

# Serum-free culture medium and IL-7 costimulation increase the sensitivity of ELISpot detection

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## Abstract

The identification of parameters maximizing detection sensitivity in ELISpot assays is important to transfer this technology into the clinical setting for identifying rare Ag-specific CD8<sup>+</sup> T cells. We have therefore considered human IFN- $\gamma$  CD8<sup>+</sup> T cell responses against viral epitopes to analyze different variables which could be critical during the epitope-specific stimulation period. Two parameters were found to greatly enhance detection sensitivity (i.e., to specifically increase epitope-driven signal while keeping background noise to a minimum): use of human serum-free vs. serum-supplemented culture medium (2.4-fold median increase) and addition of low dose IL-7 (1.5-fold increase). Incorporating both of these parameters into the ELISpot procedure proved capable of greatly amplifying (35.1-fold increase) the low grade CD8<sup>+</sup> T cell responses directed against  $\beta$ -cell epitopes of type 1 diabetes patients, as compared to a previously optimized procedure using human serum-supplemented medium and low dose IL-2. Implementation of this ELISpot procedure should expedite development of "immune staging" protocols for autoimmune as well as tumor and infectious diseases.

**MESH Keywords** Adult ; Aged ; Antibodies ; Monoclonal ; pharmacology ; Antigens ; CD28 ; immunology ; CD8-Positive T-Lymphocytes ; immunology ; Cell Culture Techniques ; methods ; Culture Media ; Serum-Free ; Diabetes Mellitus ; Type 1 ; immunology ; Enzyme-Linked Immunosorbent Assay ; methods ; Epitopes ; T-Lymphocyte ; analysis ; immunology ; Female ; HLA-A2 Antigen ; immunology ; Humans ; Insulin-Secreting Cells ; immunology ; Interferon-gamma ; immunology ; Interleukin-2 ; immunology ; pharmacology ; Interleukin-7 ; immunology ; pharmacology ; Male ; Middle Aged ; Statistics ; Nonparametric

## Introduction

Detection and quantification of antigen-specific T cell responses has represented a milestone advance in the immunology field. Not only it has allowed to address fundamental questions about the dynamics of T cell responses, but it has also offered key clinical applications for infectious, tumoral and autoimmune diseases.

Of the two T cell arms of adaptive immunity, CD8<sup>+</sup> T cells have received more attention due to their well established role in mounting immune responses against viral and cancerous threats. Not denying the contribution of CD4<sup>+</sup> T cells in these settings (Perez-Diez et al., 2007), progress with their study has lagged behind due to several technical limitations, including more scattered HLA Class II distribution (Nepom, 1995), more difficult epitope identification (Kwok et al., 2001), troublesome HLA Class II tetramer production (Mallone and Nepom, 2004) and lower Ag-specific T cell frequencies (Homann et al., 2001). Furthermore, more recent studies in type 1 diabetes (T1D) (Amrani et al., 2000; Toma et al., 2005; Mallone et al., 2007) and multiple sclerosis (Crawford et al., 2004) have highlighted the fundamental contribution of CD8<sup>+</sup> T cells in autoimmunity (Liblau et al., 2002; Walter and Santamaria, 2005), thus challenging the paradigm depicting these diseases as predominantly CD4<sup>+</sup> T helper 1-mediated.

Among the various techniques which can be used to detect CD8<sup>+</sup> T cell responses, two main strategies share the mainstream. On one side, flow cytometry-based technique allow for direct visualization of Ag-specific cells by a variety of structural (i.e., peptide-HLA tetramer binding) or functional (e.g. IFN- $\gamma$  secretion, CD107a/b upregulation) readouts (Kern et al., 2005). On the other side, ELISpot-based techniques give an indirect detection of Ag-specific T cells by means of their cytokine (mostly IFN- $\gamma$ ) secretion, but are endowed with at least a 10-fold higher sensitivity, as they can detect as few as 0.001% Ag-specific cells (Meierhoff et al., 2002).

The detection sensitivity reached by ELISpot is particularly useful in the autoimmune context, since autoreactive T cell responses are typically of much lower grade (i.e., lower intensity and precursor frequency) than those found in the viral and tumor setting (Mallone and Nepom, 2005). For example, our recent ELISpot studies on T1D patients vs. healthy controls indicate that the  $\beta$ -cell epitope-specific CD8<sup>+</sup> T cell fractions which can be measured in peripheral blood are in the order of 0.004% of total PBMCs (Mallone et al., 2007). Optimization

of current ELISpot protocols is therefore highly needed to maximize the chances of detecting such rare events and to eventually reach clinical application. Such optimization should aim at obtaining the highest specific signal, while keeping the background noise of the system to a minimum. We here address this issue by considering different costimulatory parameters during the ELISpot culture incubation, in order to achieve preferential amplification of the epitope-specific T cell responses.

