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UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDECINE
Dermatologie

QUANTIFICATION OF LL-37-SPECIFIC CD4(+) T CELLS IN PSORIASIS USING ELISPOT

THESE

Préparée sous la direction du Professeur Michel Gilliet

et présentée à la Faculté de biologie et de médecine de
l'Université de Lausanne pour l'obtention du grade de

DOCTEUR EN MEDECINE

par

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Quantification of LL-37-Specific CD4(+) T cells in psoriasis using ELISPOT

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Monsieur le Professeur John Prior
Vice-Directeur de l'Ecole doctorale

Quantification of LL-37-specific CD4(+) T cells in psoriasis using ELISPOT

Introduction

Le psoriasis, avec une prévalence de 2-3% dans le monde, est considérée comme une pathologie chronique d'origine auto-immune. La maladie se présente principalement avec des plaques cutanées érythémato-squameuses épaisses. Le degré de sévérité est variable pouvant aller jusqu'à l'érythrodermie. Une atteinte inflammatoire systémique y est souvent associée, notamment au niveau articulaire. Les plaques psoriasiques résultent d'une prolifération kératinocytaire, médiée par l'activation anormale du système immunitaire innée et adaptatif. Cette activation dépend d'une prédisposition génétique et de la présence de facteurs environnementaux tels que des traumatismes cutanés [1].

Avec la découverte de cellules T CD4+ et CD8+ produisant IL-17 dans les lésions psoriasiques, ainsi que la présence de cytokines dérivées du Th17, le rôle central de l'axe IL-23-IL-17 dans l'immunopathogénèse du psoriasis a été établi [2]. Ceci a été prouvé par l'efficacité des nouveaux traitements biologiques visant cet axe [3-6]. Toutefois, la nature exacte des déclencheurs de cette réponse immune reste mal élucidée. Plusieurs groupes de recherche ont abouti à identifier des autoantigènes dérivés des kératinocytes qui sont reconnus par les cellules T pathologiques dans le psoriasis [7-9]. Notre groupe de recherche a découvert le peptide antimicrobien LL37 qui est également un autoantigène surexprimé dans les lésions psoriasiques [10]. En effet, les cellules T CD4+ et CD8+ isolées du sang des patients souffrant de psoriasis reconnaissent le peptide antimicorbien LL37 et produisent de l'interféron-γ et des cytokines Th17 [10].

Le but de ce travail a été de développer un protocole basé sur la technique ELISPOT, afin de détecter la fréquence des lymphocytes T spécifiques au peptide LL37 circulants dans le sang des patients souffrants de psoriasis. Les cellules mononuclées du sang périphérique (PBMCs) isolées des patients ont été stimulées avec le peptide LL37 et placées sur des plaques ELISPOT ou la production d'interféron-γ a pu être détectée. Malgré le nombre limité de patients analysés, on a retrouvé un taux de cellules T spécifiques à LL37 supérieur en comparaison au taux des donneurs sains. Ce travail a abouti à la réalisation d'un protocole avec une bonne reproductibilité de détection et de quantification de cellules T répondantes à LL37, qui est une base pour des études prospectives futurs sur la pathogenèse du psoriasis ou qui pourrait trouver une application clinique dans le suivi des patients.

