ELSEVIER

Journal of Immunological Methods 270 (2002) 99–108

JIM Journal of Immunological Methods

www.elsevier.com/locate/jim

Enhanced ELISPOT detection of antigen-specific T cell responses from cryopreserved specimens with addition of both IL-7 and IL-15—the Amplispot assay

Wim Jennes[a,b,*], Luc Kestens[b], Douglas F. Nixon[a], Barbara L. Shacklett[a]

[a] Gladstone Institute of Virology and Immunology, University of California-San Francisco, San Francisco, CA, USA
[b] Laboratory of Immunology, Department of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium

Received 11 April 2002; received in revised form 8 July 2002; accepted 8 July 2002

Abstract

The importance of the enzyme-linked immunosorbent spot (ELISPOT) assay as a tool for studying immune responses in vitro is becoming increasingly apparent. However, there remains a need for enhanced sensitivity for the detection of low frequency antigen-specific T cell responses. We reasoned that the addition of a combination of the cytokines interleukin (IL)-7 and IL-15 would selectively increase interferon-gamma (IFN-γ) production from antigen-stimulated CD4+ and CD8+ effector memory T cells. Freshly isolated or cryopreserved peripheral blood mononuclear cells (PBMC) from four healthy donors were analysed by ELISPOT for the frequency of purified protein derivative (PPD)-specific CD4+ T cells or cytomegalovirus (CMV) peptide-specific CD8+ T cells. Addition of IL-7 and IL-15 increased the number of PPD-specific CD4+ T cells up to 2.4-fold in fresh PBMC and up to 18-fold in cryopreserved PBMC. The cytokines also increased the number of CMV peptide-specific CD8+ T cells in fresh PBMC up to 7.5-fold. No additional increases were seen when antibodies to co-stimulatory molecules CD28 and CD49d were applied together with the cytokine combination. These data demonstrate that the sensitivity of the ELISPOT assay may be significantly augmented by addition of the cytokines IL-7 and IL-15 to antigen-stimulated cells. This method will be particularly useful for the assessment of antigen-stimulated cytokine production by T cells in cryopreserved biological specimens.
© 2002 Elsevier Science B.V. All rights reserved.

Keywords: ELISPOT; IL-7; IL-15; Cytokine production; T cells

1. Introduction

The enzyme-linked immunosorbent spot (ELISPOT) assay has become widely utilised as a tool to study cellular and humoral immune responses in vitro (Czerkinsky et al., 1983, 1988). ELISPOT has been used to determine the frequency of specific CD4+ and CD8+ T cell responses to self (Fiorillo et al., 2000; Pelfrey et al., 2000), tumor (Jager et al., 2000;

Abbreviations: BCG, Bacillus Calmette-Guérin; CMV, cytomegalovirus; ELISPOT, enzyme-linked immunosorbent spot assay; HIV, human immunodeficiency virus; IFN-γ, interferon-gamma; IL, interleukin; PBMC, peripheral blood mononuclear cells; PPD, purified protein derivative; SEB, staphylococcal enterotoxin B; S.E.M., standard error of the mean.

* Corresponding author. Laboratory of Immunology, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium. Tel.: +32-3-247-6227; fax: +32-3-247-6231

E-mail address: wjennes@itg.be (W. Jennes).

0022-1759/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0022-1759(02)00275-2

Nagorsen et al., 2000), viral (Larsson et al., 1999; Tan et al., 1999; Brown et al., 2000), bacterial (Smith et al., 2000; Geginat et al., 2001a) and other antigens (King et al., 1993; Elghazali et al., 1997). Secretion of a range of cytokines (interferon-gamma (IFN-γ), TGF-β, TNF-α, interleukin (IL)-4, IL-5, IL-6, IL-10, IL-12) (Ozenci et al., 2000; Kouwenhoven et al., 2001; Ostrowski et al., 2001; Reece et al., 2002), β-chemokines (MIP-1α, MIP-1β, RANTES) (these authors, unpublished) and cytotoxins (granzymes) (Rininsland et al., 2000) may be measured. The sensitivity of the ELISPOT assay for detecting CD8+ T cell responses has been estimated to be at least 1 $\log_{10}$ higher than traditional limiting dilution analysis or bulk ^{51}Cr release assay (Miyahira et al., 1995; Murali-Krishna et al., 1998; Larsson et al., 1999; Tan et al., 1999).

Although the sensitivity and technical ease of the ELISPOT assay make it a useful alternative to traditional analytical methods, there remains a need for increased sensitivity in detection of low frequency antigen-specific T cell responses. In addition, cytokine production may be compromised in cryopreserved samples (these authors, unpublished; Larsson et al., 2002; Mwau et al., 2002). Use of autologous dendritic cells as antigen-presenting cells may augment the sensitivity of ELISPOT (Subklewe et al., 1999; Larsson et al., 2002; Schmitz et al., 2002). However, this method requires large amounts of fresh peripheral blood and in vitro maturation of monocytes over a period of 5 to 7 days. Thus, it is desirable to identify a combination of cytokines and/or co-stimulatory molecules that might substitute for the potent antigen-presenting capacity of mature dendritic cells.

Naive T cells require interactions with self-MHC molecules in order to survive and proliferate under lymphopenic conditions (Ernst et al., 1999; Goldrath and Bevan, 1999; Murali-Krishna et al., 1999). Memory T cells do not require these signals, but depend upon cytokines including IL-7 and IL-15 for homeostatic proliferation (Lau et al., 1994; Nakajima et al., 1997; Ku et al., 2000; Schluns et al., 2000; Geginat et al., 2001b; Goldrath et al., 2002; Tan et al., 2002) and can be expanded in vitro with addition of these cytokines (Hickman et al., 1990; Kos and Mullbacher, 1993; Kanai et al., 1996; Lalvani et al., 1997). IL-7, which may be produced in vivo in response to T cell depletion (Napolitano et al., 2001), can enhance proliferation of both naive and memory CD8+ T cells (Welch et al., 1989). IL-15 is produced by a wide variety of cells, including monocytes and dendritic cells, and induces CD4+ and CD8+ T cell proliferation (Kuniyoshi et al., 1999; Hasan et al., 2000; Mattei et al., 2001; Mohamadzadeh et al., 2001; Yajima et al., 2002). If the TCR is activated by antigen, IL-15 can augment cytokine production (Niedbala et al., 2002). We reasoned that a combination of antigen, IL-7 and IL-15 would selectively increase cytokine production from CD4+ and CD8+ effector memory T cells, leading to increased sensitivity of detection in the ELISPOT assay.

