Characterization of the Human CD8⁺ T Cell Response following Infection with 2009 Pandemic Influenza H1N1 Virus[▽]

M. L. B. Hillaire,¹ S. E. van Trierum,¹ R. Bodewes,¹ C. A. van Baalen,³ R. S. van Binnendijk,²
M. P. Koopmans,^{1,2} R. A. M. Fouchier,¹ A. D. M. E. Osterhaus,^{1,3}
and G. F. Rimmelzwaan^{1,3*}

Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands¹; Department of Infectious Disease Control, National Institute of Public Health, Bilthoven, The Netherlands²; and Viroclinics Biosciences BV, Rotterdam, The Netherlands³

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The 2009 H1N1 influenza pandemic provided an opportunity to study human virus-specific T cell responses after infection with a novel influenza virus against which limited humoral immunity existed in the population. Here we describe the magnitude, kinetics, and nature of the virus-specific T cell response using intracellular gamma interferon (IFN- γ) staining and fluorochrome-labeled major histocompatibility complex (MHC) class I-peptide complexes. We demonstrate that influenza virus-infected patients develop recall T cell responses that peak within 1 week postinfection and that contract rapidly. In particular, effector cell frequencies declined rapidly postinfection in favor of relatively larger proportions of central memory cells.

Influenza viruses are an important cause of respiratory tract infections. It is well-known that virus-specific antibodies to the viral hemagglutinin (HA) induced by previous infections or vaccination can protect individuals from contracting influenza, provided that the specificities of these antibodies match the virus causing the infection and reached protective levels. In general, strain-specific serum antibody titers of ≥ 40 measured in the hemagglutination inhibition assay are considered protective (5). In situations where preexisting antibodies are not present or do not match the strain causing the infections, e.g., in a pandemic outbreak when a novel strain or subtype of influenza A virus is introduced into the human population, other arms of the adaptive immune system may afford some degree of protection. It has been demonstrated that infections with influenza A viruses can induce protective immunity to influenza virus of another subtype to a certain extent. It is generally accepted that virus-specific T cells contribute to this type of immunity. In particular, virus-specific CD8⁺ T cells that can recognize and eliminate virus-infected cells play a role in this so-called heterosubtypic immunity (1, 11, 14, 15, 18, 20, 21, 25, 26). Indeed, it has been shown that the majority of virus-specific CD8⁺ T cells are directed to the relatively conserved internal viral proteins like nucleoprotein and the matrix protein (9, 10, 29) and that these cytotoxic T cells display strong cross-reactivity with influenza A viruses of other subtypes (7, 16, 25, 28, 29). Thus, infection with a novel (pandemic) influenza A virus may induce an recall CD8⁺ T cell response that could result in a more rapid clearance of the infection and afford clinical protection.

Although the CD8⁺ T cell responses have been studied at the epitope level extensively in mouse models, very little is

known about human CD8⁺ T cell responses after natural infection with influenza viruses. Knowledge of protective CD8⁺ T cell responses induced after infection may aid in defining the T cell responses that novel (universal) vaccines need to mount for the induction of protective immunity to (pandemic) influenza virus infections.

Here we investigated the magnitude, kinetics, and nature of the human CD8⁺ T cell response after infection with pandemic 2009 H1N1 influenza virus. To this end, we collected peripheral blood mononuclear cells (PBMC) from eleven 2009 H1N1 virus-infected patients at various time points after the onset of clinical symptoms (Table 1).

Eleven patients were selected for this study, four males and seven females ranging in age from 16 to 57 years. They had been recruited as part of a national cohort study to assess clinical impact and risk factors for 2009 pandemic H1N1 influenza A virus (I. H. M. Friesema, A. Meijer, A. B. van Gageldonk-Lafeber, M. van der Lubben, J. van Beek, G. A. Donker, J. M. Prins, M. D. de Jong, S. Boskamp, L. D. Isken, M. P. G. Koopmans, and M. A. B. van der Sande, submitted for publication). For each patient, the infection with 2009 H1N1 virus was confirmed by PCR, which was performed according to standard methods (19). Nine of these patients also displayed a rise in serum antibodies specific for 2009 H1N1 virus as detected by hemagglutination inhibition assay (Table 1). Except for one patient, all patients developed mild upper respiratory tract disease and recovered without complications. The patients consented to be included in the study, which had been subjected to and approved by the medical ethical board.