Contents

1 Introduction

Psoriasis is a chronic T cell-mediated autoimmune skin disease with prevalence of 2-3% worldwide, equally among men and women. The disease is characterized by the formation of thick erythematous scaly plaques, mainly in areas of the body that are exposed to friction such as the elbows, the knees, the scalp or the lower back. Chronic plaque psoriasis is the most common form of psoriasis, affecting about 85% to 90% of patients, but other subforms including pustular psoriasis or psoriasis erythroderma exist. Psoriasis can also be associated with arthritis and systemic inflammation [1]. As for other chronic inflammatory diseases, no definitive cure is available for psoriasis. Treatments are used to control disease manifestations with the goal to achieve complete remissions as assessed by the Psoriatic Area severity Index (PASI). The PASI score measures the severity of erythema, induration, thickness, and desquamation of a skin plaque, along with the percentage of affected skin areas [2]. Standard treatments for mild-to-moderate psoriasis PASI<10 include topical therapies, such as corticosteroids and vitamin D analogues. UVA/UVB phototherapy and systemic agents, including methotrexate, cyclosporine, fumaric acid esters or systemic retinoids, are preferred for moderate-to-severe specific cases PASI>7.5-10 [3-5]. Better understanding of psoriasis immunopathogenesis, has led in the past decades to the development of novel immunomodulatory drugs, known as biologics. Biologics target specific key mediators of the immunological pathways in psoriasis and are usually used as an effective second-line therapy, when non-biological drugs are unresponsive [3, 5]. Psoriatic plaques are histologically characterized by a hyperproliferation of epidermal keratinocytes, driven by an abnormal activation of both the innate and adaptive immune systems [1]. This pathological immune activation relies on environmental factors and genetic predisposition [1, 3, 4]. Environmental factors disrupting the skin barrier such as mechanical skin friction or trauma, irritants and infections, can trigger the activation of the immune system in genetically predisposed individuals [1, 6, 7]. Psoriasis was initially described as a Th1-mediated disease, due to the high frequency of Th1 cytokines such as TNF-α and INF-γ found in psoriatic lesions and in peripheral blood of patients [8-10]. The discovery of IL-17-producing CD4+ and CD8+ T cells infiltrating the psoriatic skin, along with the presence of Th17-derived cytokines such as IL-22 and IL-17 suggested a central role of the Th17 response in psoriasis pathogenesis [11-14]. Accumulation of Th17 cells in the dermal compartment of psoriatic skin was shown to depend on the increased production of the Th17-polarizing cytokine IL-23 [14, 15]. In line with a key role of Th17 cells in the pathogenesis of psoriasis, numerous studies have demonstrated clinical efficacy of biologic therapies targeting the IL-23–IL-17 immune axis [16-19]. Regarding the genetic basis of psoriasis, an increasing number of studies have identified genomic associations with a risk to develop psoriasis. Genetic polymorphisms have been found in genes involved in the regulation of the main immunological pathways and key mediators found in psoriasis pathogenesis [1, 20-22]. These genetic predispositions result in an enhanced response to type I IFN signaling, a reduced threshold for T cell activation, and a favored differentiation of T cells towards the Th17 subset, that ultimately leads to an increased production of anti-microbial peptides (AMPs) by proliferating keratinocytes [1, 23-26]. AMPs sustain the inflammation by stimulating plasmacytoid dendritic cells (pDCs) that produce type I IFNs, triggering the activation of

dermal dendritic cells (DCs) and autoreactive Th17 cells [4]. A variant of the human leukocyte antigens (HLA) named HLA-Cw6, was shown to have the strongest association with psoriasis, with a prevalence of about 20% to over 50% in psoriatic patients, depending on the studied population, and only 4-16% in healthy controls [27-29]. As psoriatic skin lesions are clinically not infected, it is thought that psoriatic antigens derive from host cells and several groups tempted to identify them in psoriatic patients based on their capacity to bind HLA-Cw6. Using such a strategy, Keratin 17 and ADAMTSL5, two antigens expressed by keratinocytes and melanocytes respectively, were found to be recognized by psoriatic skin-infiltrating T cells [30, 31]. Given the abnormal expression of antimicrobial peptide LL-37 in psoriatic skin and its capacity to trigger inflammatory responses, our group investigated whether it could also be an autoantigen in psoriasis [32]. LL-37 is a linear peptide of 37 amino acids belonging to the cathelicidins, a proteins family that plays a role in the first line defense against bacterial infections in epithelial cells, as well as in resolving inflammation after infection [33]. Indeed, LL-37 due to its inherent cationic amphipathic nature, can insert into bacterial membranes leading to their lysis [33]. Like other AMPs, LL-37 can be expressed by damaged epithelial cells such as skin keratinocytes, or by activated granulocytes, such as neutrophils and mast cells [33]. It has been previously showed that the strong cationicity of LL-37 confers it with the capacity to bind extracellular nucleic acids and to form complexes leading to the activation of innate immune cells through the trigger of DNA and RNA sensors [34, 35]. Upon skin injury, stimulation of innate immune cells like DCs or monocytes by nucleic acids complexed with LL-37, leads to inflammatory responses that are important for wound healing [36]. In psoriatic skin, the overexpression of LL-37 promotes activation of DCs and their maturation into potent stimulators of skin infiltrating Th17 cells that trigger proliferation of keratinocytes [32]. In our previous work we found that LL-37 can be recognized by both CD4 (+) and CD8 (+) T cells isolated from blood and skin of psoriatic patients [32]. The T cells responding to LL-37 produced both INF-γ and Th17 cytokines. However, the analysis of the frequency of circulating LL-37-specific T cells and its relation to disease severity has not been done. Therefore, the aim of this study is to develop an ELISPOT-based assay to detect and measure the frequency of LL-37-specific T cells and to correlate it to the clinical status of the patients.