2. Methods

2.1. Peripheral blood mononuclear cells (PBMC) and donors

Heparinised blood samples were obtained, with informed consent, from healthy donors among some were known to be vaccinated with the Bacillus Calmette-Guérin (BCG) vaccine (a live vaccine prepared from an attenuated strain of *Mycobacterium bovis*), or had a known cellular response against cytomegalovirus (CMV). PBMC were isolated by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Cells were either used fresh or were kept frozen at − 140 °C (vapour phase of liquid nitrogen) in foetal calf serum (FCS) (Gemini Bioproducts, Woodland, CA, USA) containing 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Protein and peptide preparations

IFN-γ production by CD4+ or CD8+ T cells was assessed in response to stimulation with protein antigen or synthetic peptides, respectively. Purified protein derivative (PPD) (Staten Serum Institute, Copenhagen, Denmark) was used as protein antigen. Synthetic peptides included HLA-A*0201-restricted immunodominant epitopes from cytomegalovirus (CMV) matrix protein pp65 (495–503, NLVPMVATV) and human immunodeficiency virus type-1 (HIV-1) Gag (77–85,

SLYNTVATL), and an HLA-B*35-restricted epitope from HIV-1 Pol (329–337, HPDIVIYQY) (peptides provided by G. Ogg, Oxford University). As a negative control, PBMC were stimulated with media alone. As a positive control, PBMC were stimulated with staphylococcal enterotoxin B (SEB) (Sigma-Aldrich).

2.3. ELISPOT assay

For ELISPOT assays, 96-well nitrocellulose-bottom plates (Multiscreen-HA, Millipore, Molsheim, France) were coated with 50 μl/well of anti-human IFN-γ at a concentration of 5 μg/ml (1-D1K, Mabtech, Nacka, Sweden) and incubated at 4 °C overnight. The following day, plates were washed four times in PBS and blocked with 50 μl/well of culture medium (RPMI containing 15% FCS, 1% L-glutamine, 1% penicillin–streptomycin and HEPES buffer). Cytokine cocktails were prepared containing IL-7 and IL-15 (R&D Systems, Minneapolis, MN, USA) in concentrations ranging from 1 to 100 ng/ml. For some experiments, co-stimulatory antibodies anti-CD28 and anti-CD49d were added at 1 μg/ml (Becton Dickinson, San Jose, CA, USA). One hundred microliters of medium, with or without cytokines and co-stimulatory antibodies, was distributed to replicate wells of the ELISPOT plate. Antigens were added such that final concentrations were 10 μg/ml for PPD and 10 μg/ml for synthetic peptides. PBMC (2×10^5) were then added in a volume of 50 μl, bringing the total volume in each well to 200 μl. Incubation was continued overnight (16–18 h) at 37 °C, 5% CO_2.

ELISPOT plates were developed as previously described (Larsson et al., 1999). Briefly, plates were washed four times with PBS containing 0.05% Tween-20 (Sigma-Aldrich) and incubated for 2 h with 50 μl/well of biotinylated anti-human IFN-γ at a concentration of 1 μg/ml (7-B6-1, Mabtech) at 37 °C. Plates were then washed four times in PBS with 0.1% Tween-20 and incubated for 1 h with avidin-bound biotinylated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) at room temperature. ELISPOT plates were washed again four times in PBS with 0.1% Tween-20 and developed by incubating for 5 min with stable diaminobenzadine substrate (DAB, Research Genetics, Huntsville, AL, USA), followed by rinsing in tap water. Spots were counted using an AID ELISPOT reader (Cell Technology, Jessup, MD, USA), totals for duplicate or triplicate wells were averaged and normalised to numbers of IFN-γ spotforming cells per 1×10^6 PBMC. Average values for negative medium control wells were subtracted from the average values from antigen-stimulated wells.

3. Results

3.1. Enhanced ELISPOT detection of CD4+ T cell responses in fresh and cryopreserved PBMC by addition of both IL-7 and IL-15

Frequencies of CD4+ T cells reactive against PPD were analysed by ELISPOT in fresh and cryopreserved PBMC from two donors (donors 1 and 2) known to be vaccinated with BCG. PBMC were incubated overnight in duplicate wells in the presence of media alone, PPD, media supplemented with 50 ng/ml of both IL-7 and IL-15, and PPD supplemented with 50 ng/ml of both IL-7 and IL-15 (Fig. 1, panels A–D for fresh PBMC from donor 1, panels F–I for cryopreserved PBMC from donor 1).

For fresh PBMC, addition of both IL-7 and IL-15 to the PPD-stimulation resulted in an increase in the number of PPD-specific IFN-γ-secreting cells per million PBMC by a factor of 1.6 for donor 1 (mean ± S.E.M.: 2635 ± 77.5 vs. 1670 ± 155.0, Fig. 1E) and by a factor of 2.4 for donor 2 (605 ± 122.5 vs. 250 ± 25.0, data not shown). Addition of IL-7 and IL-15 enhanced spontaneous IFN-γ release in medium control wells by a factor of 9.7 (145 ± 60.0 vs. 15 ± 7.5, Fig. 1E) for donor 1 and by a factor of 19 (95 ± 40.0 vs. 5 ± 5.0, data not shown) for donor 2.

In the absence of IL-7 and IL-15, the number of PPD-specific IFN-γ-secreting cells was significantly lower in cryopreserved PBMC compared to fresh PBMC (420 ± 27.5 vs. 1670 ± 155.0 for donor 1, factor 0.25, Fig. 1J and E; 55 ± 25.0 vs. 250 ± 25.0 for donor 2, factor 0.22, data not shown). Addition of IL-7 and IL-15 to PPD in cryopreserved PBMC augmented the number of PPD-specific IFN-γ-secreting cells by a factor of 4.7 for donor 1 (1970 ± 150.0

