activity of virus-specific polyclonal CD8$^+$ T cells against BLCLs infected with the homologous or H7N9 virus. Seasonal influenza virus-specific polyclonal CD8$^+$ T cells from study subjects 1, 3, and 5 were isolated after stimulation with sH3N2 (A, D, and G), sH1N1 (B, E, and H), or pH1N1 (C, F, and I) virus, as indicated. Lytic activity against CFSE-labled BLCLs infected with the homologous virus (sH3N2, sH1N1, or pH1N1) (solid squares) or the heterologous novel H7N9 virus (open squares) was assessed as lytic background activity against uninfected cells (open circles). Experiments were performed in triplicate. The error bars indicate standard deviations for the triplicates.

A similar trend was observed for virus-specific CD8$^+$ T cells obtained from subject 3. Again, the lytic activity to H7N9 virus-infected cells exceeded that to cells infected with the homologous viruses to various extents (Fig. 3D, E, and F). Virus-specific CD8$^+$ T cells of subject 5 displayed minor lytic activity to target cells infected with the respective homologous viruses. Again, the lytic activity to target cells infected with H7N9 virus exceeded that to cells infected with the homologous viruses and uninfected control cells (Fig. 3G, H, and I). The background lytic activity of T cells derived from subjects 1 and 5 was high, which may be related to bystander proliferation of Epstein-Barr virus (EBV)-specific T cells.

DISCUSSION

Here, we assessed the cross-reactivity of seasonal influenza A virus-specific CD8$^+$ T cells with the newly emerging H7N9 virus. This study showed that a significant proportion of the polyclonal CD8$^+$ T cells specific for sH3N2 (A/Netherlands/384/07), sH1N1 (A/Netherlands/26/07), and pH1N1 (A/Netherlands/602/09) cross-react with the novel H7N9 virus (A/Anhui/1/2013).

Comparison of epitope sequences revealed that the majority of the currently known HLA class I epitopes are conserved in the novel H7N9 viruses. Several studies have shown that the conservation of these HLA class I epitopes is responsible for cross-reactivity of influenza A virus-specific CD8$^+$ T cells with influenza A viruses of another subtype (43, 45, 49–51). However, variation in some of the known epitopes was observed. We demonstrated that there is very little cross-reactivity of seasonal influenza A virus-specific CD8$^+$ T cells with four individual H7N9 variant epitopes, although CD8$^+$ T cells of subject 1 displayed some cross-reactivity with the H7N9 $NP_{418-426}$ (HLA-B*35-restricted) epitope (Fig. 1A). The magnitudes of the responses to individual peptides varied between study subjects (Fig. 1). These differences may reflect differences in HLA class I makeup (64) and/or differences in the history of influenza A virus infections.

FIG 2 Virus-specific IFN-γ production by polyclonal CD8$^+$ T cells after stimulation with BLCLs infected with homologous or H7N9 virus. (A to Q) Seasonal influenza virus-specific polyclonal CD8$^+$ T cells were isolated from PBMCs stimulated with sH3N2 (A, D, G, J, M, and P), sH1N1 (B, E, H, K, N, and Q), or pH1N1 (C, F, I, L, and O). PBMCs of subject 6 were not stimulated *in vitro* with pH1N1, since they were isolated prior to the pH1N1 outbreak. The CD8$^+$ T cells were subsequently cocultured with BLCLs infected with homologous virus (sH3N2, sH1N1, or pH1N1) (black bars) or the heterologous novel H7N9 virus (gray bars). The number of IFN-γ-producing cells per 5,000 polyclonal CD8$^+$ T cells was determined by ELISpot assay. Uninfected BLCLs were used as negative controls (white bars). Experiments were performed in triplicate. The error bars indicate standard deviations for the triplicates. (R, S, and T) The symbols represent the averages of triplicate experiments for each individual subject, and the horizontal bars represent the average responses of all study subjects combined.

Although it has been suggested that the novel H7N9 virus is poorly immunogenic based on the *in silico* predictions of T cell epitopes in HA (66), we clearly demonstrate that the presence of most conserved HLA class I epitopes in the novel H7N9 virus contributes to the high cross-reactivity of the polyclonal $CD8^+$ T cell populations with the H7N9 virus (Fig. 2 and 3). The low IFN-γ responses of study subjects 5 and 6 (A*0101, -A*0301, -B*0801, and -B*3501) to stimulation with the homologous seasonal influenza viruses and the H7N9 virus (Fig. 2) might be attributed to the absence of the HLA-A*0201 allele, which is required for a dominant $CD8^+$ T cell response to the conserved and $M1_{58-66}$ epitopes (64). All study subjects displayed cross-reactive responses to H7N9 virus equal to or greater than those against the homologous viruses (Fig. 2 and 3), which could not be attributed to differences in infection rates (data not shown). These results correspond to previous assessments of cross-reactive $CD8^+$ T cells with avian influenza A viruses of the H5N1 subtype (49). The strong reactivity to avian influenza A viruses might be the result of differences in antigen processing in infected cells, allowing more peptides to be liberated and presented from viral proteins of avian viruses than from those of human influenza viruses. It can be hypothesized that since these avian viruses have not circulated in the human population extensively, they have not yet had a chance to acquire mechanisms to escape from human epitope processing (33, 67–72).