2 Material and Methods

2.1 Human peripheral blood samplers

Human peripheral blood samples are drawn at the Service of Dermatology of the CHUV (Centre Hospitalier Universitaire Vaudois). Samples are drawn from patients diagnosed with psoriasis according to the clinical criteria, with the condition of no undergoing systemic treatment at the moment of blood sampling. Some of the enrolled patients were taking advantage of a topic treatment, though. Control blood samples are taken from healthy donors. Peripheral blood mononuclear cells (PBMCs) were isolated after centrifugation on a density gradient and they were either used as fresh PBMCs or kept frozen at -80°C. When possible, Psoriasis Area Severity Index (PASI) was assessed on the day of blood test.

2.2 Antigens

Antigens used for stimulation are LL-37 from Innovagen and four recombinant LL-37 peptides from Proteogenix. LL-37 is a 37 amino acid (AA) protein, while recombinant LL-37 peptides consist in 13 to 15-mer peptides with 4-amino acid overlap (Figure 1). As a positive control, PBMCs were stimulated with phytohaemagglutinin (PHA) to assess polyclonal T cells activation and proliferation. As a positif control for an MHC restricted peptide presentation, we used PepTivator® CMV pp65, a peptide pool that consists of 15-mer peptides with 11 amino acids overlap, covering the complete sequence of the pp65 protein of human CMV. As negative control, PBMCs are incubated in the absence of stimulation. Concentration of antigens used is based on previous studies and is 10 µg/ml for LL-37, 2 µM for recombinant LL-37 peptides. PHA and CMV pp65 are used at a concentration of 10 µg/ml and 0.25 µg/ml, respectively.

Peptide	Sequences	Length (aa)
P1	LLGDFFRKSKEKIGK	(15)
P2	FFRKSKEKIGKEFKR	(15)
P3	SKEKIGKEFKRIVQR	(15)
P4	IGKEFKRIVQRIKDF	(15)
P5	FKRIVQRIKDFLRNL	(15)
P6	VQRIKDFLRNLVPRT	(15)
P7	KDFLRNLVPRTES	(13)

Figure 1: List of LL-37 shorter recombinant peptides used to stimulate PMBCs.

2.3 Antigen-specific T cell expansion assay

PBMCs are purified on Ficoll-Hypaque and incubated (10^5 cells/well) in a 96-well-U- bottom plate. T cell medium used is RPMI 1640 with 1% of PenStrep (100 U/mL Penicilium and 100 µg/mL Streptomycin) and 8% of heat-inactivated Human AB serum. PBMCs are stimulated for 7 days at 37°C, they are then re- stimulated with the same antigens for 24 hours on a 96-well-ELISPOT plate before the readout of INF-γ production. Initially, to improve antigen presentation to T cells, autologous monocytes were isolated from patients PBMCs and culture differentiated into monocytes-derived dendritic cells (moDCs) for 7 days with 20 ng/ml GM-CSF and 20 ng/ml IL-4. MoDCs were then matured with 10 µg/ml CD40L for 24 hours. As a negative control, moDCs were not maturated with CD40L. MoDCs were then pulsed during one hour with antigens, before being added to PBMCs on ELISPOT plate.

2.4 ELISPOT Kit

We tried two INF-γ ELISPOT kits, the first one was from eBioscience© and we then switched to Diaclone© kit because of better quality results and better reproducibility of analysis. ELISPOT plates were scanned with the AID ELISpot reader machine. The number of spots counted for each well was double-checked and recounted by hand looking at the taken picture.

2.5 Statistical analysis

Statistical analysis is performed to validate correlations made between the frequency of LL-37-specific T cells and the clinical status of the disease. We set a cut-off of spot forming cells (SFC) based on the mean of spots all negative controls +/- 2 standard deviations (SD).
Alternatives approaches: if statistical analysis results in non significative differences for LL-37, we plan to reproduce the same protocol testing other cationic AMPs previously found to be additional triggers of pDCs activation in psoriatic skin: human beta-defensin (hBD2, hBD3 and lysozyme) [37]. Another alternative approach would be to increase the number of patients, including other forms of psoriasis such as psoriatic arthritis.

3 Results

Whereas around 30% of psoriatic patients' PBMCs have T cells that proliferate in response to LL-37, there is no information on the number of circulating LL-37-specific T cells [37]. In order to measure this frequency, our goal was to develop an INF-γ ELISPOT-based assay. ELISPOT assay is widely described in the literature as a very sensible technique to evaluate quantitatively specific T cell responses to an antigen by measuring cytokine production at a single cell level. ELISPOT has been used to measure immune responses in infections, allergies, autoimmune diseases, and more recently in transplantations and antitumor responses in cancer (e.g. melanoma) [38-46].