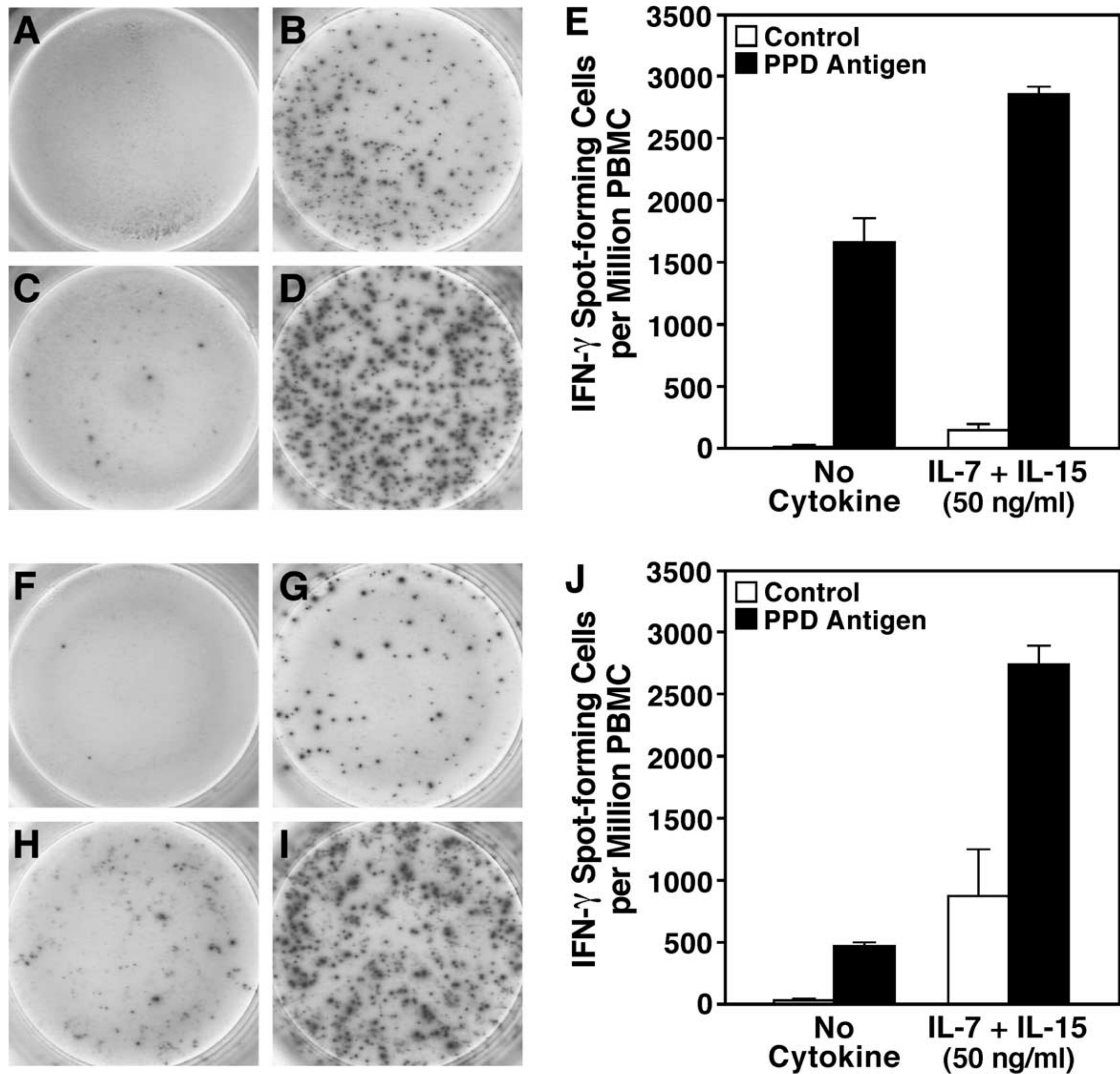

Fig. 1. ELISPOT detection of PPD-specific CD4+ T cell responses in fresh and cryopreserved PBMC from donor 1 with addition of both IL-7 and IL-15. For fresh PBMC (A–E), pictures are shown from ELISPOT wells containing 2×10^5 PBMC stimulated with medium alone (A), 10 μg/ml of PPD (B), medium supplemented with 50 ng/ml of IL-7 and IL-15 (C) and 10 μg/ml of PPD supplemented with 50 ng/ml of IL-7 and IL-15 (D). (E) shows averaged values from duplicate wells normalised to numbers of IFN-γ spotforming cells per 1×10^6 PBMC for the different stimulation conditions; error bars represent the standard error of the mean. For cryopreserved PBMC (F–J), idem.

vs. 420 ± 27.5, Fig. 1J) and by a factor of 18 for donor 2 (980 ± 242.5 vs. 55 ± 25.0, data not shown), restoring these responses to levels near those observed in fresh PBMC. The combination of IL-7 and IL-15 also enhanced spontaneous IFN-γ release by cryopreserved PBMC in medium control wells, by a factor of 77 (765 ± 362.5 vs. 10 ± 2.5, Fig. 1J) for donor 1 and by a factor of 38 (190 ± 72.5 vs. 5 ± 5.0, data not shown) for donor 2.

For both donors, PPD-specific IFN-γ-producing lymphocytes were CD4+ T cells as demonstrated by intracellular cytokine flow cytometry using monoclonal antibodies specific for IFN-γ and CD3, and CD4 cell depletion (data not shown).

3.2. Enhanced ELISPOT detection of CD8+ T cell responses in fresh PBMC by addition of IL-7 and IL-15

Frequencies of CD8+ T cells reactive against the HLA-A*0201-restricted immunodominant epitope from CMV matrix protein pp65 (NLVPMVATV) were analysed by ELISPOT in freshly isolated PBMC from an HLA-A*02-positive donor (donor 3). PBMC were incubated overnight in triplicate wells in the presence of media alone, CMV peptide, media supplemented with 50 ng/ml of IL-7 and/or IL-15, and CMV peptide supplemented with 50 ng/ml of IL-7 and/or IL-15 (Fig. 2, panels A–D).

Addition of both IL-7 and IL-15 to CMV peptide resulted in a 7.5-fold increase in the number of IFN-γ-secreting cells per million PBMC as compared to CMV peptide alone (mean ± S.E.M.: 2285 ± 675.5 vs. 305 ± 1.7, Fig. 2E). Addition of both IL-7 and IL-15 enhanced the spontaneous release of IFN-γ in medium control wells by a factor of 10 (200 ± 58.5 vs. 20 ± 1.7, Fig. 2E).

As opposed to the combined addition of the cytokines, the individual addition of IL-15 to CMV peptide only resulted in a marginal increase in the number of IFN-γ-secreting cells compared to CMV peptide alone (mean ± S.E.M.: 370 ± 117.6 vs. 305 ± 2.9, factor 1.2), while the addition of IL-7 individually did not have any effect at all (300 ± 70.1 vs. 305 ± 2.9, factor 0.98) (data not shown).