## Materials and methods

### Study subjects

Eight HLA-A2<sup>+</sup> (HLA-A\*0201) healthy donors (Table I) were selected by FACS screening of lysed whole blood with the anti-HLA-A2 mAb BB7.2. Subsequent genotyping was performed using the Olerup SSP HLA\*02 kit (GenoVision/Qiagen, Vienna, Austria). All subjects gave written informed consent and the study was approved by the Local Ethics Committee. PBMCs were isolated by density gradient centrifugation using lymphocyte separation medium (PAA, Les Mureaux, France), and immediately used or stored frozen (10% DMSO in pooled human male AB serum). In selected experiments, immunomagnetically CD4-depleted or CD8-enriched (>90% pure) PBMCs were prepared using Miltenyi CD4 positive and CD8 negative selection kits, respectively (Miltenyi Biotec, Paris, France).

To test detection of  $\beta$ -cell-specific CD8<sup>+</sup> T cell responses in T1D vs. healthy subjects, PBMCs were obtained from three adult (29-, 40- and 63-yo) HLA-A2<sup>+</sup> male patients with new onset T1D (1, 5 and 30 days after diagnosis, respectively) positive for anti-GAD antibodies. T1D diagnosis was formulated according to consensus criteria (American Diabetes Association, 2006).

### Peptides

The following HLA-A2-restricted viral peptides (>80% pure; Schafer-N, Copenhagen, Denmark) were used: Flu matrix protein (MP)<sub>58-66</sub> (GILGFVFTL), cytomegalovirus (CMV) pp65<sub>495-503</sub> (NLVPMVATV) and Epstein-Barr virus (EBV) BMLF1<sub>280-288</sub> (GLCTLVAML). Negative controls were HIV gag<sub>77-85</sub> (SLYNTVATL) and DMSO diluent alone. A PHA positive control (1  $\mu$ g/ml; Sigma, Lyon, France) was included in all experiments. The response of each subject to these peptides (Flu, CMV, EBV or a pool of the three) was titrated in preliminary experiments to identify a suboptimal concentration for each donor giving a positive response (> basal +3SD, see below), but with low numbers of epitope-specific spots (<100 spot-forming cells/10<sup>6</sup> PBMCs after basal subtraction). This suboptimal concentration was subsequently used in all experiments.

The immunodominant  $\beta$ -cell epitopes proinsulin (PI)<sub>A12-20</sub>, glutamic acid decarboxylase (GAD)<sub>114-123</sub>, insulinoma-associated protein 2 (IA-2)<sub>206-214</sub> and islet glucose-6-phosphatase catalytic subunit related protein (IGRP)<sub>265-273</sub> were previously described (Mallone et al., 2007; Blancou et al., 2007; Takaki et al., 2006).

### mAbs, cytokines and culture media

Anti-CD28 mAb (clone CD28.2) was purchased from BD Pharmingen (Le Pont de Claix, France). Recombinant human IL-2 and IL-7 were from R&D Systems (Lille, France). RPMI, AIM-V (both from Invitrogen, Cergy Pontoise, France) or X-VIVO 15 culture medium (BioWhittaker-Lonza, Levallois-Perret, France) were used for ELISpot assays, as specified for each experiment.

### ELISpot

Ninety-six well PVDF plates (Millipore, Saint-Quentin-en-Yvelines, France) were coated overnight with an anti-IFN- $\gamma$  Ab (U-CyTech, Utrecht, The Netherlands). After blocking with RPMI + 10% human serum (HS; PAA) for 1 h at 37°C, peptides were distributed into wells at the suboptimal concentrations (0.5–500 nM) previously determined for each donor, along with the appropriate co-stimuli, as detailed for each experiment. PBMCs were seeded in triplicates at 3 $\times$ 10<sup>5</sup> cells/well and cultured for 20–24 h. Following PBMC removal, IFN- $\gamma$  secretion was visualized with a biotin-conjugated anti-IFN- $\gamma$  mAb (U-CyTech), alkaline phosphatase-conjugated ExtrAvidin and Sigmafast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) tablets (both from Sigma). Spots were counted using an AID reader (Strassberg, Germany), means of triplicate wells calculated and results expressed as spot-forming cells (SFC)/10<sup>6</sup> PBMCs after background subtraction. The cut-off for a positive response was set at 3SD above the average basal reactivity (i.e., reactivity against HIV gag<sub>77-85</sub> and DMSO diluent alone). This was chosen as the cut-off allowing for the best diagnostic sensitivity (i.e., highest number of positive responses to  $\beta$ -cell epitopes in T1D patients) and specificity (i.e., lowest number of positive responses in healthy controls), as determined by receiver-operator characteristics (ROC) analysis (Mallone et al., 2007). The variability of our ELISpot assay is of 14.1% intra-assay, 4.2% at the analytical inter-assay level (i.e., using thawed PBMC samples frozen on the same occasion) and 9.2% at the pre-analytical and analytical level (i.e., using separate blood draws from the same donor) (Mallone et al., 2007). Coefficients of variation between fresh and frozen samples are typically <10%.

