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Vagaries of the ELISpot assay: Specific detection of antigen responsive cells requires purified CD8$^+$ T cells and MHC class I expressing antigen presenting cell lines

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Abstract
Quantification of antigen-specific CD8$^+$ T cells is important for monitoring infection, vaccination, and response to therapy in cancer and immune-mediated diseases. Cytokine enzyme-linked-immunospot (ELISpot) assays are often used for this purpose. We found that substantial spot formation in IFNγ ELISpot assays occurred independently of CD8$^+$ T cells even when classical MHC class I restricted peptides are used for stimulation. Using fractionated cells and intracellular cytokine staining, the non-CD8$^+$ T cell IFNγ production was attributed to the CD4$^+$ T cell fraction. We therefore refined a cell line-based ELISpot assay combining HLA-A*0201 expressing K562 cells for antigen presentation with purified CD8$^+$ T cells and demonstrated that it specifically detected CD8$^+$ T cell responses with detection limits comparable to traditional ELISpot assays and dextramer-based quantification. The assay was further adapted to whole antigen responses with antigen (pre-proinsulin)-expressing HLA-A*0201K562 cells. Thus, we revealed and corrected a weak spot of the CD8$^+$ ELISpot assay.

Abbreviations: PBMC, Peripheral Blood Mononuclear Cells; ELISpot, Enzyme-Linked Immuno Spot; IFNγ, Interferon-γ; MHC, Major Histocompatibility Complex; HLA, Human Leukocyte Antigen.

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1. Introduction

Antigen-specific CD8+ T cells are classic responders in disease with substantial changes in their numbers seen in periods of active viral infection. They are actively involved in tumor clearance and autoimmune disease and their quantification is therefore an important part of immune monitoring in these settings. Of particular relevance to disease is the ability to discriminate disease-related and benign responses on the basis of different cytokine responses [1,2]. Enzyme-linked immunospot (ELISpot) assays are often used for the measurement of antigen-specific responses in infection, vaccination and autoimmunity [1,3–7]. They are considered short-term assays that identify antigen-primed memory T cells on the basis of cytokine production from individual responsive cells visualized as spots [8,9]. ELISpot assays generally use whole mononuclear cell preparations or whole blood [1,2,4,5,10], and are determined to be specific for CD4+ or CD8+ T cell responses on the basis of the peptides used to stimulate the cells. Important requisites for allocating responses to CD4+ or CD8+ T cells are that the peptides bind specifically to HLA class II alleles for CD4+ T cell responses or HLA class I alleles for CD8+ T cell responses, and that the antigen-presenting cells express the allele bound by the peptide. Various methods have been used to identify peptides that are relevant to man in this context. This includes immunization of HLA allele transgenic mice [11], elution of peptides from specific HLA molecules [1,12], or predictive algorithms together with peptide binding studies (reviewed in [13]). Specific binding to the class I or class II alleles is important for distinguishing CD8+ T cell and CD4+ T cell responses. Since there are important differences in the lengths of peptides presented by HLA class I and class II alleles [14–20], binding specificity is often checked within alleles for either HLA class I or class II alleles and not both. This may be a dangerous assumption; however, since the peptide length distinction is not absolute [21,22], CD8+ T cells can be stimulated by antigen presented on HLA class II molecules [23], and there is substantial promiscuity in the binding of T-cell receptor to HLA–peptide complexes [24]. In the course of measuring T cell responses to peptides bound to the HLA A*0201 allele, we have noticed that there is substantial non-CD8+ T cell response observed in the ELISpot assay in certain donors. Here, we report and investigate this phenomenon, and provide evidence that the CD8+ T cell ELISpot assay can be improved by the introduction of artificial antigen-presenting cells.

2. Material and methods

2.1. Subjects

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient. For CD4+ and CD8+ proportions, samples from healthy children enrolled in the TEENDIAB clinical study (n = 50, median age 9 years, range 7–11 years, 25 males) were used. Samples from 8 patients with T1D (median age 19 years, range 12–20 years, 3 males) within median 1.9 months (range 0.7–11.6 months) after disease onset and healthy blood donors at the Deutsches Rotes Kreuz Blutspendedienst Ost GmbH Dresden (n = 17, median age 30, range 20–45 years) were used for functional T cell and multimer assays. All donors and patients had the HLA-A*0201 allele. Samples were collected and processed with informed consent and ethical committee approval.

2.2. Mice

NOD.$\beta 2m^{null}$.HHD mice were kindly provided by Prof. David V. Serreze and were housed in the animal facility of the Medical Theoretical Centre Dresden under specific pathogen-free conditions. Animal studies were performed in accordance with German Animal Welfare legislation and approved by the regional animal welfare license body (Landesdirektion Dresden, Germany; permit numbers 24-9168.11-1/2010-39).

2.3. PBMC cryopreservation and thawing

Cells were resuspended in human AB supplemented with 10% dimethyl sulphoxide (DMSO) at a concentration of $1–2 \times 10^7$ PBMC/ml. Pre-cooled cryovials (4 °C) were filled with up to 1 ml of cell suspension and tubes were cooled to −80 °C in controlled-rate freezing containers. Cryovials were transferred to the vapor phase of liquid nitrogen and stored until use. For thawing, cryovials were placed in a 37 °C water bath until the frozen cell suspension was partly dissolved. 1 ml DMEM was added dropwise and the cell suspension slowly adjusted to a volume of 10 ml with DMEM. After centrifugation and an additional washing step with 10 ml DMEM, cells were counted and resuspended in cell culture medium. Where indicated, cells were rested overnight in X-VIVO15 medium supplemented with 5% human AB serum. Overnight resting resulted in slight loss of the non-CD3+ population, but did not activate CD4+ or CD8+ T cells (supplementary figure A).

2.4. Peptides

Peptides used in this study were influenza matrix protein peptide 58–66 (Flu MP $_{58–66}$: GILGFVFTL), islet specific glucose-6-phosphatase catalytic subunit-related protein peptides 228–236, 265–273 (IGRP$_{228–236}$: LNIDLLWSV; IGRP$_{265–273}$: VLFGLGFAI), pre-proinsulin peptides 15–23, 42–51,101-109, (PPI$_{15–23}$: ALWGPDPAA; PPI$_{42–51}$: VCGERGFFYT; PPI$_{101–109}$: SLYQLENYC), glutamic acid decarboxylase 65 peptide 114–122 (GAD65$_{114–122}$: VMNILLQYV), insulinoma-associated 2 peptide 797–805 (IA-2$_{797–805}$: MVWESGCTV); zinc transporter 8 peptide 185–194 (ZnT8$_{185–194}$: AVAANIVLTV) and pre-pro islet amyloid polypeptide peptide 5–13 (pplAPP$_{5–13}$: KLQVFLIVL). Viral peptide mix consisted of the following peptides: Epstein–Barr virus latent membrane protein 2A peptide 426–434 (CLGGLLTMV); cytomegalovirus 65 kDa phosphoprotein peptide 495–503 (NLVPMVATV); measles virus hemagglutinin glycoprotein peptide 250–259 (SMYRVFEVGV) and Flu MP$_{58–66}$. For experiments involving murine CD8+ T cells, murine IGRP peptide 228–236 (FGIDLLWSV) and control peptide lymphocytic choriomeningitis virus glycoprotein peptide 33–41 (KAVYNFATM) were used. Peptides were synthesized by Pro Immune (Oxford; United Kingdom), Mimotopes (Clayton Victoria, Australia) or PANATecs GmbH