3.3. Combination of both IL-7 and IL-15 with co-stimulatory antibodies provides no additional enhancement

Addition of co-stimulatory antibodies anti-CD28 and anti-CD49d to PPD stimulation, in the absence of cytokines, resulted in increased numbers of PPD-specific IFN-γ-secreting cells compared to PPD stimulation alone, by a factor of 1.5 for fresh PBMC from donor 1 (mean ± S.E.M.: 2550 ± 70 vs. 1670 ± 155, Figs. 3A and 1E) and by a factor of 1.5 for cryopreserved PBMC from donor 1 (625 ± 120 vs. 420 ± 27.5, Figs. 3B and 1J). For fresh PBMC from

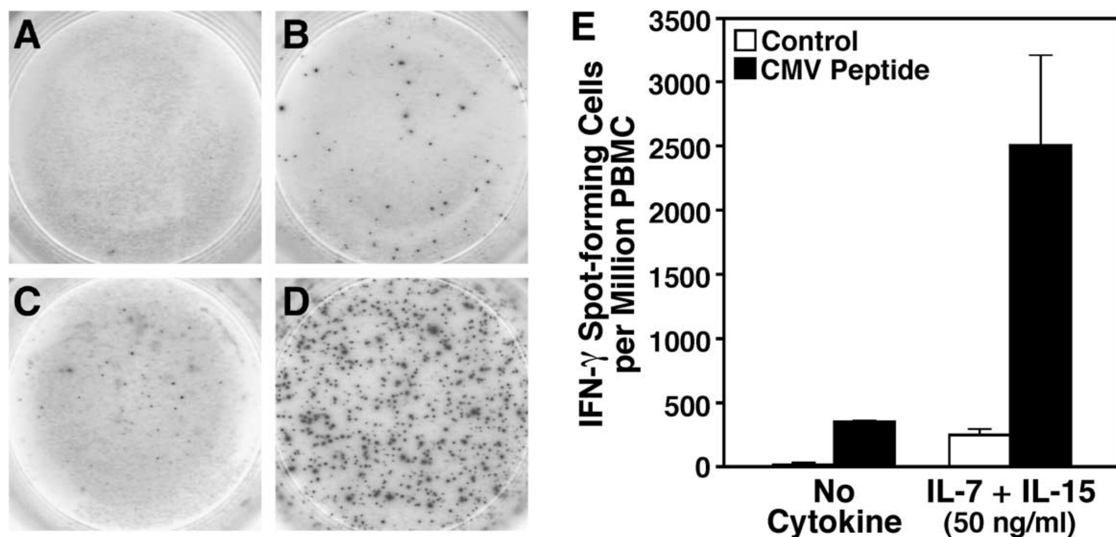

Fig. 2. ELISPOT detection of CD8+ T cell responses specific for an immunodominant HLA-A*0201-restricted epitope from CMV matrix protein pp65 in fresh PBMC from donor 3 with addition of both IL-7 and IL-15. Pictures are shown from ELISPOT wells containing 2×10^5 PBMC stimulated with medium alone (A), 10 μg/ml CMV peptide (B), medium supplemented with 50 ng/ml of IL-7 and IL-15 (C) and 10 μg/ml of CMV peptide supplemented with 50 ng/ml of IL-7 and IL-15 (D). Bars represent averaged values from triplicate wells normalised to numbers of IFN-γ spotforming cells per 1×10^6 PBMC for the different stimulation conditions; error bars represent the standard error of the mean (E).

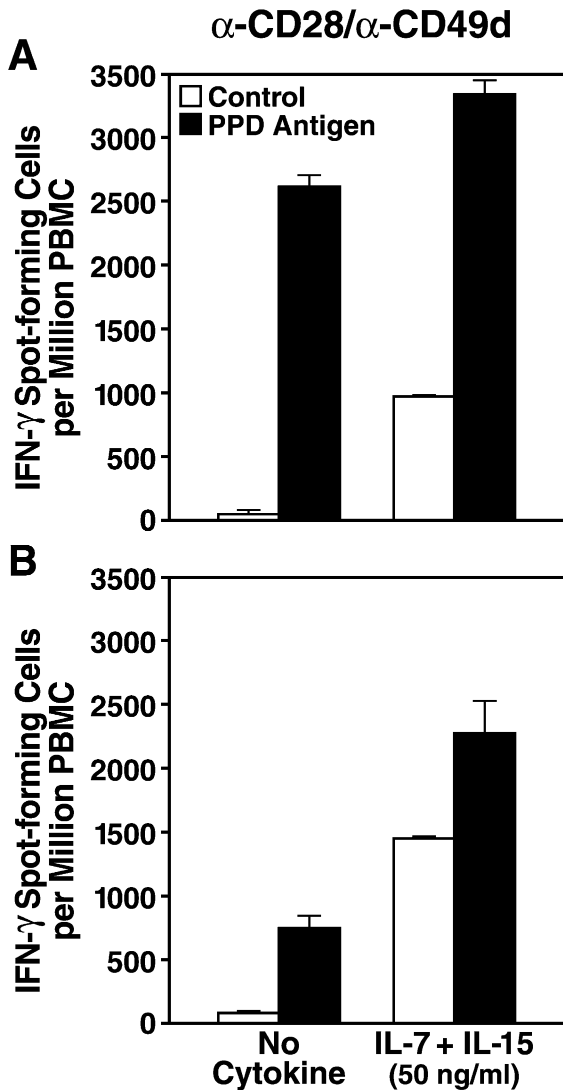

Fig. 3. Combination of IL-7 and IL-15 with antibodies to co-stimulatory molecules CD28 and CD49d in ELISPOT detection of PPD-specific CD4+ T cell responses in fresh (A) and cryopreserved (B) PBMC from donor 1. Bars represent averaged values from duplicate wells normalised to numbers of IFN-γ spotforming cells per 1×10^6 PBMC for the different stimulation conditions; error bars represent the standard error of the mean. Concentration of anti-CD28 and anti-CD49d antibodies was 1 μg/ml.

donor 1, the enhancement of PPD-specific IFN-γ secretion provided by co-stimulatory antibodies equalled that induced by IL-7 and IL-15 (factor 1.5 vs. 1.6), but was much lower for cryopreserved PBMC from donor 1 (factor 1.5 vs. 4.7). The same conclusions were valid for donor 2 (data not shown).