### Statistical analysis

All graphs are displayed as means of triplicate wells  $\pm$ SEM. Since spot numbers are normally distributed within each triplicate, comparisons of means within the same donor were carried out with two-tailed Student's t test, while the non-parametric Wilcoxon matched pairs test was used for comparing two ELISpot conditions across the whole cohort of donors. Analysis of variance (ANOVA) was used for comparisons among multiple groups.  $P < 0.05$  was considered to be of statistical significance.

## Results

### Titration of epitope-specific responses

In order to better visualize the influence of different culture conditions on the ELISpot performance, we preliminarily titrated the reactivity of each donor to one viral epitope chosen among Influenza, CMV or EBV ones, or to a pool of the three (Tab. I). We thus identified for each subject a suboptimal (0.5–500 nM) peptide concentration achieving a positive response but with no more than 100 SFC/ $10^6$  PBMCs when tested without cytokine addition and in RPMI+ 10% HS (Fig. 1A). As we plate  $3 \times 10^5$  PBMCs/well, this corresponds to  $<30$  spots counted in each well before normalization to  $10^6$  PBMCs, a number which avoids counting inaccuracies due to excessive spot densities. All experiments (Fig. 1 to 5) were subsequently performed with the indicated peptide(s) and suboptimal concentrations specific to each donor (Tab. I).

### Culture media

As differences in human serum (HS) batches may affect the reproducibility of the ELISpot technique, we first investigated whether substituting HS-supplemented RPMI with AIM-V medium would improve assay performance. As shown in Fig. 1A, AIM-V performed consistently better than HS-supplemented RPMI, as all but one donor displayed increased responses with this latter culture condition. The median increase in specific signal was of 2.4-fold (range 1 – 6.4-fold;  $P < 0.03$  by Wilcoxon test), while background noise was low in all instances ( $<15$  SFC/ $10^6$  PBMCs).

The lower specific signals obtained with HS-supplemented medium were not due to poor performance of the particular HS batch used, as different lots – either produced locally or commercially available – gave similar results (Fig. 1B). However, the absence or presence of HS did not account for the difference between AIM-V and RPMI-HS, as HS-supplemented and non-supplemented AIM-V did not differ significantly in their effect (Fig. 1C). A second HS-free synthetic medium (i.e., X-VIVO 15) gave similar results, i.e., it performed as well as AIM-V, better than HS-supplemented RPMI, and not differently with and without HS supplementation (Fig. 1C).

The AIM-V also performed better than HS when used as a freezing medium (10% DMSO/90% AIM-V or HS). At thawing, the specific signal did not differ significantly (not shown), but the AIM-V freezing medium allowed an average recovery of 17% more cells ( $P < 0.04$ ; Fig. 1D).

### Anti-CD28 costimulation

We next tested the effect of adding an anti-CD28 mAb to the ELISpot culture, as it has previously been reported that such costimulation increases detection sensitivity (Ott et al., 2004). However, we did not observe any beneficial effect (Fig. 2): addition of anti-CD28 mAb (1  $\mu$ g/ml) to AIM-V medium marginally increased signal detection in only 1 of the 4 donors tested (39.7% increase;  $P = 0.29$ ). This was moreover obtained at the expense of a much higher background. Even in those subjects where the specific signal was not improved, the increase in noise was quite important (median increase 7.3-fold; range 1 – 13.3;  $P < 0.03$  by Student's t test for 3 of 4 donors).

### IL-2 costimulation

We have previously reported that addition of low dose (0.5 U/ml) IL-2 leads to a small but consistent improvement in ELISpot sensitivity, as assessed by measuring low grade CD8<sup>+</sup> T cell responses against  $\beta$ -cell autoantigens in T1D patients (Mallone et al., 2007; Blancou et al., 2007). This was indeed still the case when HS-supplemented RPMI medium was enriched with low dose IL-2 as per our previous protocol to detect viral epitope-specific responses (Fig. 3; median signal increase 2.3-fold, range 1.1 – 3.8;  $P < 0.06$  by Wilcoxon test). However, when this IL-2 supplement was added to the HS-free AIM-V medium, this improvement was no longer observed (Fig. 4). Rather, a marginal increase in specific signal (median 1.05-fold, range 0.52 – 1.4-fold;  $P = 0.56$ ) was counterbalanced by a larger increase in background noise (median 2.4-fold, range 0.83 – 3.6-fold;  $P = 0.16$ ). These increases were even more important when higher IL-2 doses were used. IL-2 doses lower than 0.5 U/ml did not induce any significant effect as compared to unsupplemented HS-free AIM-V (not shown).