The frequencies of virus-specific CD8⁺ T cells were assessed by intracellular gamma interferon (IFN- γ) staining using standard techniques (3). In short, 1×10^6 PBMC were stimulated with influenza virus A/Netherlands/602/09 (pH1N1), which had been propagated in MDCK cells and purified by density centrifugation in sucrose gradients, at a multiplicity of infection of 3 in duplicate for 16 h. The cells were then incubated for another 6 h in the presence of Brefeldin A (Sigma-Aldrich,

* Corresponding author. Mailing address: Department of Virology, Erasmus Medical Center, P. O. Box 2040, 3000 CA Rotterdam, The Netherlands. Phone: 31 10 7044243. Fax: 31 10 7044760. E-mail: g.rimmelzwaan@erasmusmc.nl.

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TABLE 1. PCR-confirmed 2009 H1N1 patients used in the present study

Patient no.	Gender	Age (yr)	Time point of blood sample collection (days) ^a			Antibody titer rise ^b
			Sample 1	Sample 2	Sample 3	
89	Male	25.1	3	12	36	+
60	Male	53.6	5	17	39	+
46	Female	25	6	16	34	+
44	Male	19.6	5	15	35	+
15	Female	55.2	10	20	40	+
2	Female	51.9	NA	13	31	+
21	Female	57.1	2	12	40	+
241	Female	55.3	2	5	NA	—
31	Female	16.6	4	NA	32	+
14	Male	28.4	6	17	39	—
123	Female	53.1	2	14	43	+

^a Days after the onset of clinical symptoms. NA, not available.
^b Antibody titers to the hemagglutinin (HA) of 2009 H1N1 influenza virus were measured in the initial and convalescent-phase serum samples by hemagglutination inhibition assay. Symbols: +, detection of a rise in the titer of ≥ 4 ; —, no rise in the titer detected.

Zwijndrecht, The Netherlands), and the frequency of CD69⁺ IFN- γ ⁺ CD8⁺ or CD69⁺ IFN- γ ⁺ CD4⁺ cells was determined as shown in Fig. 1A using fluorochrome-labeled antibodies to CD3, CD4, CD69 (Becton & Dickinson, Alphen a/d Rijn, The Netherlands), CD8, and IFN- γ (eBioscience, San Diego, CA). To exclude dead cells during analysis, cells were also stained with Live/Dead fixable dead cell stain (Invitrogen, Breda, The Netherlands). For a positive control, we used the superantigen staphylococcus enterotoxin B. With this control, strong responses were always detected, confirming the functional integrity of the cells after thawing.

In six of the subjects, a decline in the frequency of virus-specific CD8⁺ T cells was observed (Fig. 2A). In four of the patients, the frequency of CD8⁺ IFN- γ ⁺ T cells did not change, and in one person, a modest increase was observed. When the data of all patients, except for patients 44 and 14 who did not respond, were analyzed together, a rapid decline in the frequency of virus-specific CD8⁺ T cells was observed after the onset of clinical symptoms (Fig. 2C). Similar results were obtained with CD4⁺ T cells (Fig. 2D). This suggests that

FIG. 1. Example of the analysis of virus-specific T cells. (A) The frequency of IFN- γ ⁺ CD8⁺ T cells was determined by intracellular IFN- γ staining after stimulation with influenza virus A/Netherlands/602/09 (pH1N1) virus (left panel) and subtracting the background values of nonstimulated control cells (right panel). (B) The subsets of Tm⁺ cells were quantified on the basis of expression of CCR7, CD45RA, CD28, and CD27 using flow cytometry. Phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein (PerCP), FITC, and Cy7 fluorophore were used to label CD27, CD28, CCR7, etc.