When both cytokines (IL-7 and IL-15) and co-stimulatory antibodies (anti-CD28 and anti-CD49d) were combined with PPD stimulation of PBMC from donor 1, no significant additive effects were observed. Although the total number of IFN-γ-secreting cells was increased compared to stimulation with PPD alone (2470 ± 107.5 vs. 1670 ± 155 for fresh PBMC, factor 1.5, Figs. 3A and 1E; 805 ± 270 vs. 420 ± 27.5 for cryopreserved PBMC, factor 1.9, Figs. 3B and 1J), the magnitude of the increase was comparable to or lower than that observed when cytokines or co-stimulatory antibodies were added individually to PPD. This effect was explained by a sharp increase in background release of IFN-γ in wells treated with both cytokines and co-stimulatory antibodies, as shown in medium control wells (935 ± 10 vs. 15 ± 7.5 for fresh PBMC, factor 62, Figs. 3A and 1E; 1340 ± 2.5 vs. 10 ± 2.5 for cryopreserved PBMC, factor 134, Figs. 3B and 1J). The same conclusions were valid for donor 2 (data not shown).

3.4. Optimal cytokine concentrations

The cytokines IL-7 and IL-15 were added to PPD in the ELISPOT assay using cryopreserved PBMC from donors 1 and 2, in concentrations ranging from 1 to 100 ng/ml for both cytokines. For donor 1, addition of IL-7 and IL-15 at a concentration as low as 1 ng/ml resulted in a 7.3-fold increase in the number of PPD-specific IFN-γ-secreting cells compared to PPD alone (mean ± S.E.M.: 1315 ± 107.6 vs. 180 ± 10, Fig. 4). The magnitude of enhancement was not significantly increased when higher concentrations of cytokines were added. The maximal enhancement observed was 7.9-fold, at IL-7 and IL-15 concentrations of 50 ng/ml (Fig. 4). However, augmenting the concentrations of IL-7 and IL-15 in medium control wells also resulted in increased spontaneous release of IFN-γ, thereby reducing the net enhancement of PPD-specific responses. Concentrations in the 1–5 ng/ml range appeared to provide maximal enhancement of antigen-specific responses with minimal increases in background spontaneous IFN-γ release. The same results were obtained for donor 2 (data not shown).

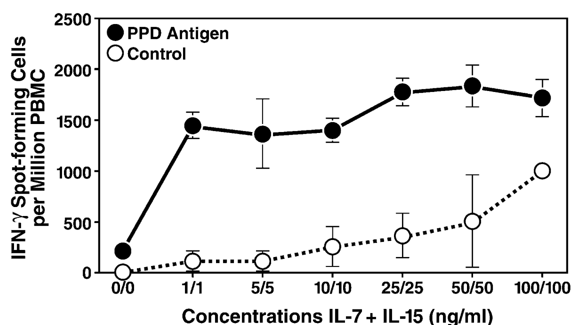

Fig. 4. Titration of cytokine concentrations. Cytokines IL-7 and IL-15 at concentrations ranging from 1 to 100 ng/ml were applied to ELISPOT detection of PPD-specific CD4+ T cell responses in cryopreserved PBMC from donor 1. Averaged values from duplicate wells normalised to numbers of IFN-γ spotforming cells per 1×10^6 PBMC are shown; error bars represent the standard error of the mean.

3.5. Induction of primary responses against HIV-1 peptides by addition of both IL-7 and IL-15

Frequencies of CD8+ T cells reactive against an HLA-A*0201-restricted human immunodeficiency virus type-1 (HIV-1) Gag peptide epitope (77–85, SLYNTVATL) and an HLA-B*35-restricted HIV-1 Pol peptide epitope (329–337, HPDIVIYQY), were analysed by ELISPOT in cryopreserved PBMC from two donors (donors 3 and 4) without known exposure to HIV. Both donors were HLA-A*02-positive, donor 3 was HLA-B*35-negative and donor 4 was HLA-B*35 positive. PBMC were incubated overnight in duplicate wells in the presence of media alone, HIV-1 peptides, media supplemented with 50 ng/ml of both IL-7 and IL-15, and HIV-1 peptides supplemented with 50 ng/ml of both IL-7 and IL-15.

In the absence of IL-7 and IL-15, neither donor mounted a response against the Gag peptide or the Pol peptide (mean ± S.E.M.: 15 ± 2.5 and 15 ± 2.5, respectively, for donor 3; 25 ± 15 and 15 ± 5, respectively, for donor 4). In the presence of IL-7 and IL-15, however, a significant increase was observed in the response of donor 3 against the Gag peptide (120 ± 5 vs. 15 ± 2.5, factor 8). No significant increases were seen for the Pol peptide in donor 3, or for either peptide in donor 4 (data not shown).

4. Discussion

In this report, we demonstrate that simultaneous addition of the cytokines IL-7 and IL-15 enhances detection of antigen-specific CD4+ and CD8+ T cells in the IFN-γ ELISPOT assay by factors ranging from 1.6- to 7.5-fold for fresh PBMC and 4.7- to 18-fold for cryopreserved PBMC. Antibodies to co-stimulatory molecules CD28 and CD49d applied together with the cytokine combination increased background IFN-γ release and did not result in additional enhancement. Optimal enhancement of antigen-specific responses with minimal increases in background IFN-γ release was provided at cytokine concentrations as low as 1 ng/ml.

This method may prove particularly useful for the quantification of antigen-specific CD4+ and CD8+ T cells in the setting of longitudinal clinical studies, in which only cryopreserved PBMC samples are available. Detection of antigen-specific cells by ELISPOT using cryopreserved PBMC samples has shown variable sensitivity (Larsson et al., 2002; Mwau et al., 2002). This is probably the result of decreased cell viability (Betensky et al., 2000; Weinberg et al., 2000), and the selective depletion of monocytes after freezing and thawing (Makino and Baba, 1997; Lund et al., 2001). The presence of viable antigen-presenting cells (i.e., monocytes and/or dendritic cells) is believed to be critical for efficient induction of antigen-specific T cell responses in ELISPOT assays (Schmittel et al., 2001; Larsson et al., 2002). In addition, the method described here may be relevant for subjects with low-level HIV-specific T cell responses that have been difficult to detect by standard methods. These subjects include individuals exposed to HIV but who remain uninfected (Kaul et al., 2000; Schmechel et al., 2001; Skurnick et al., 2002), HIV-infected patients on HAART with suppressed viral load and subsequent diminished T cell responses (Kalams et al., 1999; Kuhn et al., 2001; Larsson et al., 2002), and patients with very low CD4+ T cell counts resulting in antigen unresponsiveness in both CD4+ and CD8+ T cell subsets (Spiegel et al., 2000). This method will also be useful for measuring responses to experimental HIV vaccines. Recently, a phase III efficacy trial testing the canarypox-based ALVAC vCP1452 vaccine with gp120 boost was postponed because of low-level T cell

responses against HIV Env and Gag in the phase II trial (Bass and Jefferys, 2002). Finally, the method may be applied to detect T cell responses to other infectious agents (viral, bacterial or other), as well as tumor or "self" antigens, in instances where low-level responses may be overlooked by standard methods (Larsson et al., 2002).