### IL-7 costimulation

As IL-7 is thought to be an important co-stimulation factor for memory T cells (Bielekova et al., 1999), we hypothesized that it could also have a more beneficial effect than IL-2 on the ELISpot-detected signal. Indeed, low dose (0.5 ng/ml) IL-7 induced a small yet highly reproducible increase in specific signal, while increasing the basal levels of reactivity only marginally (Fig. 5A). The median increase in

net signal was of 50% (range 20 – 250%;  $P < 0.03$  by Wilcoxon test), while background noise was unaffected in most cases (median increase 0%, range -11 – 63.9%;  $P = 0.31$ ). Higher IL-7 doses (1–5 ng/ml) only gave a marginal increase in specific signal (median 21.7 and 7.6%, respectively;  $P = 0.22$  and  $0.44$ ), while the increase in background level was much higher (median 49.1 and 267.0%;  $P = 0.06$  and  $0.03$ ). IL-7 supplements lower than 0.5 ng/ml did not have any effect as compared to unsupplemented AIM-V (not shown). The effect of low dose IL-7 was exerted on CD8<sup>+</sup> T cells, as it was still present when CD4-depleted PBMCs or purified CD8<sup>+</sup> cells were used (Fig. 5B).

### **IL-7-supplemented AIM-V medium allows for easier detection of low grade autoimmune CD8<sup>+</sup> responses**

Finally, we looked at whether this optimized protocol was suitable to detect the low grade CD8<sup>+</sup> T cell responses which characterize autoimmune diseases such as T1D. Indeed, use of either AIM-V medium or of IL-7 costimulation increased the specific signal obtained upon challenging PBMCs from a T1D patient with the HLA-A2-restricted GAD<sub>114–123</sub> epitope (Fig. 6A). Interestingly, use of both AIM-V and IL-7 achieved a synergistic effect, with a 13-fold increase in signal as compared to HS-supplemented RPMI ( $P < 0.04$ ).

HLA-A2<sup>+</sup> T1D patients and healthy controls were subsequently compared for their responses against immunodominant  $\beta$ -cell epitopes by using either our previously described protocol (HS-supplemented RPMI + IL-2) (Mallone et al., 2007) or the newly optimized condition with HS-free AIM-V and IL-7 (Fig. 6B). Such responses were detected in T1D but not in healthy subjects, as previously reported (Mallone et al., 2007). Some rise in the background levels was observed with IL-7-supplemented AIM-V in 4 of 6 cases, but it was overall not statistically significant (median increase 2.6-fold; range 0.34 – 13.0-fold;  $P = 0.44$  by Wilcoxon test). More importantly, this rise was outweighed by a much higher amplification of the  $\beta$ -cell epitope-specific responses in 4 of 4 cases (median increase 35.1-fold; range 3.6 – 162.2;  $P < 0.001$ ). Of these 4 responses, 3 which would have remained undetected with the IL-2 protocol (being lower than the basal mean + 3SD cut-off) were instead ranked as positive with AIM-V + IL-7. Of note, this amplification effect was specific, as the  $\beta$ -cell-directed responses of healthy subjects did not rise significantly in 91.7% (11/12) of cases, and remained negative in all instances.

## **Discussion**

We provide evidence that optimized culture and costimulation conditions during ELISpot incubation greatly improve epitope-specific stimulation. Small increases in background levels were outweighed by a much higher increase in specific signal. Two parameters were found to be critical: substitution of HS-supplemented RPMI with HS-free AIM-V medium; and addition of low dose (0.5 ng/ml) IL-7.

The higher efficacy of the AIM-V medium could have been due either to exclusion of HS from the culture condition or to the composition of the AIM-V medium itself. The former hypothesis was excluded, as addition of HS to AIM-V or X-VIVO did not bring a significant change. The latter hypothesis is therefore the most likely, but the proprietary formulation of AIM-V does not allow further speculations. However, excluding HS from the culture media brings the advantage of avoiding lot-to-lot variabilities.

The costimulatory effect of IL-7 was found to be quite selective on the epitope-specific fraction of interest, while only marginally increasing basal reactivity. Other cytokines previously found to increase detection sensitivity such as IL-15 (Jennes et al., 2002) did not have the same effect, as they greatly increased background noise, with little advantage on the net signal revealed (not shown). The advantage of IL-7 vs. IL-2 could be due to a preferential effect on the IL-7 receptor-positive (CD127<sup>+</sup>) effector/memory compartment as compared to the CD127<sup>-</sup> Treg subset (Liu et al., 2006; Seddiki et al., 2006). It has indeed been shown that exclusion of Treg cells from *in vitro* cultures eases detection of autoimmune responses for CD4<sup>+</sup> T cells (Danke et al., 2004), and the same is likely to be true for CD8<sup>+</sup> T cells (Piccirillo and Shevach, 2001). Alternatively, the IL-7 effect may be due to a preferential action on memory vs. naïve T cells (Bielekova et al., 1999; Welch et al., 1989; Surh et al., 2006; Melchionda et al., 2005; Tan et al., 2002). In a therapeutic perspective, it is this memory subset which contains the fraction of interest, i.e., T cells which have already encountered the Ag and are therefore more suitable to be boosted or quenched, depending on the clinical setting. Although the preferential action of IL-7 on memory vs. naïve T cells is a matter of debate (Bielekova et al., 1999; Tan et al., 2001), the preferential targeting of the memory compartment could be more effective for IL-7 than for IL-2 or anti-CD28 mAb. As naïve T cells are more dependent on costimulation (Viglietta et al., 2002) and in light of the importance of both CD28 and IL-2 signals for Treg biology (Tang et al., 2003), these two regimens may actually exert their effect in the opposite direction, preferentially boosting naïve or Treg responses rather than memory/effector ones. Both hypotheses (i.e., preferential activation of effector/memory vs. regulatory T cells or of memory vs. naïve T cells) are open, as the IL-7 effect was maintained when CD4-depleted PBMCs or purified CD8<sup>+</sup> T cells were used.