In our first tests we noticed that the reproducibility and the quality of our results were not consistent, and frequencies of LL-37 specific T cells seemed to be very low. Therefore we switched from ELISPOT eBioscience© kit to the Diaclone© kit, which gave a better quality of spots, allowing reproducible counting of spot forming cells (SFC).

Activation of antigen specific T cells is driven by APCs that present antigens to CD8 (+) cells via MHC class I molecules, or CD4 (+) T cells via MHC class II-restricted antigens. Whereas MHC class I molecules present short peptides of around 9 amino acids, MHC class II molecules rather hold 15 aa-long antigenic peptides. Since we previously found LL-37 specific CD4 (+) T cells to be more frequent than CD8 (+) T cells [37], we decided to focus on detection of LL-37 CD4 (+) T cells responses by using 13 to 15-mere peptides. Because of the low frequencies of LL-37-specific T cells, we amplified this population by two approaches. The first approach was based on culturing PBMCs with either the full LL-37 antigen or the 13-15-mer overlapping LL-37 peptides for 7 days at 37°C. The second approach was based on a 7-day stimulation of PBMC with purified APCs pulsed with antigens in order to enhance antigen presentation. We used autologous monocytes differentiated into monocyte-derived dendritic cells (moDCs) for 7 days with GM-CSF and IL-4. MoDCs were used directly as immature DC or further differentiated into mature DC by a 24-h stimulation with CD40L. Immature or mature DC were pulsed for one hour with antigens before being added to the T cell-containing PBMCs. As a negative control, DCs were not pulsed with antigens. The two approaches yielded similar results (Figure 2). In some patients, INF-γ production by T cells was even better in the bulk PBMC stimulation, without moDCs. For this reason, we stopped adding moDCs to the cultures assuming that patients' PBMCs contained a sufficient number of APCs to present antigens.

In the literature, there is no general agreement on a SFC cut-off, as ELISPOT has formally no inferior limit of detection. We set an arbitrary positivity cut-off based on the mean of SFCs of all negative controls (unpulsed) plus two standard deviations (SD) resulting in a value of 2.36 as the positivity threshold for SFC. The maximal SFC count was set to 300.

Having validated the expansion protocol, we then tested 18 patients (Table 1) along with 7 healthy donors. Demographic information of the patients are given in Table 1. Patients' mean of age was 53.5 years old and the percentage of male and female was 67% and 33% respectively. None of the patients had systemic treatment at the time when blood was drawn. Because part of the samples were collected prior to the current study, PASI was not obtainable for all patients. The ELISPOT

results for 18 psoriasis patients and 7 healthy donors are given in Table 2 and Table 3 respectively. Representative pictures of an ELISPOT plate for psoriasis patients and healthy donors are shown in Figure 3A and Figure 3B, respectively. As a negative control, PBMCs were left untreated, positive controls included polyclonal stimulation of PBMC with phytohemagglutinin (PHA) to verify cell viability, and with CMV pp65-derived peptides to verify MHC restricted antigen presentation. All donors responded to PHA. CMV responses were detected in 13 out of 18 psoriasis patients (72.2%), and 3 out of 7 healthy donors (42,8%) (Figure 4). Stimulation with LL-37 or its peptides, showed responses in 3 out of 18 patients (16.7%): two responded to LL-37-derived 15-mere peptides (patients #4 and #5) and one patient weakly responded to the full LL-37 (patient #7). The frequency of LL-37-specific T cells ranged from $6/10^5$ PBMC in patient #7, to $15/10^5$ PBMC in patient #5, to $57/10^5$ PBMC in patient #4 (Table 2). Importantly, none of the healthy donors responded to full LL-37 or the LL-37-derived peptides.

The secondary aim of this study was to correlate frequency of circulating LL-37-specific T cells with the psoriasis disease activity score.

Our hypothesis is that a higher frequency of circulating LL-37-specific T cells would be observed in patients with more severe disease. Of the three responding patients, patient #5 with a frequency of 15 LL-37-specific T cells/10^5 PBMC presented the highest PASI score of 26.3 among all tested patients. Unfortunately, PASI information was lacking for the other 2 patients showing significant LL-37-specific T cells in the circulation and therefore a correlation could not be achieved.