Fresh and cryopreserved PBMC samples from all four donors included in the study showed elevated background IFN-γ release in the presence 50 ng/ml of both IL-7 and IL-15. In addition, 50 ng/ml of both IL-7 and IL-15 induced an apparently nonspecific response against the HIV-1 Gag peptide in donor 3, suggesting that primary in vitro responses may be generated in a proportion of donors by this cytokine combination. Accordingly, it will be important to establish a statistically valid range of positive and negative responses by testing irrelevant peptides in the presence of the cytokine combination in HIV-unexposed, seronegative individuals. However, for cryopreserved PBMC from two donors, it was shown that cytokine concentrations as low as 1 ng/ml can preserve the enhancement of antigen-specific responses in the absence of significant background IFN-γ release. Accordingly, the use of lower concentrations of cytokines (i.e., 1–5 ng/ml rather than 50 ng/ml, as shown in Fig. 4) might also prevent the amplification of primary responses.

The results presented in this study demonstrate that combined addition of the cytokines IL-7 and IL-15 to antigen-stimulated PBMC can significantly enhance IFN-γ production. Thus, the presence of these two cytokines significantly enhances the sensitivity of the ELISPOT assay. This method will be particularly useful for the assessment of antigen-stimulated cytokine production by CD4+ and CD8+ T cells in cryopreserved specimens and in subjects with low-level antigen-specific T cell responses that have been difficult to detect by standard methods.

Acknowledgements

We thank Einar Martin Aandahl and Patrick A. Haslett for performing cytokine flow cytometry analyses, Graham S. Ogg for providing peptides, John Carroll and Chris Goodfellow for preparing the figures, Guido van der Groen for helpful discussion. Funding for this research was provided by the National Institutes of Health (R21-AI47746 to B.L.S. and R01-AI46254 to D.F.N.). W.J. was supported by a research grant (G.0396.99) and a travel grant from the Nationaal Fonds voor Wetenschappelijk Onderzoek (NFWO)-Vlaanderen. D.F.N. is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation.

References

Bass, E., Jefferys, R., 2002. Warming trends at Keystone Vaccine Conference. IAVI Rep. 6 (2), 1.

Betensky, R.A., Connick, E., Devers, J., Landay, A.L., Nokta, M., Plaeger, S., Rosenblatt, H., Schmitz, J.L., Valentine, F., Wara, D., Weinberg, A., Lederman, H.M., 2000. Shipment impairs lymphocyte proliferative responses to microbial antigens. Clin. Diagn. Lab. Immunol. 7, 759.

Brown, K.A., Kriss, J.A., Moser, C.A., Wenner, W.J., Offit, P.A., 2000. Circulating rotavirus-specific antibody-secreting cells (ASCs) predict the presence of rotavirus-specific ASCs in the human small intestinal lamina propria. J. Infect. Dis. 182, 1039.

Czerkinsky, C.C., Nilsson, L.A., Nygren, H., Ouchterlony, O., Tarkowski, A., 1983. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. J. Immunol. Methods 65, 109.

Czerkinsky, C., Andersson, G., Ekre, H.P., Nilsson, L.A., Klareskog, L., Ouchterlony, O., 1988. Reverse ELISPOT assay for clonal analysis of cytokine production: I. Enumeration of gamma-interferon-secreting cells. J. Immunol. Methods 110, 29.

Elghazali, G., Perlmann, H., Rutta, A.S., Perlmann, P., Troye-Blomberg, M., 1997. Elevated plasma levels of IgE in *Plasmodium falciparum*-primed individuals reflect an increased ratio of IL-4 to interferon-gamma (IFN-gamma)-producing cells. Clin. Exp. Immunol. 109, 84.

Ernst, B., Lee, D.S., Chang, J.M., Sprent, J., Surh, C.D., 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. Immunity 11, 173.

Fiorillo, M.T., Maragno, M., Butler, R., Dupuis, M.L., Sorrentino, R., 2000. CD8(+) T-cell autoreactivity to an HLA-B27-restricted self-epitope correlates with ankylosing spondylitis. J. Clin. Invest. 106, 47.

Geginat, G., Schenk, S., Skoberne, M., Goebel, W., Hof, H., 2001a. A novel approach of direct ex vivo epitope mapping identifies dominant and subdominant CD4 and CD8 T cell epitopes from *Listeria monocytogenes*. J. Immunol. 166, 1877.

Geginat, J., Sallusto, F., Lanzavecchia, A., 2001b. Cytokine-driven proliferation and differentiation of human naive, central memory, and effector memory CD4(+) T cells. J. Exp. Med. 194, 1711.

Goldrath, A.W., Bevan, M.J., 1999. Low-affinity ligands for the TCR drive proliferation of mature CD8+ T cells in lymphopenic hosts. Immunity 11, 183.

Goldrath, A.W., Sivakumar, P.V., Glaccum, M., Kennedy, M.K.,

Bevan, M.J., Benoist, C., Mathis, D., Butz, E.A., 2002. Cytokine requirements for acute and Basal homeostatic proliferation of naive and memory CD8+ T cells. J. Exp. Med. 195, 1515.

Hasan, M.S., Kallas, E.G., Thomas, E.K., Looney, J., Campbell, M., Evans, T.G., 2000. Effects of interleukin-15 on in vitro human T cell proliferation and activation. J. Interferon Cytokine Res. 20, 119.

Hickman, C.J., Crim, J.A., Mostowski, H.S., Siegel, J.P., 1990. Regulation of human cytotoxic T lymphocyte development by IL-7. J. Immunol. 145, 2415.