Our observation that anti-CD28 costimulation did not have any beneficial effect on ELISpot detection sensitivity is at variance with the report of Ott et al., who reached opposite conclusions (Ott et al., 2004). This may be due to the different culture medium used (AIM-V in our study vs. HS-supplemented RPMI in the quoted report). It is possible that the richer composition of the AIM-V medium makes T cells more sensitive to further costimulation. This was indeed our observation with regard to IL-2, which was effective when added to

HS-supplemented RPMI (Fig. 3), but much less with HS-free AIM-V due to background increase (Fig. 4). The culture medium used should therefore be taken into account when titrating costimulatory reagents. Keeping the noise of the system to a minimum is particularly important when looking at low grade T cell responses, as small changes can easily remain undetected over a high background.

The final advantage of this optimized stimulation scheme on the ELISpot detection sensitivity was shown by looking at  $\beta$ -cell epitope-specific CD8<sup>+</sup> responses in T1D patients vs. healthy controls. While maintaining specificity with regard to disease status, IL-7-supplemented HS-free AIM-V allowed for a ~35-fold median increase in the magnitude of the  $\beta$ -cell epitope-specific responses. Such an increase is unlikely to solely reflect an expansion of the epitope-specific precursors, given the short (24 h) stimulation period. It probably also reflects increased bystander activation, a mechanism with a much higher potential for amplifying T cell responses. It may be argued that such amplification does not anymore reflect the ex-vivo precursor frequency. However, the main scope of ELISpot techniques in the clinical setting – especially in the autoimmunity field – is to allow for disease monitoring, i.e., for early diagnosis, prognostic stratification and therapeutic follow-up (so called “immune staging”) (Mallone and Nepom, 2005). Such clinical applications rely on sensitive and quantitative detection of the relevant T cell responses rather than on precise counting of the actual T cell frequencies. The proposed ELISpot procedures should greatly facilitate this task.

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## Abbreviations

ANOVA: analysis of variance

CMV: cytomegalovirus

EBV: Epstein-Barr virus

GAD: glutamic acid decarboxylase

HS: human serum

IA-2: insulinoma-associated protein 2

IGRP: islet glucose-6-phosphatase catalytic subunit related protein

MP: matrix protein

PI: proinsulin

PPI: preproinsulin

SFC: spot-forming cells

T1D: type 1 diabetes

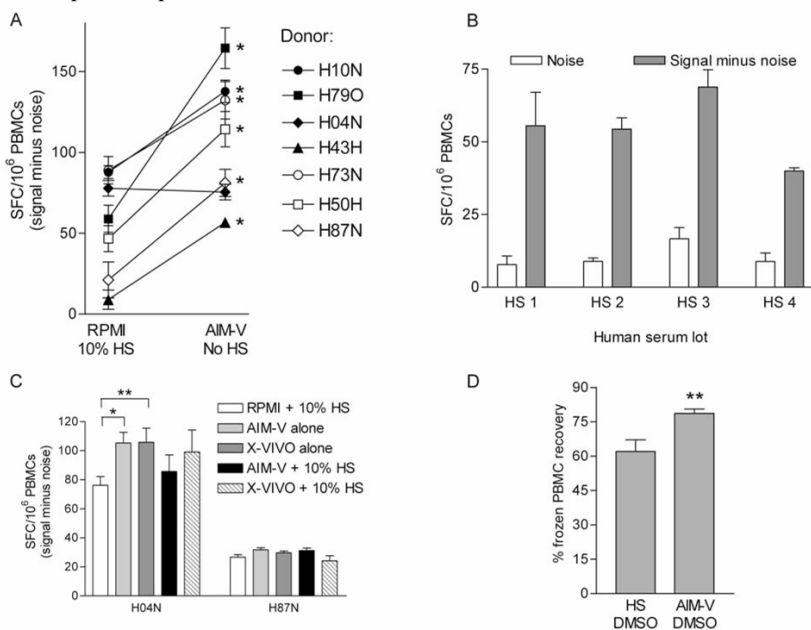
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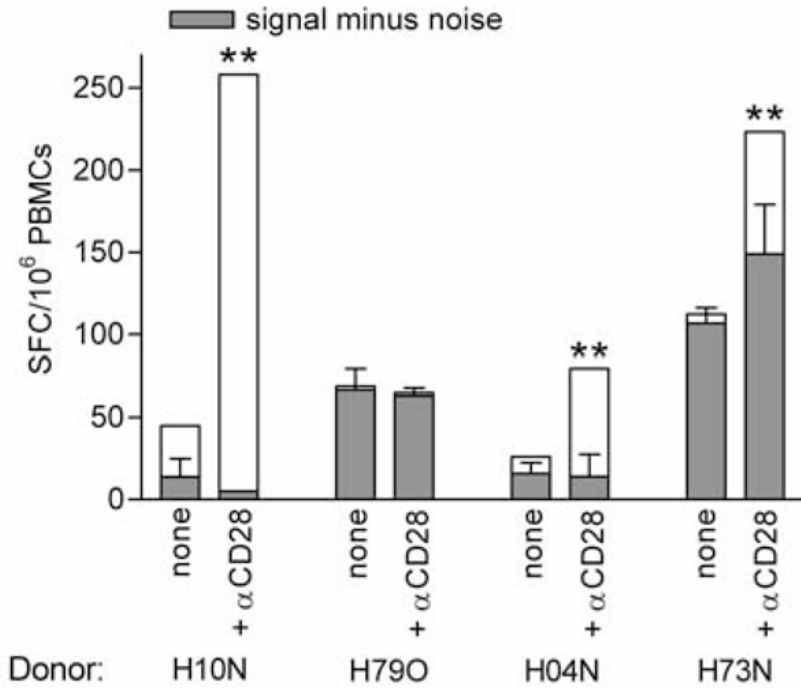
**Fig. 1**

