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Research paper

A culture amplified multi-parametric intracellular cytokine assay (CAMP-ICC) for enhanced detection of antigen specific T-cell responses

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ABSTRACT

Ex-vivo T-cell responses to vaccines and some viral antigens are not always detectable by conventional functional T-cell assays such as lympho-proliferation assays, IFN-γ ELIspot and intracellular cytokine staining (ICC). In this study we describe the development, optimisation and utilisation of a culture amplified multiparametric intracellular cytokine assay (CAMP-ICC) to detect antigen specific T-cells that may be present at low frequencies and small primed responses typical of those induced by DNA vaccines in humans. CFSE labelled PBMCs are cultured for 10 days with antigens of interest. Low concentrations of exogenous proliferative and anti-apoptotic cytokines are added to assist in amplification of the antigen specific, but not background responses. On day 10 the cultured cells are re-challenged with or without antigen as for the standard ICC and then stained with fluorescent monoclonal antibodies of interest. Various conditions, including concentration, day of administration and length of incubation were tested employing the cytokines, IL-2, IL-7, IL-15 and IL-21 alone or in combination. CMV lysate, CEF peptides and measles viral lysate were used in the optimisation of the CAMP-ICC. We found that addition of 0.5 ng/ml of IL-15 in combination with 0.1 ng/ml of IL-21 at day 5 of culture enhanced proliferation of antigen specific responses whilst maintaining low background. The CAMP-ICC provides much more information than conventional functional T-cell assays allowing simultaneous determination of proliferative capacity and cytokine production by both CD4 and CD8+ T cells.

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Abbreviations: CAMP-ICC, culture amplified intracellular cytokine assay; ICC, intracellular cytokine assay; LPA, lymphoproliferation assay; HIV, human immunodeficiency virus; MV, measles virus; CMV, cytomegalovirus; EBV, Epstein Barr virus; CEF, cytomegalovirus, Epstein Barr virus, influenza virus; CFSE, carboxyfluorescein diacetate succinimidyl ester; PBMC, peripheral blood mononuclear cells; ACD, acid-citrate dextrose; HI, heat inactivated; FBS, foetal bovine serum; DMSO, dimethyl sulphoxide; LN2, liquid nitrogen; MAb, monoclonal antibodies; PerCP, Peridinin–chlorophyll–protein; PE, phycoerythrin; APC, allophycocyanin; IFN-γ, gamma interferon; IL-, interleukin-; TNF-α, tumor necrosis factor alpha; ECD, energy coupled dye; SEB, staphylococcal enterotoxin B; PHA, phytohemagglutinin; FPV, fowl pox virus; DNA, deoxyribonucleic acid; PPD, purified protein derivative; SHIV, simian-human immunodeficiency virus; PHAEDRA, Primary HIV and Early Disease Research: Australian Cohort Study; IMDM, Iscove's Modified Dulbecco's medium; EDTA, ethylenediaminetetra-acetic acid; PBS, phosphate buffered saline; B-LCL, B-lymphoblastoid cell lines; DC, dendritic cells; LTNP, long-term non-progressors; PHI, primary HIV infection.

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1. Introduction

The detection of antigen specific T-cells responding to micro-organisms is important in understanding the immunopathogenesis of infection, informs vaccine design, reflects their immunogenicity and perhaps provides a correlate of their efficacy. Functional T-cell assays such as lympho-proliferation assays (LPA) based upon incorporation of tritiated [^{3}H]-thymidine, IFN-γ ELIspot assays and intracellular cytokine staining assays (ICC) are routinely used to detect responses of antigen specific T-cells. However, ex-vivo T-cell responses to vaccines, some viral antigens such as HIV and Hepatitis C (reviewed in (Lloyd et al., 2007)) and abacavir specific T-cells in hypersensitive individuals (Martin et al., 2007) are not always detectable when these conventional functional T-cell assays are used.

Studies of DNA and fowlpox virus (FPV) prime/boosting vaccine strategies for simian-human immunodeficiency virus, performed in pigtail macaques (Dale et al., 2004), revealed equivalent efficacy of a two dose DNA prime FPV boost regimen and a three dose DNA strategy in terms of viral control when these macaques were challenged mucosally with virulent $SHIV_{mn}$229. Determination of vaccine immunogenicity by IFN-γ ELIspot, ICC and LPA revealed that the macaques vaccinated with the former regimen had much larger T-cell responses than those receiving the latter regimen. The standard conventional functional T-cell assays deemed the DNA vaccine had negligible immunogenicity, despite outcome data. Therefore, we aimed to develop a novel assay to boost small primed responses typical of those induced by DNA vaccines that are not always detectable by the routine assays described above. This was performed as part of a prophylactic HIV vaccine development program based on a DNA prime, recombinant fowlpox virus boost strategy that has proceeded to clinical trial (Kelleher et al., 2006).

