Journal of Immunological Methods 398–399 (2013) 60–67

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

A real-time killing assay to follow viral epitope presentation to CD8 T cells

Pauline Gourdain, Julie Boucau, Georgio Kourjian, Nicole Y. Lai, Ellen Duong, Sylvie Le Gall *

Ragon Institute of MGH, MIT and Harvard, Massachusetts General Hospital, Harvard Medical School, Cambridge, MA, USA

ARTICLE INFO

Article history:
Received 23 May 2013
Accepted 13 September 2013
Available online 20 September 2013

Keywords:
HIV
Antigen processing
CD8 T cells
Real-time killing assay
Cytotoxicity
Kinetics

ABSTRACT

The ability of cytotoxic T lymphocytes (CTL) to clear virus-infected cells requires the presentation of viral peptides intracellularly processed and displayed by major histocompatibility complex class I. Assays to measure CTL-mediated killing often use peptides exogenously added onto target cells – which does not account for epitope processing – or follow killing of infected cells at a single time point. In this study we established a real-time fluorogenic cytotoxic assay that measures the release of the Glucose-6-phosphate-dehydrogenase by dying target cells every 5 min after addition of CTL. It has comparable sensitivity to ^{51}chromium-based killing assay with the additional advantage of incorporating the kinetics of epitope presentation. We showed that HIV infection of immortalized or primary CD4 T cells leads to asynchronous killing by two CTL clones specific for epitopes located in different proteins. Real-time monitoring of killing of virus-infected cells will enable identification of immune responses efficiently preventing virus dissemination.

1. Introduction

Cytotoxic T lymphocytes (CTL) are one of the key components of host defense against viral infection or tumor cell elimination. Virus-specific CTL responses are elicited in most viral infections, including influenza, HIV, EBV, CMV and HCV (Bangham, 2009). HIV-specific CTL play a critical role in containing HIV viremia in acute infection or in situation of spontaneous control (Hersperger et al., 2011). However in chronic infections such as HIV and HCV the virus is not cleared despite the presence of CTL responses (Migueles and Connors, 2001; Rowland-Jones et al., 2001). The identification of criteria defining protective immune responses is paramount to the design of vaccine immunogens (Burton et al., 2012). Several parameters including peptide sequence and avidity for MHC and T cell receptor (TCR), proliferative capacity, production of cytokines and cytolytic granule release contribute to the antiviral capacity of the CTL responses (Almeida et al., 2007; Migueles et al., 2008; Hersperger et al., 2010; Ndhlovu et al., 2013). However one parameter often disregarded in the identification of efficacious immune responses is the timing of epitope presentation.

The killing of an infected cell by epitope-specific T cells is the culminating event of a multistep process. MHC-I epitopes displayed in the surface of cells come from the degradation of proteins in a multistep pathway involving peptidases located in the cytosol, in the endoplasmic reticulum, and for exogenous antigens peptidases from the endo-lysosomal pathway (Neefjes et al., 2011). The presentation of MHC-I-epitope is a prerequisite to the interaction between target and effector cells, activation of T cells, release of cytolytic granules content by T cells, and eventually disintegration of the target cell.

Multiple assays have been developed to assess killing of target cells or activation of effector T cells at a given time point following infection (Shacklett, 2002; Lemonnier, 2013). Radioactive isotope ^{51}chromium (^{51}Cr) or tritium (^{3}H) killing

Abbreviations: CFSE, carboxyfluorescein succinimidyl ester; MTG, MitoTrackerGreen; CAM, calceinacetoxymethylester; 7-AAD, 7-amino-actinomycin D; G6PDH, glucose 6-phosphate dehydrogenase.

* Corresponding author at: Ragon Institute of MGH, MIT and Harvard, Massachusetts General Hospital, Harvard Medical School, 400 Technology Square, Cambridge, MA 02139, USA. Tel.: +1 857 268 7010; fax: +1 857 268 7142.

E-mail address: sylvie_legall@hms.harvard.edu (S. Le Gall).

0022-1759/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jim.2013.09.009

assays are the traditional methods to assess virus-specific T cell-mediated cytotoxicity (Brunner et al., 1968; Usharauli et al., 2006). They are based on generating infected target cell populations and labeling them with ^{51}Cr or ^{3}H. The addition of virus-specific T cells leads to killing of target cells and release of radioactivity into the culture supernatant. Quantification of ^{51}Cr- or ^{3}H-release provides a measure of the specificity and the cytotoxic capacity of T cells in the effector cell population at a single time point typically measured several hours after addition of CTL.