FIG. 2. Analysis of the magnitude of the T cell response upon infection with 2009 H1N1 virus. (A to D) The frequencies of virus-specific CD8⁺ (A and C) and CD4⁺ (B and D) T cells were determined by intracellular IFN- γ staining in individual patients at various time points after the onset of clinical symptoms as indicated. The data are averages of duplicate values. In panels C and D, trend lines and their R^2 values were added using Excel software.

FIG. 3. Subset analysis of Tm⁺ T cells. PBMC were obtained from individual patients at various time points after the onset of clinical symptoms, and Tm⁺ cells were tested for the expression of CCR7, CD45RA, CD28, and CD27 to determine the proportion of effector T cells, naïve T cells, T_{EM}, T_{CM}, T_{EMRA} and CD45RA⁺ CCR7⁺ CD28⁻ cells. Cells not belonging to any of these subsets are shown in white. The data are averages of duplicate values.

after infection, a rapid recall cytotoxic T lymphocyte (CTL) response was induced in most patients that subsequently contracted within 3 weeks postinfection. Five of the six patients in which the virus-specific CD8⁺ T cell response declined also displayed a decline in the frequency of virus-specific CD4⁺ T cells (Fig. 2B).

To further analyze the nature of the virus-specific CD8⁺ T cells, they were tested for their expression of chemokine receptor 7 (CCR7), CD28, CD27, and CD45 receptor A (CD45RA) to distinguish naïve, effector T cells, effector memory T cells (T_{EM}), effector memory RA T cells (T_{EMRA}), and central memory T cells (T_{CM}). To this end, we used a cocktail of fluorescein isothiocyanate (FITC)-labeled tetramers (Tm) consisting of HLA-A*01 (NP₄₄₋₅₂ [amino acids 44 to 52 of nucleoprotein NP] CTEKLSDY and PB₅₉₁₋₅₉₉ VSDGGPN LY), HLA-A*0201 (M1₅₈₋₆₆ GILGFVFTL), HLA-A*03 (NP₂₆₅₋₂₇₃ ILRGSVAHK), and HLA-B*0801 (NP₃₈₀₋₃₈₈ ELR SRYWAI) to identify virus-specific CD8⁺ T cells, since the HLA background of the patients was unknown. This cocktail of tetramers included common HLA alleles and corresponding influenza virus CTL epitopes and covered a majority of subjects in the population (59%) (17). The relative differences in immunodominance of the respective epitopes were not taken into account (4). These epitopes are fully conserved in seasonal influenza A viruses and the 2009 pandemic influenza virus Netherlands/602/09 that we used in the present study. The cells were also incubated with antibodies directed to the respective differentiation markers and a dead cell staining and analyzed by flow cytometry (between 1×10^6 to 1.5×10^6 cells per sample). Cells with an effector phenotype were characterized as CCR7⁻ CD45RA⁻ CD28⁻ CD27⁻, naïve T cells were characterized as CCR7⁺ CD45RA⁺ CD28⁺, T_{CM} were characterized as CCR7⁺ CD45RA⁻ CD28⁺, T_{EM} were characterized as CCR7⁻ CD45RA⁻ CD28⁺, and T_{EMRA} were characterized as CCR7⁻ CD45RA⁺ CD28⁻. Also, CCR7⁺ CD45RA⁺ CD28⁻ CD27⁺ cells were identified as a separate subset of T cells and quantified. This subset has never been described to our knowledge.

For six patients, sufficient PBMC were available for analysis. Prior to investigating the PBMC from the patients, we opti-

mized the Tm⁺ staining by using PBMC from healthy blood donors from which virus-specific T cells were expanded *in vitro* by stimulation with virus or no stimulation. Using these cells, we were able to set the gates for virus-specific CD8⁺ T cells (using the nonstimulated PBMC as negative controls).