(A) Basal-subtracted epitope-specific responses obtained by ELISpot using either RPMI + 10% HS (left) or HS-free AIM-V (right) as culture medium. Basal reactivities (responses against an HIV gag<sub>77-85</sub> control epitope and DMSO diluents alone) were <15 SFC/10<sup>6</sup> PBMCs in all instances and did not differ significantly between the two culture conditions. \*P<0.04 for the comparison between the two culture media by Student's t test (P<0.03 when comparing cumulative data for all donors by Wilcoxon test). (B) Epitope-specific responses measured by ELISpot using RPMI medium supplemented with different HS batches. The background-subtracted signal (grey bars) and the basal reactivity levels (white bars) are shown. Differences in background and in basal-subtracted signals among HS lots are not statistically significant, as assessed by ANOVA. HS 1 is the batch used for the experiments depicted in panel A and in all other experiments. Results refer to a representative experiment performed in duplicate. (C) Effect of different HS-free media and of HS supplementation on ELISpot responses. Two donors were tested with different media as indicated. \*P<0.02 by Student's t test (P<0.02 when comparing cumulative data for both donors by Wilcoxon test). \*\*P<0.05 (P=0.11 for cumulative data). (D) Percent cell recovery upon thawing of PBMCs frozen either in 10% DMSO/90% HS (left) or 10% DMSO/90% AIM-V (right). \*\*P<0.04 for the comparison between the two conditions. Results are means of three separate experiments.