Several non-radioactive techniques have been developed to assess the killing of target cells (Cholujova et al., 2008) or apoptosis in various culture conditions (McMillian et al., 2002). Dyes to label target cells include carboxyfluorescein succinimidyl ester (CFSE) (Wierda et al., 1989), MitoTrackerGreen (MTG), calceinacetoxymethylester (CAM), Vybrant DiO (DiO), 7-amino-actinomycin D (7-AAD) (Lecoeur et al., 2001; Sheehy et al., 2001) or caspase-3 substrates (Liu et al., 2002; He et al., 2005). They measure cell apoptosis (7-AAD; caspase-3 substrates), disintegration of dying cells after recognition by T cells (release of CFSE or MTG), the proportion of live/dead cells with two substrates such as CFSE/PKH26 (Lee-MacAry et al., 2001; Sheehy et al., 2001), or track surviving cells (CAM; (Roden et al., 1999)). All these dyes are intracellular markers which may leak out of the cells and can be toxic to primary cells such as dendritic cells. Extracellular substrates cleaved by enzymes released by dying cells such as resazurin cleaved by glucose 6-phosphate dehydrogenase (G6PDH) have been used to measure apoptosis and drug-induced cytotoxicity but not to follow T cell-mediated lysis of target cells (Batchelor and Zhou, 2004).

Indirect assays to assess recognition of target cells by effector T cells measure CD8 T cell activation by measuring the production of cytokines such as interferon gamma or Interleukin 2 (Miyahira et al., 1995; Maino and Picker, 1998; Mwau et al., 2002; Janetzki et al., 2005; Nomura et al., 2008), the release of cytolytic granules through exposure of CD107a (Betts et al., 2003) or cytotoxic molecules (perforin and granzymes) (Snyder et al., 2003; Snyder-Cappione et al., 2006; Hersperger et al., 2010). However these parameters do not address whether killing and clearance of infected cells occurred, a critical issue in chronic infections such as HIV and HCV where T cells can be partly functionally impaired (Hersperger et al., 2011).

Despite their advantages, all these approaches provide limited information about the timing of presentation of an epitope to its cognate CTL, a factor critical to ensure efficient clearance of infected cells — that is independent of the antiviral function of T cells. Here we present a new non-radioactive cytotoxic assay with a low toxicity and a low cell number requirement that allows us to sensitively measure the killing of target cells in real-time after addition of CTL, and to compare the kinetics of presentation of endogenously processed HIV epitopes to HIV-specific CTL.

2. Material and methods

2.1. Study participants

HIV-negative and HIV-infected donors were recruited at Massachusetts General Hospital (MGH) in Boston. Partners Human Research Committee (Boston, MA) approved the use of anonymous buffy coats under protocol 2005P001218, the use of samples from HIV-negative coded donors under protocol 2010P002121 and the use of coded HIV-infected samples under protocols 2010P002463 and 2003P001894. All participants provided written informed consent for participation in the study.

2.2. Peptides

Highly purified peptides (>98% pure) were purchased from MGH peptide core facility.

2.3. Cell culture

EBV-immortalized B cells were maintained in RPMI 10% FCS. KF11-, TW10-, ATK9- and RK9-specific CTL clones were isolated by limiting dilution and maintained in the presence of 50 U/ml IL-2 (R10-IL2) using the CD3-specific mAb 12 F6 and irradiated PBMC as stimulus for T cell proliferation (Le Gall et al., 2007).

2.4. Cell sorting

CD4 T cells were enriched from freshly isolated peripheral blood mononuclear cells (PBMCs) by magnetic immunodepletion of cells expressing CD8, CD14, CD16, CD19, CD20, CD36, CD56, CD66b, CD123, TCRγ/δ, glycophorin A and dextran-coated magnetic particles, according to the manufacturer's instructions (StemCell). The percentage of CD4 T cells assessed by flow cytometry was >90%. PHA-stimulated CD4 T cells were obtained by incubating CD4 T cells at 1×10^6/ml in R10-IL2 with 0.25 μg/ml PHA for 4–6 days.

2.5. HIV infection

VSV-G-pseudotyped viral stocks were prepared by co-transfection of 293 T cells with NL4-3 proviral DNA (or with a GFP-encoding provirus) along with a CMV-VSV-G plasmid and titrated as previously described (Miura et al., 2009). B cells or PHA-activated CD4 T cells were harvested and incubated at 2×10^6/well in R10 in 24-well plates. VSV-G-pseudotyped virus was added at a concentration of 100 ng p24 per well for 5 h. Cells were washed twice and plated again. Every other day until day 10, cells were monitored for infection rate and used as targets in real-time or ^{51}Cr killing assays.

2.6. Flow cytometry

Cells were washed and incubated with CD4-PE and HLA-DR-APC antibody (pre-titrated volume, BD Pharmingen). Cells were then permeabilized with the Cytofix/Cytoperm Plus kit (BD Biosciences) for 20 min following manufacturer's instructions and stained for intracellular p24 with p24-FITC antibody (pre-titrated volume, Santa Cruz Biotech) for 30 min. Cells were fixed and acquired on a two-laser Calibur flow cytometer using CellQuestPro software (BD Biosciences) and data were analyzed using FlowJo software (TreeStar).