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Fluorescent dye labeled influenza virus mainly infects innate immune cells and activated lymphocytes and can be used in cell-mediated immune response assay

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Abstract

Early results have recognized that influenza virus infects the innate and adaptive immune cells. The data presented in this paper demonstrated that influenza virus labeled with fluorescent dye not only retained the ability to infect and replicate in host cells, but also stimulated a similar human immune response as did unlabeled virus. Influenza virus largely infected the innate and activated adaptive immune cells. Influenza B type virus was different from that of A type virus. B type virus was able to infect the immature lymphocytes, but in lower amounts when compared to activated lymphocytes. Protection from influenza is tightly associated with cellular immunity. Traditional methods of cellular immunity assay had limitations to imitate the natural human cell-mediated responses to influenza virus. Labeled viruses could be used in the assay of virus-specific cytotoxicity, which might reflect the natural process more closely. Furthermore, human immune cells activated by one influenza subtype virus could kill the cells infected by other subtype virus. These results implied the human immune cells could directly handle and remove free virus using similar mechanism that was used to remove virus-infected non immune cells, which might help to simplify the design and production of influenza vaccine, thereby reduce the cost.

Keywords

influenza virus; fluorescence; cytotoxicity; granzyme B; T cell

1. Introduction

Influenza virus is controlled by both humoral and cell-mediated immune responses (Doherty et al., 2006; Thomas et al., 2006). The traditional detection of human cell-mediated responses used ^{51}Cr -release assays. Enzyme-linked immunospot, intracellular cytokine staining, CD107 staining, and specific T cell receptor staining by the use of fluorescent major histocompatibility class (MHC) I peptide complexes are alternative methods for the detection of CTL responses (Altman et al., 1996; Rubio et al., 2003; Sun et al., 2003; Tomaru et al., 2003). These methods are aimed at the detection of virus-specific CTLs in lymphocyte populations (Yang, 2003; Lieberman, 2004). As non-radioactive alternatives for the ^{51}Cr assay, several assays used

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fluorescent dyes to discriminate between target and effector cells and to assess target-cell elimination (Lecoeur et al., 2001; Lee-MacAry et al., 2001; Liu et al., 2002). In order to measure the outcome of the cytolytic cascade, the detection methods of cytolytic activity have been developed; they include sets of overlapping peptides pulsing and directly transfecting target cells with DNA vector encoding antigen-green fluorescent protein (GFP) fusion protein (Maecker et al., 2001; van Baalen et al., 2005). A disadvantage to this approach is that the infection of cells may differ from that identified by exogenously added peptides and DNA vector (Addo et al., 2001). It has been reported that conjugates of HA protein of influenza virus are suitable for obtaining a strong immune response (Huebner, 1997). Fluorescent isothiocyanate (FITC) labeled influenza virus was able to infect both innate and adaptive immune cells, and kept the ability of infection and replication as unlabeled virus (Nichols et al., 1993). However, whether there is a difference between native and activated lymphocytes in the infection of influenza virus and whether influenza virus labeled with fluorescent dye stimulates the same immune response as unlabeled virus remains unclear. The data presented in this paper demonstrated that influenza virus labeled with fluorescent dyes kept their abilities of infection, replication and stimulation to immune response. Labeled influenza viruses had a selective infection for innate and activated adaptive immune cells. The target cells, which were infected with fluorescent dye labeled influenza virus, were co-cultured with CTL effector cells, and their killing by CTLs was monitored by flow cytometry.

2. Materials and methods

2.1. Human subjects and virus

Human peripheral blood was collected in heparinized tubes from healthy donors. Influenza virus strains: A/Puerto Rico/8/34/[H1N1], A/Wyoming/03/2003 [H3N2], A/New Caledonia/20/99 [H1N1], A/Panama/2007/99 [H3N2], and B/Hong Kong/330/2201 were purchased from the Centers for Diseases Control and Prevention (CDC), USA. This work was approved by the University of Connecticut Health Center.