Jager, E., Jager, D., Karbach, J., Chen, Y.T., Ritter, G., Nagata, Y., Gnjatic, S., Stockert, E., Arand, M., Old, L.J., Knuth, A., 2000. Identification of NY-ESO-1 epitopes presented by human histocompatibility antigen (HLA)-DRB4*0101-0103 and recognized by CD4(+) T lymphocytes of patients with NY-ESO-1-expressing melanoma. J. Exp. Med. 191, 625.

Kalams, S.A., Goulder, P.J., Shea, A.K., Jones, N.G., Trocha, A.K., Ogg, G.S., Walker, B.D., 1999. Levels of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte effector and memory responses decline after suppression of viremia with highly active antiretroviral therapy. J. Virol. 73, 6721.

Kanai, T., Thomas, E.K., Yasutomi, Y., Letvin, N.L., 1996. IL-15 stimulates the expansion of AIDS virus-specific CTL. J. Immunol. 157, 3681.

Kaul, R., Plummer, F.A., Kimani, J., Dong, T., Kiama, P., Rostron, T., Njagi, E., MacDonald, K.S., Bwayo, J.J., McMichael, A.J., Rowland-Jones, S.L., 2000. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. J. Immunol. 164, 1602.

King, C.L., Low, C.C., Nutman, T.B., 1993. IgE production in human helminth infection. Reciprocal interrelationship between IL-4 and IFN-gamma. J. Immunol. 150, 1873.

Kos, F.J., Mullbacher, A., 1993. IL-2-independent activity of IL-7 in the generation of secondary antigen-specific cytotoxic T cell responses in vitro. J. Immunol. 150, 387.

Kouwenhoven, M., Ozenci, V., Teleshova, N., Hussein, Y., Huang, Y.M., Eusebio, A., Link, H., 2001. Enzyme-linked immunospot assays provide a sensitive tool for detection of cytokine secretion by monocytes. Clin. Diagn. Lab. Immunol. 8, 1248.

Ku, C.C., Murakami, M., Sakamoto, A., Kappler, J., Marrack, P., 2000. Control of homeostasis of CD8+ memory T cells by opposing cytokines. Science 288, 675.

Kuhn, L., Meddows-Taylor, S., Gray, G., Trabattoni, D., Clerici, M., Shearer, G.M., Tiemessen, C., 2001. Reduced HIV-stimulated T-helper cell reactivity in cord blood with short-course antiretroviral treatment for prevention of maternal–infant transmission. Clin. Exp. Immunol. 123, 443.

Kuniyoshi, J.S., Kuniyoshi, C.J., Lim, A.M., Wang, F.Y., Bade, E.R., Lau, R., Thomas, E.K., Weber, J.S., 1999. Dendritic cell secretion of IL-15 is induced by recombinant huCD40LT and augments the stimulation of antigen-specific cytolytic T cells. Cell. Immunol. 193, 48.

Lalvani, A., Dong, T., Ogg, G., Patham, A.A., Newell, H., Hill, A.V., McMichael, A.J., Rowland-Jones, S., 1997. Optimization of a peptide-based protocol employing IL-7 for in vitro restimulation of human cytotoxic T lymphocyte precursors. J. Immunol. Methods 210, 65.

Larsson, M., Jin, X., Ramratnam, B., Ogg, G.S., Engelmayer, J., Demoitie, M.A., McMichael, A.J., Cox, W.I., Steinman, R.M., Nixon, D., Bhardwaj, N., 1999. A recombinant vaccinia virus based ELISPOT assay detects high frequencies of Pol-specific CD8 T cells in HIV-1-positive individuals. AIDS 13, 767.

Larsson, M., Wilkens, D.T., Fonteneau, J.F., Beadle, T.J., Merritt, M.J., Kost, R.G., Haslett, P.A., Cu-Uvin, S., Bhardwaj, N., Nixon, D.F., Shacklett, B.L., 2002. Amplification of low-frequency antiviral CD8 T cell responses using autologous dendritic cells. AIDS 16, 171.

Lau, L.L., Jamieson, B.D., Somasundaram, T., Ahmed, R., 1994. Cytotoxic T-cell memory without antigen. Nature 369, 648.

Lund, P.K., Westvik, A.B., Joo, G.B., Ovstebo, R., Haug, K.B., Kierulf, P., 2001. Flow cytometric evaluation of apoptosis, necrosis and recovery when culturing monocytes. J. Immunol. Methods 252, 45.

Makino, M., Baba, M., 1997. A cryopreservation method of human peripheral blood mononuclear cells for efficient production of dendritic cells. Scand. J. Immunol. 45, 618.

Mattei, F., Schiavoni, G., Belardelli, F., Tough, D.F., 2001. IL-15 is expressed by dendritic cells in response to type I IFN, double-stranded RNA, or lipopolysaccharide and promotes dendritic cell activation. J. Immunol. 167, 1179.

Miyahira, Y., Murata, K., Rodriguez, D., Rodriguez, J.R., Esteban, M., Rodrigues, M.M., Zavala, F., 1995. Quantification of antigen specific CD8+ T cells using an ELISPOT assay. J. Immunol. Methods 181, 45.

Mohamadzadeh, M., Berard, F., Essert, G., Chalouni, C., Pulendran, B., Davoust, J., Bridges, G., Palucka, A.K., Banchereau, J., 2001. Interleukin 15 skews monocyte differentiation into dendritic cells with features of Langerhans cells. J. Exp. Med. 194, 1013.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D.J., Zajac, A.J., Miller, J.D., Slansky, J., Ahmed, R., 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity 8, 177.

Murali-Krishna, K., Lau, L.L., Sambhara, S., Lemonnier, F., Altman, J., Ahmed, R., 1999. Persistence of memory CD8 T cells in MHC class I-deficient mice. Science 286, 1377.

Mwau, M., McMichael, A.J., Hanke, T., 2002. Design and validation of an enzyme-linked immunospot assay for use in clinical trials of candidate HIV vaccines. AIDS Res. Hum. Retrovir. 18, 611.

Nagorsen, D., Keilholz, U., Rivoltini, L., Schmittel, A., Letsch, A., Asemissen, A.M., Berger, G., Buhr, H.J., Thiel, E., Scheibenbogen, C., 2000. Natural T-cell response against MHC class I epitopes of epithelial cell adhesion molecule, her-2/neu, and carcinoembryonic antigen in patients with colorectal cancer. Cancer Res. 60, 4850.