As shown in Fig. 3, in five of these patients (patients 60, 89, 123, 21, and 46), a decline in the number of Tm⁺ cells was observed, which in general coincided with the decline in the frequency of IFN- γ ⁺ cells. Of note, for patient 89, the percentage of CD8⁺ IFN- γ ⁺ cells increased, which did not correlate with the decrease in the frequency of effector cells. However, the frequency of CD8⁺ IFN- γ ⁺ cells was relatively low, which may preclude drawing firm conclusions for this particular patient. In particular, the relative contribution of effector cells decreased in three of these patients in favor of an increase in the relative proportion of T_{CM}. Of note, the first time points of sampling for patients 21 and 15 were 12 and 19 days after the onset of clinical symptoms, respectively. This may explain why only a small proportion of effector cells was detected in these samples collected relatively late after infection, which was also observed by others (27). The observed kinetics fit with the notion that after clearance of the infection, the effector cell population declines and diverse subsets of memory T cells persist. T_{EM} cells retain effector cell function, like lytic activity, reside in peripheral tissue, have poor recall ability, and eventually will decay from the memory pool of T cells (13, 23). The T_{CM} form the bona fide memory cell population, which is long-lived, resides in secondary lymphoid organs, and give rise to recall T cell responses upon renewed encounter with the same pathogen (24).

We also found a proportion of Tm⁺ T_{EMRA} cells in the acute phase of the CTL response. For patients 60, 123, and 89, for which PBMC were available shortly after the onset of clinical symptoms, it was observed that the relative proportion of these cells decreased quickly. These cells have properties shared by naïve and effector cells (22), and it has been hypothesized that these T cells are differentiating toward an effector or effector memory phenotype. Indeed, these cells have partial cytolytic capacities. Compared to T_{CM} or T_{EM}, they have limited ability to proliferate after stimulation *in vitro* (23).

The relative proportion of CD45RA⁺ CCR7⁺ CD28⁻ CD27⁺ cells decreases after infection. This cell population might represent naïve cells shortly after activation that lost expression of CD28 but still expressed CD45RA, CCR7, and CD27. More studies are needed to determine the properties and biological significance of this subset.

Taking all our data together, we conclude that upon infection with influenza A virus, humans, who most likely have experienced one or more influenza virus infections in the past (2), respond with a rapid recall T cell response. The first time points at which PBMC were analyzed in the present study was 2 to 4 days after the onset of clinical symptoms. Considering an incubation time of 1 or 2 days, this would mean that the virus-specific CD8⁺ T cell response reached a peak within 1 week postinfection. This rapid response most likely contributed to rapid clearance of the infection, as was also demonstrated in animal models (for a review, see reference 13). Once the virus infection is cleared, the CTL response contracts rapidly, but memory T cells persist, which can be recalled upon a subsequent infection with influenza A virus (8). The rapid decrease of virus-specific CD8⁺ T cells from the blood may reflect migration of these cells to the site of infection (12). It has been reported that the frequency of Tm⁺ CD8⁺ T cell is low after 2009 H1N1 influenza virus infection (6). However, T cell responses were analyzed with Tm in two patients only and at a late time point after the onset of clinical symptoms. In contrast, we examined the frequency of virus-specific CD8⁺ T cells at various time points postinfection which allowed assessment of the kinetics of the T cell responses after infection.

Since the majority of the virus-specific CD8⁺ T cells are directed against conserved internal viral proteins and are directed to epitopes that are shared by different subtypes of influenza A virus, the induction of these cross-reactive T cells could be a venue for the development of vaccines that could induce broadly protective immunity. The magnitude, kinetics, and nature of T cell responses that were observed after infection with influenza A viruses could be the benchmark for the development of novel vaccines. Most likely, vaccines that induce T cell responses similar to those induced by natural infections will afford heterosubtypic immunity, which would be advantageous in the face of continuous pandemic threat caused by influenza A viruses of novel subtypes.

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