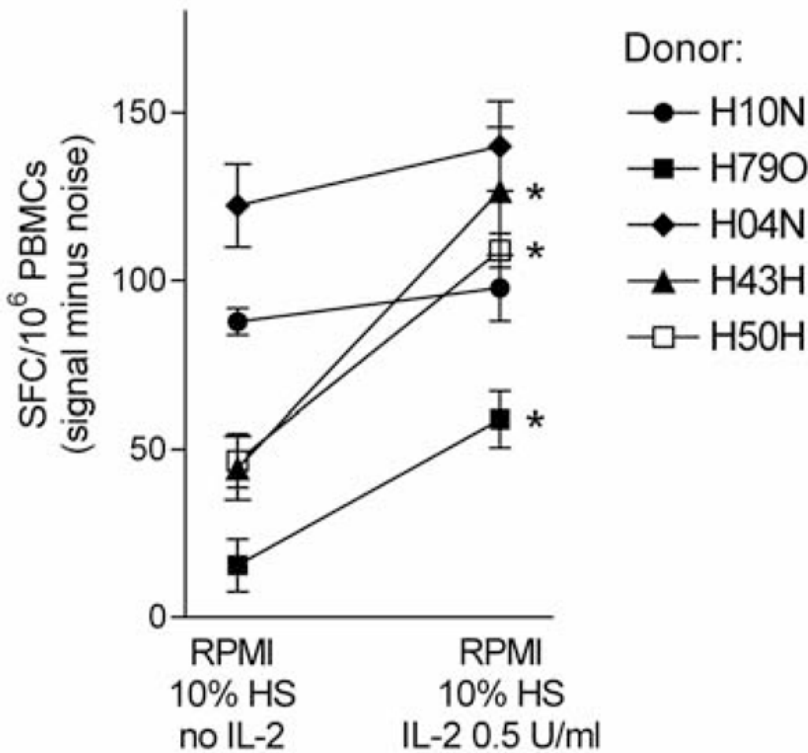
**Fig. 2**

Effect of anti-CD28 costimulation on the epitope-specific T cell responses measured by ELISpot. PBMCs from the indicated donors were tested using HS-free AIM-V medium with or without addition of anti-CD28 mAb (1  $\mu\text{g}/\text{ml}$ ). Grey bars represent the basal-subtracted ELISpot signal, while the basal reactivity is shown in the superimposed white bar. Total bar height (grey plus white) therefore represents raw spot counts before basal subtraction.  $**P < 0.03$  for the comparison between noise levels of anti-CD28-stimulated vs. unstimulated conditions by Student's t test. All differences in basal-subtracted ELISpot signals are not statistically significant.



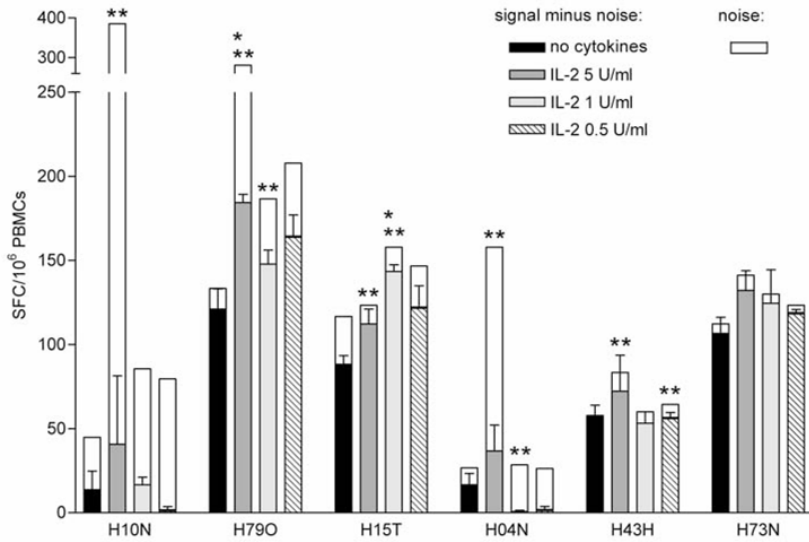
**Fig. 3**

Effect of low dose (0.5 U/ml) IL-2 addition to HS-supplemented medium on ELISpot responses. PBMCs from different donors were subjected to ELISpot testing using RPMI + 10% HS with (left) or without (right) IL-2. Basal-subtracted responses are shown, where background levels did not differ significantly between the two culture conditions.  $*P < 0.03$  for the comparison between the two conditions ( $P < 0.06$  when comparing cumulative data for all donors by Wilcoxon test).

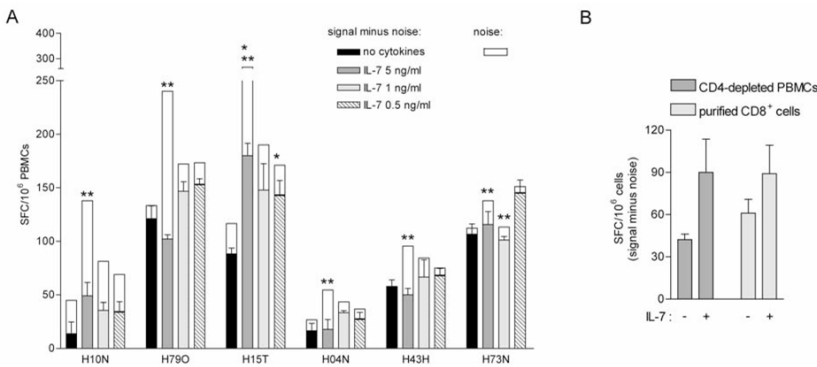


**Fig. 4**

Effect of IL-2 addition to HS-free AIM-V medium on ELISpot responses. PBMCs from different donors were subjected to ELISpot testing using HS-free AIM-V without IL-2 (black bars) or with 5, 1 or 0.5 U/ml IL-2 (dark grey, light grey and hatched bars, respectively). The superimposed white bars represent background reactivities for each condition, while total bar heights (colored plus white portion) correspond to raw spot counts before basal subtraction, as for Fig. 2. \* $P < 0.02$  for the difference in basal-subtracted responses as compared to the “no cytokines” condition. \*\* $P < 0.04$  for the difference in background noise as compared to the “no cytokines” condition.