Patient	Age (y.o.)	Sex	Diagnosis	Time since diagnosis (years)	Treatment	PASI	BSA
1	56	F	Pso*	-	none	-	-
2	82	F	Psoriasis vulgaris	20	none	-	-
3	45	M	Psoriasis vulgaris + joints	15	none	-	-
4	54	M	Inverse and nail psoriasis	6	topic	-	-
5	15	M	Psoriasis vulgaris	11	topic	26.3	50%
6	68	F	Psoriasis vulgaris	3	phototherapy	-	-
7	57	M	Psoriasis vulgaris + joints	31	none	-	-
8	84	F	Psoriasis vulgaris + guttate psoriasis	9	none	-	-
9	36	M	Psoriasis vulgaris	-	topic	16.7	20%
10	31	F	Pso*	-	none	-	-
11	72	M	Pso*	-	none	-	-
12	68	M	Pso*	-	none	-	-
13	53	M	Psoriasis vulgaris + guttate psoriasis	1	topic	-	4-5%
14	47	F	Psoriasis vulgaris + inverse psoriasis	-	none	19	-
15	55	M	Psoriasis vulgaris + inverse psoriasis	6	topic	12	9%
16	51	M	Psoriasis vulgaris	-	none	9.2	20%
17	38	M	Pso*	-	none	-	-
18	44	M	Psoriasis vulgaris	-	none	8.2	8%

Table 1: Baseline characteristics of the studied population. When possible, severity of psoriasis was assessed clinically with PASI (psoriatic area severity index) and BSA (body surface area) scores. PASI is an index evaluating the severity of erythema, induration, thickness, desquamation and the percentage of affected skin areas with rates going from 0 to 72 (72 = most severe psoriasis) and BSA (Body surface area) is a score evaluating the surface of the affected skin by psoriatic plaques expressed in percentage of surface affected.
*Pso = psoriatic patients with no specified type of psoriasis in their diagnosis.

Patients	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Med	0	0	2	0	2	1	2	0	0	x	0	0	0	0	0	0	0	0
P4	0	0	1	**8**	0	1	1	0	0	x	0	0	0	0	0	0	0	0
P5	0	0	0	**57**	0	2	1	1	0	x	0	0	0	0	0	0	0	1
P6	0	0	0	**3**	1	0	2	0	0	x	0	0	0	1	0	0	0	0
P7	0		0	**4**	**15**	2	0	0	0	x	0	0	0	0	0	0	0	0
LL-37	0	0	0	1	0	0	**6**	0	0	x	0	0	0	0	0	0	0	0
PHA	**300**	**300**	**300**	**300**	**300**	**300**	**300**	**300**	**300**	**x**	**300**	**300**	**300**	**300**	**300**	**300**	**300**	**300**
CMV	**300**	**100**	**57**	**180**	0	2	**300**	**300**	0	x	**80**	**40**	**300**	**300**	**4**	**80**	**5**	0

Table 2: Spot forming cells (SFC) for each well, containing a total of 10^5 PBMCs. PBMCs were culture stimulated with antigens at 37°C for 7 days, and then re-stimulated for 24h hours on ELISPOT plate before the read out of the plate. We set a cut-off of 2.36 SFC, calculating the mean of all SFC of negative controls +/- 2 standard deviations (SD). We set a maximum of 300 SFC for very positive wells. Control wells with PHA all reached maximum of positivity. In bold the wells that showed a significant number of SFCs. Med = medium; P4-P7 = recombinant LL-37 13 to 15-mer peptides; PHA = phytohaemagglutinin; CMV = PepTivator® CMV pp65, peptide pool that consists mainly of 15-mer peptides with 11–amino acid overlap, covering the complete sequence of the pp65 protein of human cytomegalovirus.

	HD1	HD2	HD3	HD4	HD5	HD6	HD7
Med	0	0	0	0	0	0	0
P4	0	0	1	0	2	0	1
P5	0	1	0	0	2	0	0
P6	0	0	0	0	0	0	1
P7	2	1	3	0	1	0	0
LL-37	0	0	0	0	3	1	1
PHA	**300**	**300**	**300**	**300**	**300**	**300**	**300**
CMV	0	1	**300**	0	**103**	**174**	0

Table 3: PBMCs of 7 Healthy donors stimulated with same antigens and at the same conditions as patients' PBMCs.