Nakajima, H., Shores, E.W., Noguchi, M., Leonard, W.J., 1997. The common cytokine receptor gamma chain plays an essential role in regulating lymphoid homeostasis. J. Exp. Med. 185, 189.

Napolitano, L.A., Grant, R.M., Deeks, S.G., Schmidt, D., De Rosa, S.C., Herzenberg, L.A., Herndier, B.G., Andersson, J., McCune, J.M., 2001. Increased production of IL-7 accompanies HIV-1-mediated T-cell depletion: implications for T-cell homeostasis. Nat. Med. 7, 73.

Niedbala, W., Wei, X., Liew, F.Y., 2002. IL-15 induces type 1 and type 2 CD4+ and CD8+ T cells proliferation but is unable to drive cytokine production in the absence of TCR activation or IL-12/IL-4 stimulation in vitro. Eur. J. Immunol. 32, 341.

Ostrowski, M.A., Gu, J.X., Kovacs, C., Freedman, J., Luscher, M.A., MacDonald, K.S., 2001. Quantitative and qualitative assessment of human immunodeficiency virus type 1 (HIV-1)-specific CD4+ T cell immunity to gag in HIV-1-infected individuals with differential disease progression: reciprocal interferon-gamma and interleukin-10 responses. J. Infect. Dis. 184, 1268.

Ozenci, V., Kouwenhoven, M., Press, R., Link, H., Huang, Y.M., 2000. IL-12 elispot assays to detect and enumerate IL-12 secreting cells. Cytokine 12, 1218.

Pelfrey, C.M., Rudick, R.A., Cotleur, A.C., Lee, J.C., Tary-Lehmann, M., Lehmann, P.V., 2000. Quantification of self-recognition in multiple sclerosis by single-cell analysis of cytokine production. J. Immunol. 165, 1641.

Reece, W.H., Plebanski, M., Akinwunmi, P., Gothard, P., Flanagan, K.L., Lee, E.A., Cortina-Borja, M., Hill, A.V., Pinder, M., 2002. Naturally exposed populations differ in their T1 and T2 responses to the circumsporozoite protein of *Plasmodium falciparum*. Infect. Immun. 70, 1468.

Rininsland, F.H., Helms, T., Asaad, R.J., Boehm, B.O., Tary-Lehmann, M., 2000. Granzyme B ELISPOT assay for ex vivo measurements of T cell immunity. J. Immunol. Methods 240, 143.

Schluns, K.S., Kieper, W.C., Jameson, S.C., Lefrancois, L., 2000. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat. Immunol. 1, 426.

Schmechel, S.C., Russell, N., Hladik, F., Lang, J., Wilson, A., Ha, R., Desbien, A., McElrath, M.J., 2001. Immune defence against HIV-1 infection in HIV-1-exposed seronegative persons. Immunol. Lett. 79, 21.

Schmittel, A., Keilholz, U., Bauer, S., Kuhne, U., Stevanovic, S., Thiel, E., Scheibenbogen, C., 2001. Application of the IFN-gamma ELISPOT assay to quantify T cell responses against proteins. J. Immunol. Methods 247, 17.

Schmitz, M., Rohayem, J., Paul, R., Weigle, B., Stein, A., Rieber, E.P., 2002. Quantification of antigen-reactive T cells by a modified ELISPOT assay based on freshly isolated blood dendritic cells. J. Clin. Lab. Anal. 16, 30.

Skurnick, J.H., Palumbo, P., DeVico, A., Shacklett, B.L., Valentine, F.T., Merges, M., Kamin-Lewis, R., Mestecky, J., Denny, T., Lewis, G.K., Lloyd, J., Praschunus, R., Baker, A., Nixon, D.F., Stranford, S., Gallo, R., Vermund, S.H., Louria, D.B., 2002. Correlates of nontransmission in US women at high risk of human immunodeficiency virus type 1 infection through sexual exposure. J. Infect. Dis. 185, 428.

Smith, S.M., Brookes, R., Klein, M.R., Malin, A.S., Lukey, P.T., King, A.S., Ogg, G.S., Hill, A.V., Dockrell, H.M., 2000. Human CD8+ CTL specific for the mycobacterial major secreted antigen 85A. J. Immunol. 165, 7088.

Spiegel, H.M., Ogg, G.S., DeFalcon, E., Sheehy, M.E., Monard, S., Haslett, P.A., Gillespie, G., Donahoe, S.M., Pollack, H., Borkowsky, W., McMichael, A.J., Nixon, D.F., 2000. Human immunodeficiency virus type 1- and cytomegalovirus-specific cytotoxic T lymphocytes can persist at high frequency for prolonged periods in the absence of circulating peripheral CD4(+) T cells. J. Virol. 74, 1018.

Subklewe, M., Chahroudi, A., Bickham, K., Larsson, M., Kurilla, M.G., Bhardwaj, N., Steinman, R.M., 1999. Presentation of epstein – barr virus latency antigens to CD8(+), interferon-gamma-secreting, T lymphocytes. Eur. J. Immunol. 29, 3995.

Tan, L.C., Gudgeon, N., Annels, N.E., Hansasuta, P., O'Callaghan, C.A., Rowland-Jones, S., McMichael, A.J., Rickinson, A.B., Callan, M.F., 1999. A re-evaluation of the frequency of CD8+ T cells specific for EBV in healthy virus carriers. J. Immunol. 162, 1827.

Tan, J.T., Ernst, B., Kieper, W.C., LeRoy, E., Sprent, J., Surh, C.D., 2002. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8(+) cells but are not required for memory phenotype CD4(+) cells. J. Exp. Med. 195, 1523.

Weinberg, A., Zhang, L., Brown, D., Erice, A., Polsky, B., Hirsch, M.S., Owens, S., Lamb, K., 2000. Viability and functional activity of cryopreserved mononuclear cells. Clin. Diagn. Lab. Immunol. 7, 714.

Welch, P.A., Namen, A.E., Goodwin, R.G., Armitage, R., Cooper, M.D., 1989. Human IL-7: a novel T cell growth factor. J. Immunol. 143, 3562.

Yajima, T., Nishimura, H., Ishimitsu, R., Watase, T., Busch, D.H., Pamer, E.G., Kuwano, H., Yoshikai, Y., 2002. Overexpression of IL-15 in vivo increases antigen-driven memory CD8+ T cells following a microbe exposure. J. Immunol. 168, 1198.