2.2. Preparation of human peripheral blood mononuclear cells (PBMC) stimulated with virus and PHA

Human PBMC were prepared with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO), washed with PBS once, treated with RBC Lysis Solution (Bio-Rad, Hercules, CA) for 5 minutes, washed with PBS once again, then resuspended in AIM-V media (Gibco Laboratories, Grand Island, NY), then adjusted into $2.0 \times 10^6/\text{ml}$. 1 ml of suspended cell was added into each well of a 48-well multiplate (Nalge Nunc, Rochester, NY), and stimulated with influenza virus ($\text{TCID}_{50}=4 \times 10^6/\text{ml}$, $\text{MOI}=2$) at 37°C in 5% CO_2 . The concentration of phytohemagglutinin (PHA, Sigma) was $1\mu\text{g}/\text{ml}$ AIM-V medium.

2.3. Antibodies and Flow Cytometry

The following antibodies used for flow cytometry were purchased from BD Pharmingen: anti-CD8-PerCP, anti-Perforin (Perf) -PE and anti-CD107a-PE antibodies. In addition, anti-CD3-PE-Cy7 antibody was purchased from eBioscience. Fv17 single chain anti-human granzyme B antibody (scFv GrB), which was a gift from C. R. Bleackley and K. P. Kane (Departments of Biochemistry and Immunology, University of Alberta, Edmonton, Alberta, Canada), was labeled with reactive fluorescent isothiocyanate (FITC, Sigma) (Rong et al., 2004). Cells for flow cytometry were prepared as previously described (McElhaney et al., 2006). In brief, cells ($0.5-1 \times 10^6$) were incubated with surface antibodies, washed with cold 0.2% BSA/PBS before and after fixing with 2% paraformaldehyde, then resuspended in a cold permeabilization buffer (0.3% saponin, 5% normal human serum PBS). Following scFv GrB intracellular staining, cells were washed with 0.1% saponin and 0.2% BSA/PBS, resuspended in 0.2% BSA/PBS, and transferred to FACS tubes for data acquisition. Data were acquired on BD LSR II, while 30,000

events were counted for each sample and analyzed using Flow Jo software (Tree Star, Ashland, OR).

2.4. ^{51}Cr release Assay

Fresh PBMC (0.7×10^6) were labeled with $0.05\text{mCi } ^{51}\text{Cr}$ (chromium, supplied as Na_2CrO_4 ; ICN) for 1 hour at 37°C . PBMC were washed with AIM-V three times, infected with A/Wyoming/03/2003 [H3N2] at $\text{MOI}=2$ as the target. $100\text{ }\mu\text{l}$ (5000cells) of labeled target cells in AIM-V were added to various PBMC, as indicated by the effector-to-target ratios in final $200\mu\text{l}$. Target cells with 5% Tween 20 or AIM-V media were used to determine the maximum release and spontaneous release. The mixtures were cultured in u-bottomed 96-well plates for 4 h. Supernatants ($100\text{ }\mu\text{l}$) were harvested, mixed with scintillation fluid, and counted in a 1450 MicroBeta Trilux liquid scintillation counter (Wallac Inc. Fort Myers, Florida). The percentage of specific lysis was calculated using the formula: % specific lysis = [(experimental release spontaneous release)/(maximum release spontaneous release)] $\times$ 100 (Powers and Belshe, 1993).

2.5. Assay of Apoptosis

Fitc-Annexin-V was purchased from BD Bioscience.

2.6. Determination of the intracellular granzyme B (GrB) activity IFN- γ

Human PBMC (1.5×10^6 cells/ml in AIM-V media) were stimulated with virus ($\text{MOI}=2$) for 5 days and PBMC lysates were prepared and analyzed by the GrB assay using the Acetyl-Ile-Glu-Pro-Asp-paranitroanilide (Ac-IEPD-pNA, Bachem) substrate according to previously described methods (Ewen et al., 2003; McElhaney et al., 2006). GrB activity was measured in PBMC lysates by cleavage of the substrate. GrB activity was calculated by A405 U/mg protein in the PBMC lysate. The lysate of YT cells was used as the standard. The supernatants were collected into new tubes as samples for the detection of IFN- γ . IFN- γ was measured with Human IFN- γ ELISA Ready-SET-Go kits (eBioscience, San Diego, CA), following the company provided protocol.