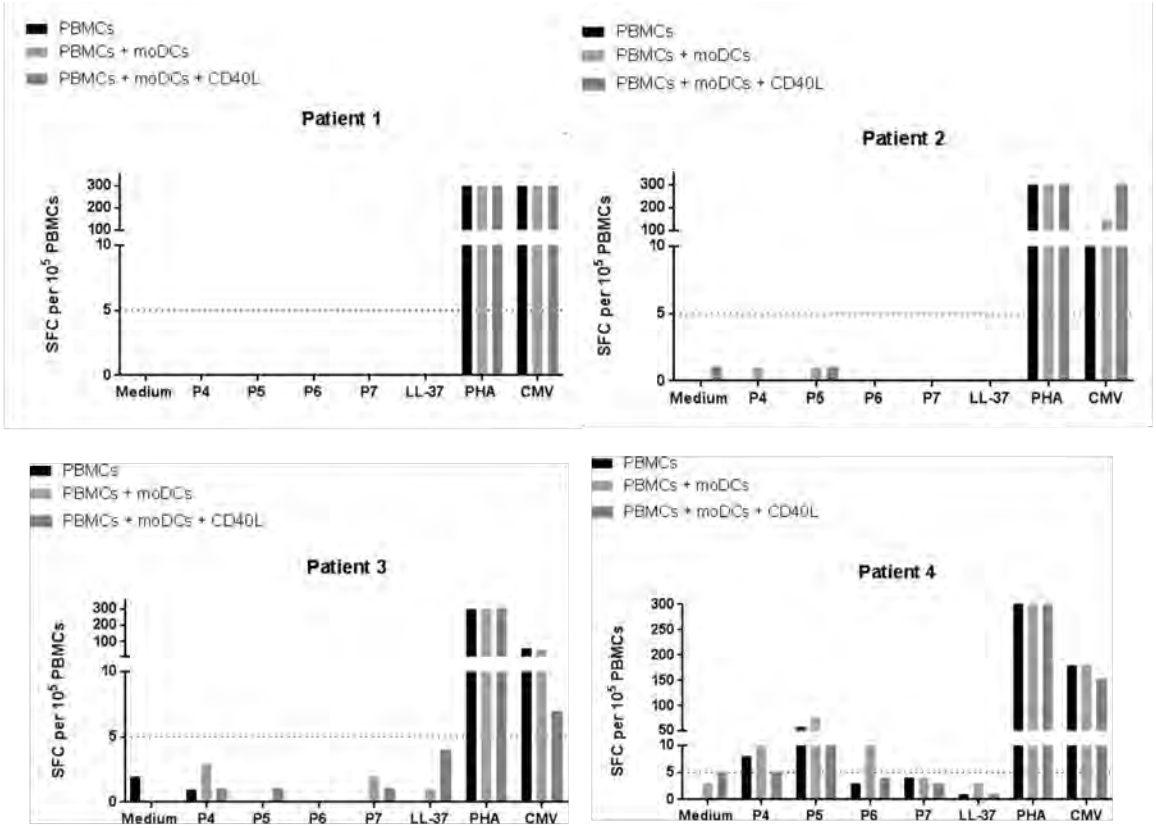


Figure 2: Comparison between stimulation of PBMCs alone, PBMCs with moDCs and PBMCs with maturated moDCs with CD40L in 4 patients. In all 4 tested there is not an amplified INF-γ production when adding moDCs or moDCs maturated with CD40L. PBMCs = Peripheral blood mononuclear cells; SFC = spot forming cells; Med = medium (negative control); P4-P5 = recombinant LL-37 peptides of 13 or 15-mer peptides, PHA = phytohaemagglutinin; CMV = PepTivator® CMV pp65.