Although we have studied the cross-reactivity of $CD8^+$ T cells of study subjects with selected HLA types, it is likely that individuals with other HLA types also possess cross-reactive $CD8^+$ T cells. The conservation of HLA class I epitopes restricted by other HLA alleles (Table 1) and the high amino acid sequence identity between the seasonal influenza viruses and the H7N9 virus underscores this (Table 2).

Cross-reactive influenza A virus-specific $CD8^+$ T cells are found in individuals who have experienced an influenza A virus infection at least once. In contrast, a seroprevalence study indicated that a large proportion of children under the age of 4 years had not experienced an influenza A virus infection and therefore may not have developed virus-specific T cell responses (73). This age group may therefore be at higher risk of developing severe disease during a pandemic outbreak than adults. This was indeed the case during the 2009 H1N1 pandemic (74) and the localized outbreaks of the H5N1 subtype (75) and the vH3N2 subtype (76, 77). However, in the case of the novel H7N9 virus, mainly older (male) individuals were at risk for developing severe disease (12–15). The reason for this discrepancy is unknown at present. It has been suggested that differences in cell-mediated immunity between different age groups are the basis for this predilection (78). Elderly people who had experienced an H1N1 infection before 1957 were serologically protected during the 2009 pandemic outbreak and in the following years, whereas many unprotected individuals, including children, suffered from a pH1N1 infection in recent years (74, 79). Recent influenza A virus infections in children and young adults most likely boosted their cellular immune responses, which may afford some protection from infection with viruses of novel subtypes, including those of the H7N9 subtype (78). Others have suggested that preexisting immunity consisting of low levels of weakly heterosubtypic antibodies may result in antibody-dependent enhancement (ADE) of the infection (14). Instead of neutralizing the virus, these antibodies would enhance uptake of the virus and thus promote its replication. The possibility that other confounding factors may have contributed to the predilection of H7N9 disease for older individuals cannot be excluded. Elderly people are more likely to suffer from underlying diseases (80) and are known to have altered T cell immunity, which is likely to influence the outcome of an influenza A virus infection (81, 82).

It is difficult to predict to what extent preexisting influenza A virus-specific $CD8^+$ T cells will afford protection against novel pandemic influenza viruses. Several animal studies have shown that virus-specific $CD8^+$ T cells contribute to heterosubtypic immunity (52–57). However, evidence for heterosubtypic protection by $CD8^+$ T cells in humans is sparse (59, 83). Epidemiologic studies showed that individuals who had experienced a seasonal H1N1 infection prior to the 1957 H2N2 pandemic were partially protected (60, 61), which could be attributed to cross-reactive T cells and/or antibodies to, e.g., the stalk region of HA. A similar trend was observed in isolated H5N1 infections (75). However, recent studies performed during the 2009 H1N1 pandemic provide better insight into the protective role of $CD8^+$ T cells during an infection with an antigenically distinct influenza virus in serologically naive humans. It was shown that patients developed less severe illness when they had a high frequency of preexisting virus-specific $CD8^+$ T cells before the onset of the pandemic (58). Another study showed that infected patients developed strong and rapid cross-reactive recall T cell responses, which in most cases coincided with the disappearance of clinical symptoms (84).

In conclusion, we have demonstrated that $CD8^+$ T cells that can cross-react with the newly emerging H7N9 influenza virus and that may afford some protection in the absence of virus-neutralizing antibodies are present in the human population. Cross-reactive $CD8^+$ T cells do not establish sterile immunity; they do, however, contribute to more rapid clearance of the H7N9 virus infection. Immunity afforded by the presence of cross-reactive $CD8^+$ T cells may not only reduce the severity of disease caused by H7N9 virus infection, it may also contribute to reduction of virus spread in the population, since infected individuals may be infectious for a shorter time. Induction of cross-reactive virus-specific T cell responses may be a promising approach for the development of universal influenza vaccines that can elicit broadly protective immunity against influenza A viruses of various subtypes.

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