2.7. Virus labeling

$2.0\mu\text{l}$ of 1M sodium bicarbonate was added into $10\mu\text{l}$ (100–500 μg) of purified virus and mixed. Fluorescent dyes including Cyanine (Cy3, Amersham), Cy5 (Amersham), Phycoerythrin (PE, Dojindo Molecular Technologies, Inc. Gaithersburg, MD) and Allophycocyanin (APC, Dojindo Molecular Technologies, Inc. Gaithersburg, MD) were dissolved in $3\mu\text{l}$ of DMSO. $3\mu\text{l}$ of fluorescent dye was added to the virus and mixed gently but thoroughly. The mixture of virus and dye was incubated in the dark for one hour at room temperature. Added $6\mu\text{l}$ of 4M hydroxylamine to the mixture, mixed thoroughly and incubated for 15 minutes in the dark at room temperature. The mixture was then loaded on the top of a 1ml G-50 column (Ambion Inc. Austin, TX), and centrifuged at $750g$ for two minutes. The dye labeled virus ran through to the 1.5ml tube. Free dye (Cy3 and Cy5) was retained in the column matrix. Virus separated from free PE and APC was centrifuged by Beckman L8–55 Ultracentrifuge at 100,000rpm for 30 minutes. The dye optical density (OD) values of the labeled virus were measured by DU® Series 650 UV/Vis Scanning Spectrophotometer. Using TCID_{50} as particle number, each virus particle could couple 2000–6500 dye molecules in all experiments.

2.8. Virus titration

Madin-Darby canine kidney (MDCK) cells were digested with 0.25% trypsin, washed with PBS, adjusted to $1 \times 10^5/\text{ml}$ in Dulbecco's Modified Eagle Medium (DMEM) (Gibco Laboratories, Grand Island, NY). Viruses were diluted sequentially in 0.002% trypsin DMEM. $100\mu\text{l}$ of the virus and $100\mu\text{l}$ MDCK cells were mixed into each well of a 96-well plate and

incubated at 37°C in 5% CO₂ for 48 hours. 100µl of supernatant and 100µl of washed chicken (0.5%) red blood cells were then mixed in a v-bottomed 96-well plate. The plates were kept at 4°C and read at 60 minutes. The aggregation of chicken red blood cells was as positive.

2.9. Statistics

Data were expressed as mean and standard error. Comparisons were analyzed using Student's paired *t*- tests. The significance level was set at 0.05 (*p*<0.05).

3. Results

3.1. Influenza virus labeled with fluorescent dye kept the ability of infection, replication in host cell and stimulation to human immune response as unlabeled virus

Early studies showed that fluorescent dye labeled influenza virus had the capacity of infection and replication in host cell (Nichols et al., 1993). It remained unclear, however, whether there are differences between labeled and unlabeled virus in the stimulation of the human immune response. In this study, influenza viruses were labeled with Cy3, Cy5, PE or APC. Labeled viruses were titrated with MDCK cell and chicken red blood cells. These results indicated that labeled influenza viruses kept the ability of infection and replication in the host cell. Labeled and unlabeled viruses were used to stimulate human PBMC for 5 days. PBMC were probed with anti human CD8, CD3, GrB and Perf antibodies. The activity of GrB of cell lysates and IFN- γ in culture media were measured. The infection with Cy5-labeled virus A/Panama/2007/99 [H3N2] enhanced the expression of GrB in CD8 T cells as did unlabeled virus did. All of the GrB⁺ CD8 T cells were Perf⁺ (Fig. 1A). Both labeled and unlabeled influenza virus stimulated a similar increase in the activity of GrB in human PBMC (Fig. 1B). The labeled and unlabeled influenza virus (Cy3-virus: A/Puerto Rico/8/34, [H1N1]) also stimulated an increase in production of IFN- γ (Fig. 1C). The viruses labeled with various fluorescent dyes showed the same results (data not shown), which suggested that labeled influenza virus not only kept the capacity of infection and replication in the host cell, but also had the ability to stimulate a similar human immune system responses as unlabeled virus.