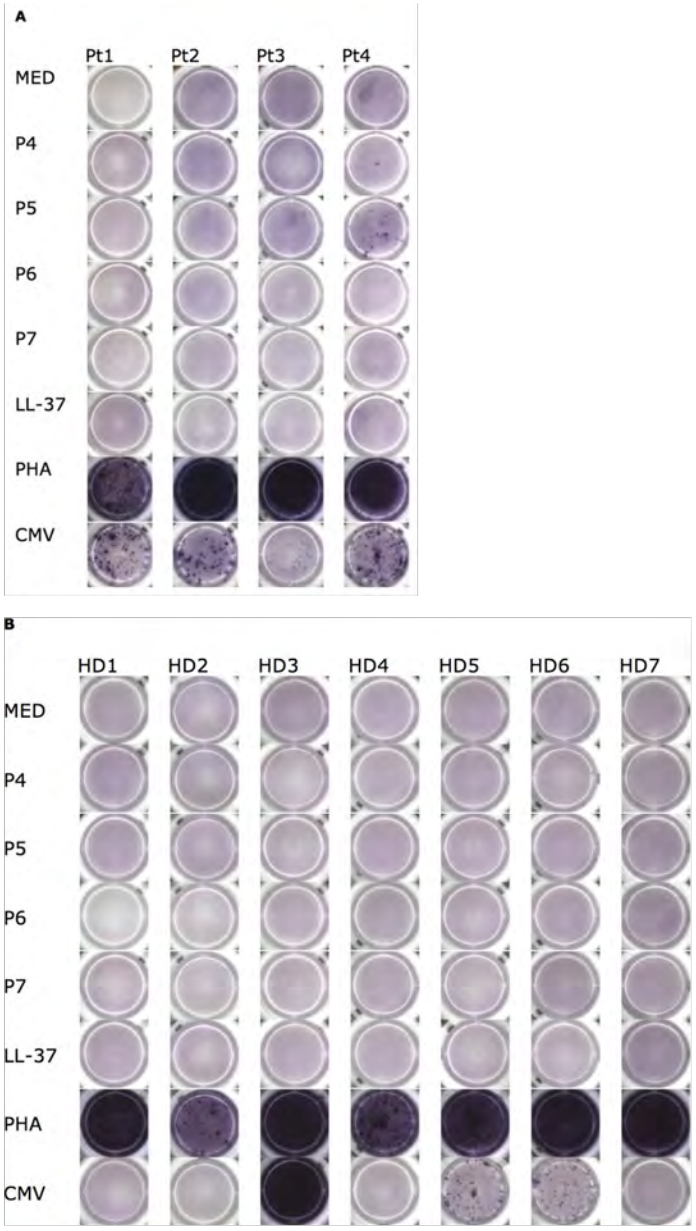
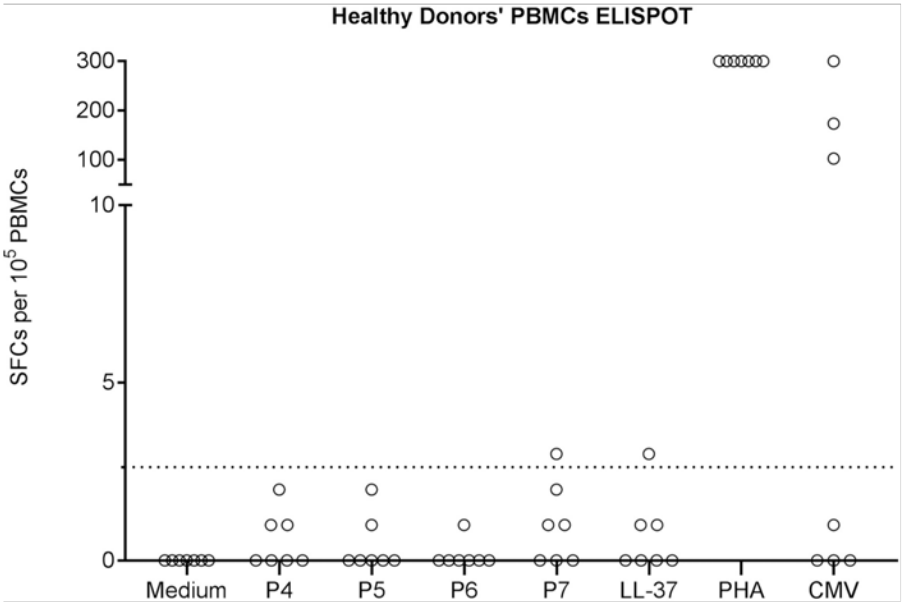


Figure 3: Scanning of single wells of four patients (A) and 7 healthy donors (B). Each spot represents an activated specific T cells producing INF-γ. Spots are counted by the AID ELISpot reader machine and double-checked by hand. We set a cut-off of 2.36 SFC, calculating the mean of all SFC of negative controls +/- 2 standard deviations (SD). We set a maximum of 300 SFC for very positive wells.