**Fig. 5**

(A) Effect of IL-7 addition to HS-free AIM-V medium on ELISpot responses. PBMCs from different donors were subjected to ELISpot testing using HS-free AIM-V without IL-7 (black bars) or with 5, 1 or 0.5 ng/ml IL-7 (dark grey, light grey and hatched bars, respectively). Data representation is the same as in Fig. 4. \* $P < 0.04$  for the difference in basal-subtracted responses as compared to the “no cytokines” condition ( $P < 0.03$  for the IL-7 0.5 ng/ml vs. “no cytokines” condition when comparing cumulative data for all donors by Wilcoxon test). \*\* $P < 0.05$  for the difference in background noise as compared to the “no cytokine” condition ( $P < 0.05$  for the IL-7 5 ng/ml and 1 ng/ml vs. “no cytokine” conditions when comparing cumulative data for all donors by Wilcoxon test). (B) Effect of low dose (0.5 ng/ml) IL-7 addition on the ELISpot responses of CD4-depleted PBMCs and purified CD8<sup>+</sup> T cells. A representative experiment out of two performed is shown.

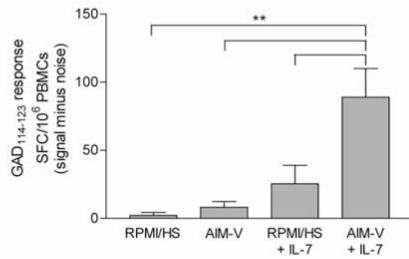




**Fig. 6**

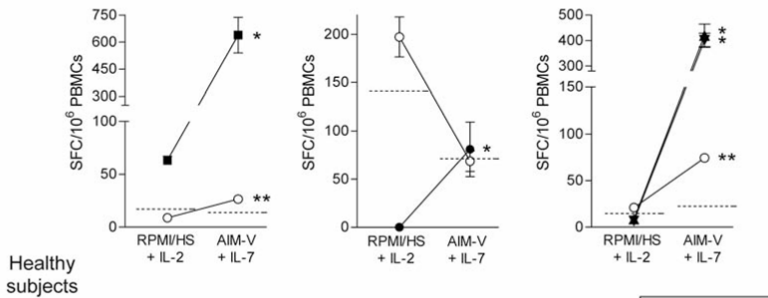
(A) Detection of GAD<sub>114-123</sub>-specific CD8<sup>+</sup> T cell responses with different culture media with or without IL-7 supplementation. PBMCs from a T1D patient were tested with different ELISpot conditions as indicated. \*\*P<0.04. (B) Comparison between IL-2- supplemented RPMI and IL-7-supplemented AIM-V media for detecting low grade autoimmune CD8<sup>+</sup> T cell responses against  $\beta$ -cell epitopes. PBMCs from HLA-A2<sup>+</sup> T1D patients (first row) and healthy controls (second row) were assayed against different  $\beta$ -cell epitopes using either HS-supplemented RPMI containing 0.5 U/ml IL-2 or HS-free AIM-V medium containing 0.5 ng/ml IL-7. All values are basal-subtracted, while the basal reactivity is shown as unsubstracted value. Dotted lines indicate the mean + 3SD cut-off for each condition. A viral mix stimulus used as further control was positive in all cases. \*P<0.04 for the difference in basal-subtracted responses. \*\*P<0.01 for the increase in background noise. Comparison of cumulative data for all  $\beta$ -cell epitope-specific responses in T1D patients gives a P<0.001 by Wilcoxon test. The difference in basal reactivities between the two stimulation protocols is not statistically significant overall (P=0.44 by Wilcoxon test).

A

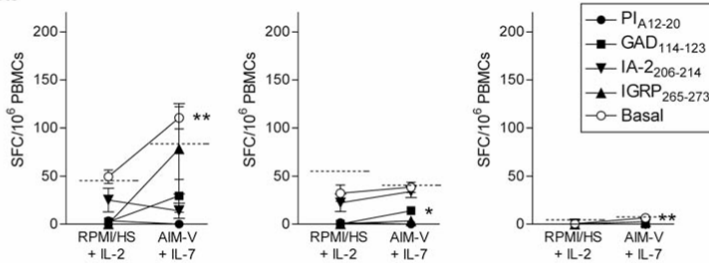


B

Type 1 diabetes patients



Healthy subjects



**Table I**

HLA-A\*0201<sup>+</sup> healthy donors enrolled in the study. The viral epitope(s) and the suboptimal concentrations used for ELISpot testing (Fig. 1 to 5) are indicated.

<b>donor</b>	<b>sex</b>	<b>age</b>	<b>epitope(s)</b>	<b>dose</b>
H10N	F	48	Flu MP <sub>58-66</sub>	12.5 nM
H79O	F	26	Flu-CMV-EBV	30.0 nM
H15T	M	22	Flu MP <sub>58-66</sub>	25.0 nM
H04N	M	33	Flu MP <sub>58-66</sub>	500 nM
H43H	F	70	CMV pp65 <sub>495-503</sub>	0.5 nM
H73N	M	27	Flu-CMV-EBV	12.5 nM
H50H	M	56	EBV BMLF1 <sub>280-288</sub>	125 nM
H87N	M	27	Flu-CMV-EBV	100 nM