3.2. Human influenza virus showed a preference for infecting monocytes and activated lymphocytes

Fresh human PBMC were infected with labeled virus. In the forward and side scatter dot plot, the inactivated lymphocytes were defined as gate 1 (G1), monocytes and activated lymphocytes as gate 2 (G2). The results showed that Cy3 labeled influenza virus, A/Panama/2007/99 [H3N2], infected almost the entire G2 population, but not the inactivated lymphocytes (G1) (Fig. 2A). The viral infection was dynamically monitored by flow cytometry (Fig. 2B, 2C). Cy5 labeled influenza A strain virus, A/Puerto Rico/8/34 [H1N1], only infected the G2 population. Labeled influenza B type virus strain B/Shanghai/361/2002 infected both G1 and G2 populations, but lower amounts of the B virus infected G1 as compared to the G2 population. Other fluorescent dyes labeled viruses showed the same results (data not shown). Furthermore, human PBMC were infected with PE labeled virus A/Wyoming/03/2003 [H3N2] and probed with anti-CD3, CD4, CD8 and GrB antibodies. Virus A/Wyoming/03/2003 [H3N2] was largely taken by activated (GrB⁺) lymphocytes virus (Fig. 2D).

3.3. Labeled influenza virus can be used in cytolytic assay

The traditional ⁵¹Cr release assay was used to test the killing caused by influenza virus A/Wyoming/03/2003 [A/H3N2]. PBMC were stimulated with virus for five days as effectors (E). PBMC that were not stimulated with virus served as a controls. Autologous PBMC, stimulated with PHA for five days followed by infection with virus, were used as the target (T). A mixture of various ratios of effector/target (E:T) were incubated for 4 hours at 37°C, in 5% CO₂. The

percentage of specific lysis ^{51}Cr release increased with E:T ratio. The results suggested that at least a 5:1 E:T ratio could be used to setup a killing test (Fig. 3A).

The labeled virus killing experiments were carried out as follows: PBMC stimulated with virus A/New Caledonia/20/99 [H1N1] for 5 days were added to autologous PBMC labeled influenza virus A/Puerto Rico/8/34 [H1N1] at an E:T ratio of 6:1. The results of flow cytometric analysis showed that the peak of the cells infected with labeled virus dropped in the mixture with PBMC stimulated with virus, but still existed in the control mixture (Fig. 3B). Most cells infected with Cy3-labeled virus were killed by PBMC stimulated with the virus.

The target cells infected with Cy3 labeled virus A/Panama/2007/99 [H3N2] were mixed with the PBMC (effector) that were stimulated with virus A/Puerto Rico/8/34, [H1N1] for five days. Fluorescence of labeled virus decreased with time after targets and effectors were mixed (Fig. 4A). Tracked with the early apoptotic marker, annexin-V, and labeled virus, the results showed that apoptosis occurred in virus infected cells (Fig. 4B).

3.4. Degranulation of GrB⁺CD8 T cells is involved in the killing process caused by influenza virus infection

The cumulative exposure of granular membrane proteins (CD107a and b) on the cell surface of responding antigen-specific T cells provides a positive marker for degranulation. Significant expression of cell surface CD107a and b can be observed as early as 30 minutes following stimulation of primary CD8 T cells, and reaches a maximum by 4 hours (Betts et al., 2003; Rubio et al., 2003). Previous intracellular staining showed that the expression of GrB and CD107a and b were identical, GrB and/or CD107a and/or b could be a marker of virus specific CTL (Xie and McElhaney, 2007). The killing process caused by influenza virus infection was probed with anti CD3, CD8, CD107a and GrB antibodies. The results showed that the killing process was accompanied by the increase of CD107a on GrB⁺ CD8 T cell surfaces (Fig. 5A). The number of GrB⁺ CD8 T cells also increased. The killing process was dynamically monitored with Annexin V and anti-CD107a antibody. Fig. 5B and 5C showed that apoptosis occurred rapidly (within 5 minutes) and almost simultaneously with degranulation, reaching a peak at about 30 minute. This result strengthened the conclusion that the degranulation of CD8 T cells was involved in the apoptosis occurring in virus infected PBMC.