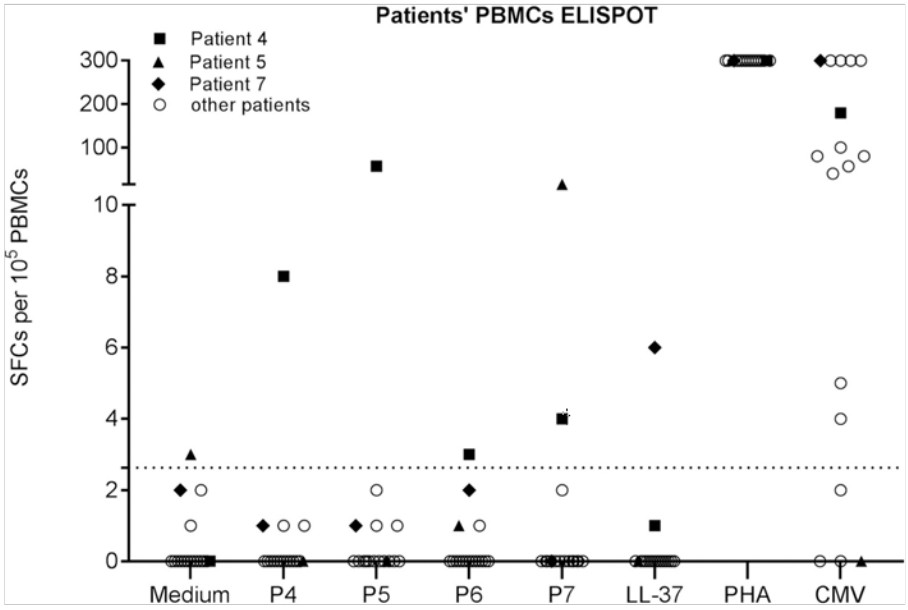


Figure 4: A Healthy donors PBMCs and B Patients PBMCs. The dotted line represents the cutoff of 2.36; SFC = spot forming cells; P4-P7 = recombinant LL-37 13 to 15-mer peptides; PHA = phytohaemagglutinin; CMV = PepTivator® CMV pp65, peptide pool that consists mainly of 15-mer peptides with 11–amino acid overlap, covering the complete sequence of the pp65 protein of human cytomegalovirus.

4 Discussion

Psoriasis is a common chronic-relapsing T cell mediated autoimmune disease of the skin. It has been shown that in lesional skin, there is an increase of IL-17A and IL-22 producing CD4+ and CD8+ T cells, suggesting a role of both T cell subsets in psoriasis pathogenesis [13] [47]. Previous studies have identified keratinocyte-derived autoantigens recognized by the pathogenic T cells in psoriasis, such as keratins that cross-react with proteins derived from group-A β-hemolytic streptococci [48-50]. Our group has previously discovered that the antimicrobial peptide LL-37 is an other auto-antigen in psoriasis. T cell responses to LL-37 were detected by measuring T cell proliferation to LL-37 stimulation and by assessing their activation and INF-γ production in the supernatants of stimulated PBMCs [32]. However, these measurements do not allow to quantify the frequency of LL-37-specific T cells. Our study aimed at developing an ELISPOT assay to measure the frequency of circulating LL-37-specific T cells and to assess the relation between this frequency and disease severity in psoriasis patients. ELISPOT is a validated and sensitive technique to quantify T cell response by measuring cytokine activation (e.g. INF-γ production) on the single cell level. In this study we aimed at establishing such a test and provide a reliable protocol that could be routinely used in the clinics to monitor psoriasis patients.

We analyzed a limited number of psoriasis patients, collected in the relatively short timeframe of the study. Nevertheless, the study served as a pilot study to establish a method to reproducibly detect and quantify LL-37-specific T cells. In a few psoriasis patients, we found low but significant frequencies of circulating LL-37-specific T cells when compared to healthy donors. The frequencies appeared to be between $10 - 57/10^5$ cells, thus higher than the described frequency of circulating autoantigen-specific CD4+ T cells in systemic lupus erythematosus (SLE), another autoimmune disease. The frequency of circulating autoantigen-specific CD4+ T cells in SLE was estimated to be between $5 - 10/10^5$ cells, which does not allow distinction from autoreactive CD4+ T cell frequencies present in the naive T cell repertoire. We used a 7-day Ag-specific T cell restimulation protocol, which allowed us to obtain higher, reliable frequencies of autoanigens specific memory T cells and a clear separation from the frequencies in the naïve T cell repertoire of healthy donors.

But why are the frequencies of LL-37-specific T cells, which are potentially pathogenic T cells so low? There are two potential explanations. Firstly, the majority of pathogenic LL-37-specific T cells may reside in the skin and recirculate only in small numbers, most likely in patient with widespread disease. In fact, higher frequencies of LL-37-specific T cells were found in lesional psoriatic skin when compared to circulating cells [13] [47]. Secondly, psoriatic T cells are oligoclonal, suggesting that other antigenic specificities exist in addition to LL-37. Indeed, the expression of other AMPs such as S100A proteins and beta defensins are elevated in psoriatic skin and potentially serve as autoantigens for psoriatic T cells. By developing a murine model of AMP overexpression, our group is currently investigating the autoantigenic role of AMPs in psoriasis.

These and many other questions remain open and represent a challenge for future studies. Nevertheless, our current study provides the validation of a protocol to reproducibly detect and quantify LL-37-specific T cells in the circulation of patients and a basis for a larger prospective study on to gain further evidence on the pathogenicity in psoriasis.

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