The assay we developed is a culture amplified multi-parametric intracellular cytokine assay (CAMP-ICC). This assay combines the carboxyfluorescein diacetate succinimidyl ester (CFSE) staining method (Lyons and Parish, 1994) to detect proliferating cells and the intracellular cytokine staining method (Suni et al., 1998; Nomura et al., 2000) to detect cells able to express cytokines such as IFN-γ, IL-2 and TNF-α. These two methodologies have previously been combined and performed by others (Fazekas de St Groth et al., 1999; Tanaka et al., 2004). However the CAMP-ICC is unique in that it utilises low doses of exogenous, proliferative and anti-apoptotic cytokines (interleukin (IL)-15 and IL-21) on day 5 of culture to specifically boost antigen specific T-cell responses.

The utilisation of cytokines to boost IFN-γ responses has been shown by a number of groups, in particular with the ELIspot assay. The Amplispot assay (Jennes et al., 2002) combines IL-7 and IL-15 to boost IFN-γ responses to purified protein derivative (PPD) and CMV. IL-15 alone has been used to boost IFN-γ responses in an ELIspot assay on samples from rhesus macaques exposed to simian-human immunodeficiency virus (SHIV) and simian immunodeficiency virus (SIV) (Calarota et al., 2003).

The cytokines that we assessed as means of amplifying responses in the CAMP-ICC were from the common cytokine receptor gamma chain (γ_c) family and included IL-2, IL-7, IL-15 and IL-21. These cytokines are known to be essential for the development and maintenance of lymphocytes (reviewed in Alves et al., 2007), play a role in homeostasis and are implicated in the generation of long-term memory antigen specific T-cells. At high doses these cytokines will induce both proliferation and cytokine production by lymphocytes. We aimed to identify concentrations of these cytokines that support these functions in purely an antigen specific manner thereby amplifying responses whilst maintaining low background, enhancing the signal to noise ratio of the assay.

In this study we describe our alternative assay, the CAMP-ICC in which we evaluate the effect of several common-γ chain cytokines on their ability to enhance proliferation of a range of antigen-specific T-cell responses that exist at low frequencies ex-vivo.

2. Materials and methods

2.1. Subjects

Healthy adult volunteers with positive CMV serology or known history of measles infection or vaccination were recruited from hospital and university staff for the optimisation of this assay. Samples from HIV infected individuals were recruited as part of the PHAEDRA/CORE01 (Hecht et al., 2006) primary HIV infection natural history study, these samples were used in proof of principle studies. Samples from HIV negative individuals that were at low risk of HIV infection and in good health were recruited as part of a phase I HIV prophylactic vaccine study (Kelleher et al., 2006). Both the PHAEDRA/CORE01 study and the phase I prophylactic vaccine study were approved by the St Vincent's Hospital research ethics committee, all individuals gave informed consent.

2.2. Cell isolation and storage

Peripheral blood mononuclear cells (PBMC) from acid-citrate dextrose (ACD; BD Biosciences) anti-coagulated venous blood were isolated by density gradient centrifugation using Ficoll-Hypaque Plus (Amersham Biosciences, Uppsala, Sweden). PBMC were used on day of separation or were cryopreserved in heat inactivated, filter sterilised, foetal bovine serum (FBS; JRH Biosciences, KA, USA) containing 10% dimethyl sulphoxide (DMSO; Sigma Aldrich, MO, USA) using a controlled rate freezer (Planer, Middlesex, UK) and stored in vapour phase liquid nitrogen (LN2) until needed.

2.3. Antibodies and reagents

The monoclonal antibodies (MAb) used were CD3-PerCP-Cy5.5,-Pacific Blue, CD4-phycoerythrin (PE)-Cy7, CD8-allophycocyanin (APC)-Cy7, gamma interferon (IFN-γ)-APC, interleukin-2 (IL-2)-PE, tumor necrosis factor alpha (TNF-α)-phycoerythrin (PE)-Cy7;(from Becton Dickinson, San Jose, CA); CD4-energy coupled dye (ECD) (phycoerythrin-Texas Red) (Beckman Coulter, Hialeah, FL). All antibodies were used according to the manufacturers' directions. Recombinant human IL-2 (Roche Diagnostics, Basel, Switzerland), IL-7, IL-15 (R&D Systems, Minneapolis, MN), and IL-21 (Biosource International, Camarillo, CA) were added to the CAMP-ICC cultures as described below.

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