4. Discussion

Although it is well-known that influenza virus can agglutinate erythrocytes by attachment to specific sialic glycoprotein receptors (Fields, 1996), the interaction between influenza virus and human immune system remains unclear. Since PBMC are mainly involved in immune system responses, these cytotoxicity assays indicated that influenza virus could infect these immune cells and the infected cells could be killed by effector cells. Current knowledge states that a virus-infected cell must be producing viral peptides and displaying them on the surface of the cell in order to be recognized by cytotoxic immune cells. However, this simplistic understanding of the interaction between the immune system and the influenza virus hardly explains how the immune system clears itself of infection. Does the interaction involve binding of virus to specific immune cells or does virus bind all immune system components? Can the human immune system directly remove free virus? Therefore, it is necessary to develop a better understanding of the immune mechanisms that are linked to influenza virus infection. Early studies showed that fluorescence labeled virus infected a majority of monocytes/macrophages and lymphocytes (Nichols et al., 1993). This study further proved that influenza A type virus largely infected the innate and mature adaptive immune cells. Influenza B type virus was different from that of A type virus. Lower amounts of B type virus infected the immature (inactivated) lymphocytes when compared to activated lymphocytes.

The results presented in this paper suggested that the human immune cells activated by one influenza subtype virus could kill the cells infected by other type virus (Fig. 3B and Fig. 4A). Even B type virus-activated immune cells killed A type virus infected cells and vice versa (data not shown). These suggest that CTL activated by one type virus can handle other type virus. In other words, activated CTL has lower target specificity. Therefore, producing enough CTLs might be the most important issue in combating virus infection. These results were different from traditional opinion.

Dynamic analysis of the killing process showed that apoptosis and GrB release (degranulation) occurred within 5 minutes after effectors and targets were mixed. Cytotoxicity assays using labeled virus might reflect the natural process more closely, and therefore offer an advantage over methods utilizing radioactivity and viral peptides. Unlike the traditional ^{51}Cr release, which is toxic to cells, this assay used no radioactivity. Combining with other surface and intracellular markers, such as CD4, CD8, CD3, CD62L, etc., this labeled virus also supports to track the fate of cells that have been infected at low MOI.

Finally, these results suggested that human immune cells could directly handle and remove free virus using a similar mechanism that was used to remove virus-infected non immune cells. These might give more options for the vaccine design and the treatment of infection diseases.

Acknowledgments

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Abbreviations

Ac-IEPD-pNA	Acetyl-Isoleucine-Glutamate-Proline-Aspartate-paranitroanalide
APC	Allophycocyanin
Cy	Cyanine
DMEM	Dulbecco's Modified Eagle Medium
FITC	fluorescent isothiocyanate
GrB	granzyme B
IFN- γ	interferon-gamma
MDCK	Madin Darby canine kidney
MOI	Multiplicity Of Infection= ratio of infectious virus particles to cells
PBMC	peripheral blood mononuclear cells
PE	Phycoerythrin
Perf	perforin

References

- Addo MM, Altfeld M, Rosenberg ES, Eldridge RL, Philips MN, Habeeb K, Khatri A, Brander C, Robbins GK, Mazzara GP, Goulder PJ, Walker BD. The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals. *Proc Natl Acad Sci U S A* 2001;98:1781–6. [PubMed: 11172028]
- Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996;274:94–6. [PubMed: 8810254]

- Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 2003;281:65–78. [PubMed: 14580882]
- Doherty PC, Turner SJ, Webby RG, Thomas PG. Influenza and the challenge for immunology. *Nat Immunol* 2006;7:449–55. [PubMed: 16622432]
- Ewen C, Kane KP, Shostak I, Griebel PJ, Bertram EM, Watts TH, Bleackley RC, McElhaney JE. A novel cytotoxicity assay to evaluate antigen-specific CTL responses using a colorimetric substrate for Granzyme B. *J Immunol Methods* 2003;276:89–101. [PubMed: 12738362]
- Fields, BN. *Virology*. Vol. 1. Lippincott-Raven Publishers; Philadelphia: 1996.
- Huebner, RC.; Harmon, MW. Influenza virus subunit conjugates. U.S. Patent (Ed) Vol. 5,612,037. 1997.
- Lecoeur H, Fevrier M, Garcia S, Riviere Y, Gougeon ML. A novel flow cytometric assay for quantitation and multiparametric characterization of cell-mediated cytotoxicity. *J Immunol Methods* 2001;253:177–87. [PubMed: 11384679]
- Lee-MacAry AE, Ross EL, Davies D, Laylor R, Honeychurch J, Glennie MJ, Snary D, Wilkinson RW. Development of a novel flow cytometric cell-mediated cytotoxicity assay using the fluorophores PKH-26 and TO-PRO-3 iodide. *J Immunol Methods* 2001;252:83–92. [PubMed: 11334968]
- Lieberman J. Tracking the killers: how should we measure CD8 T cells in HIV infection? *AIDS* 2004;18:1489–93. [PubMed: 15238766]
- Liu L, Chahroudi A, Silvestri G, Wernett ME, Kaiser WJ, Safrit JT, Komoriya A, Altman JD, Packard BZ, Feinberg MB. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. *Nat Med* 2002;8:185–9. [PubMed: 11821904]
- Maecker HT, Dunn HS, Suni MA, Khatamzas E, Pitcher CJ, Bunde T, Persaud N, Trigona W, Fu TM, Sinclair E, Brecht BM, McCune JM, Maino VC, Kern F, Picker LJ. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. *J Immunol Methods* 2001;255:27–40. [PubMed: 11470284]
- McElhaney JE, Xie D, Hager WD, Barry MB, Wang Y, Kleppinger A, Ewen C, Kane KP, Bleackley RC. T cell responses are better correlates of vaccine protection in the elderly. *J Immunol* 2006;176:6333–9. [PubMed: 16670345]
- Nichols JE, Mock DJ, Roberts NJ Jr. Use of FITC-labeled influenza virus and flow cytometry to assess binding and internalization of virus by monocytes-macrophages and lymphocytes. *Arch Virol* 1993;130:441–55. [PubMed: 8517795]
- Powers DC, Belshe RB. Effect of age on cytotoxic T lymphocyte memory as well as serum and local antibody responses elicited by inactivated influenza virus vaccine. *J Infect Dis* 1993;167:584–92. [PubMed: 8440930]
- Rong J, Xu X, Ewen C, Bleackley RC, Kane KP. Isolation and characterization of novel single-chain Fv specific for human granzyme B. *Hybrid Hybridomics* 2004;23:219–31. [PubMed: 15319069]
- Rubio V, Stuge TB, Singh N, Betts MR, Weber JS, Roederer M, Lee PP. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. *Nat Med* 2003;9:1377–82. [PubMed: 14528297]
- Sun Y, Iglesias E, Samri A, Kamkamidze G, Decoville T, Carcelain G, Autran B. A systematic comparison of methods to measure HIV-1 specific CD8 T cells. *J Immunol Methods* 2003;272:23–34. [PubMed: 12505709]
- Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection in influenza infection. *Emerg Infect Dis* 2006;12:48–54. [PubMed: 16494717]
- Tomaru U, Yamano Y, Nagai M, Maric D, Kaumaya PT, Biddison W, Jacobson S. Detection of virus-specific T cells and CD8+ T-cell epitopes by acquisition of peptide-HLA-GFP complexes: analysis of T-cell phenotype and function in chronic viral infections. *Nat Med* 2003;9:469–76. [PubMed: 12652294]
- van Baalen CA, Kwa D, Verschuren EJ, Reedijk ML, Boon AC, de Mutsert G, Rimmelzwaan GF, Osterhaus AD, Gruters RA. Fluorescent antigen-transfected target cell cytotoxic T lymphocyte assay for ex vivo detection of antigen-specific cell-mediated cytotoxicity. *J Infect Dis* 2005;192:1183–90. [PubMed: 16136460]
- Xie D, McElhaney JE. Lower GrB+ CD62Lhigh CD8 TCM effector lymphocyte response to influenza virus in older adults is associated with increased CD28null CD8 T lymphocytes. *Mech Ageing Dev* 2007;128:392–400. [PubMed: 17570460]

Yang OO. Will we be able to 'spot' an effective HIV-1 vaccine? Trends in Immunology 2003;24:6.

Fig. 1.

Influenza virus labeled with fluorescent dye had the same ability to stimulate human immune response as unlabeled virus. A, The stimulation of influenza virus (Cy5-A/Puerto Rico/8/34, [H1N1]), labeled with fluorescent dye, produced an increase of GrB⁺Perf⁺ CD3⁺ CD8 T cells, similar to unlabeled virus (A/Puerto Rico/8/34, [H1N1]). The number indicates the percentage of total CD3⁺CD8 T cells. B, Labeled influenza virus (Cy5-A/Puerto Rico/8/34, [H1N1]) (MOI=2) stimulation produced an increase in GrB activity as found in unlabeled virus. Control group was without virus treatment. Error bars indicate as standard errors. (N=5, p<0.001). C, Labeled influenza virus (Cy3-A/Panama/2007/99 [H3N2]) (MOI=2) stimulation produced an increase of IFN-gamma as did unlabeled virus. Control group was without virus treatment. Error bars indicate as standard errors. (N=5, p<0.001).

Fig. 2.

Human influenza virus showed a preference for infecting activated lymphocytes and monocytes. Influenza B type virus infected a few immature lymphocytes. A, Labeled virus (Cy3-A/Panama/2007/99 [H3N2]) preferred to infect monocytes and mature lymphocytes (G2) in the forward and side scatter dot plot. Forward and side scatter dot plot of fresh human PBMC after infected by Cy3 labeled virus A/Panama/2007/99 [H3N2] for 30 minutes. G1 refers to the immature lymphocytes population in the forward and side scatter dot plot; G2 refers to monocytes and activated lymphocytes (GrB⁺) population in the forward and side scatter dot plot. B, Dynamic assay showed that Cy3-virus (A/Puerto Rico/8/34, [H1N1]) mainly infected G2 population of fresh human PBMC. C, Cy3-B/Shanghai/361/2002 largely infected the mature lymphocytes, monocytes, with a few immature lymphocytes. D, Virus was largely taken up by activated (GrB⁺) lymphocytes. PBMC stimulated with A/New Caledonia/20/99 [H1N1] (MOI=2) for 5 days were infected with A/Wyoming/03/2003 [H3N2] (MOI=2) labeled with PE for 30 minutes, stained by anti CD3, CD4, CD8 and GrB antibodies.

Fig. 3.

The cells infected with influenza virus were killed. A, ^{51}Cr release test: PBMC (stimulated with $1\mu\text{g/ml}$ PHA for 5 days) and infected with A/Wyoming/03/2003 [H3N2] were used as target (T), autologous PBMC stimulated with A/Wyoming/03/2003 [H3N2] (MOI=2) for 5 days as effector (E). The mixtures of various ratio of effector/target (E:T) were incubated for 4 hours at 37°C , in 5% CO_2 (target: 5000 cells). ^{51}Cr release increased with E:T ratio. B, PBMC (stimulated with PHA for 5 days) infected with Cy3 labeled influenza virus strain A/Puerto Rico/8/34, [H1N1] (MOI=2) were killed by the autologous PBMC stimulated with the same virus for 5 days (E:T = 5:1).

Fig. 4.

The fluorescence of labeled virus decreased with time and virus infected cells went to apoptosis. A, Fluorescence of labeled virus decreased with time. Gated cells were as Cy3 positive. The PBMC were infected with Cy3 labeled virus A/Panama/2007/99 [H3N2] (MOI=2) and mixed with the PBMC (effector) that were stimulated with virus A/Puerto Rico/8/34, [H1N1] for five days, and incubated at 37°C, 5% CO₂. B, Virus infected cells went into apoptosis. Fresh PBMC were infected with virus A/Wyoming/03/2003 [H3N2] (PE labeled) (MOI=2), then incubated with Annexin-V (Fitec) for 2 hours.

Fig. 5.

The degranulation of CD8 T cells was involved in the killing process. A, CD107a of GrB⁺ CD8 T cells moved to the surface compared to the control (no virus infection). CD8 T cells also increased the expression of GrB with virus infection. B, C, The dynamic assay showed that apoptosis and GrB release occurred almost simultaneously, within 5 minutes and peaked at about 30 minutes.