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Evaluation of T and B lymphocyte function in clinical practice using a flow cytometry based proliferation assay

Per Marits[a,b], **Ann-Charlotte Wikström**[a,c], **Dusan Popadic**[d], **Ola Winqvist**[a,b], **Sarah Thunberg**[a,e,*]

[a] *Clinical immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden*
[b] *Dept. of Medicine, Karolinska Institutet, Stockholm, Sweden*
[c] *Dept. of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden*
[d] *Inst. of Microbiology and Immunology, University of Belgrade, Serbia*
[e] *Dept. of Oncology and Pathology, Karolinska Institutet, Stockholm, Sweden*

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Abstract The golden standard for functional evaluation of immunodeficiencies is the incorporation of [^{3}H]-thymidine in a proliferation assay stimulated with mitogens. Recently developed whole blood proliferation assays have the advantage of parallel lymphocyte lineage analysis and in addition provide a non-radioactive alternative. Here we evaluate the Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood (FASCIA) in a comparison with [^{3}H]-thymidine incorporation in four patients with severe combined immunodeficiency. The threshold for the minimum number of lymphocytes required for reliable responses in FASCIA is determined together with reference values from 100 healthy donors when stimulated with mitogens as well as antigen specific stimuli. Finally, responses against PWM and SEA + SEB stimuli are conducted with clinically relevant immunomodulatory compounds. We conclude that FASCIA is a rapid, stable and sensitive functional whole blood assay that requires small amounts of whole blood that can be used for reliable assessment of lymphocyte reactivity in patients.

Abbreviations: Con A, concanavalin A; CPM, counts per minute; CV, coefficient of variation; FASCIA, flow-cytometric assay for specific cell-mediated immune-response in activated whole blood; MMF, mycophenolate mofetil; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PID, primary immunodeficiency; PPD, purified protein derivative; PWM, pokeweed mitogen; SEA and SEB, staphylococcal enterotoxin A and B; SCID, severe combined immunodeficiency.

* Corresponding author at: Clinical immunology and Transfusion Medicine, F79, S-141 86 Stockholm, Sweden.
E-mail address: sara.thunberg@karolinska.se (S. Thunberg).

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1. Introduction

Functional evaluation of lymphocytes is part of the clinical work-up of patients with suspected immunodeficiencies. Today the golden standard is proliferation assays based on mitogen stimulation using DNA incorporation of the radioactive nucleotide [^{3}H]-thymidine [1,2]. The assay is based on the principle that activation of T-lymphocytes and/or B -lymphocytes creates intracellular signaling resulting in activation, entry into S phase of the cell cycle allowing for the incorporation of the radioactively labeled nucleotide into the DNA. However, the read out does not permit the distinction between the cell types contributing to the signal, *i.e.* T and B-cell defects cannot be separated. There is an increasing desire to find non-radioactive alternatives, since there are potential hazards linked to the use of radioactivity and scintillation liquids used in the assay. The use of non-radioactive DNA approaches such as incorporation of the nucleoside analog 5-bromodeoxyuridine (BrDU) is also possible. However, this assay is less sensitive than the [^{3}H]-thymidine incorporation assay and therefore it has not been introduced into the clinic [3]. Another option is to use the MTT assay to determine cell viability. It is based on the mitochondrial enzyme succinate dehydrogenase and its capacity to transform the tetrazolium salt of MTT into MZZ formazan, a blue compound calorimetrically detectable using ELISA. The method is comparable in sensitivity to the [^{3}H]-thymidine assay and has been available for many years [4]. However, the MTT assay is an indirect assay and only proportional to the number of living cells and not directly to dividing cells; therefore, it has not been accepted as the golden standard in clinical practice. All of the three aforementioned tests are preceded by peripheral blood mononuclear cell (PBMC) separation, usually on a density gradient, followed by multiple washing steps that may lead to activation, damage and selective loss of cell populations. Moreover, all tests mentioned are based on that the initial number of PBMCs (but not lymphocytes *i.e.* proliferating cell) in non-stimulated and stimulated samples are equal and also require more pipetting steps that may introduce further errors.

Proliferation can also be monitored using dye dilution by loading cells with carboxyfluorescein diacetate succinimidyl ester (CFSE). The trapped fluorophore is diluted at every cell division allowing enumeration of divisions [5]. The need for uniform distribution and the toxicity of the dye at higher concentrations again limits the usefulness in clinical samples from patients with low cell counts and suspected immunodeficiencies. In addition handling time for preparation and analysis is somewhat lengthy and not suitable for the clinical laboratory.

During the 1990s Cost et al. presented a non-radioactive PBMC-based assay for evaluating blast transformation of activated lymphocytes with flow cytometry [6]. The assay was further developed into "Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole blood" or "FASCIA" [7]. The whole blood assay is simple, fast with little hands on time and most importantly, it requires a very small amount of whole blood. The read out of blast transformed cells is combined with markers identifying various responding lymphocyte subpopulations. The method has been introduced into the clinic and is now routinely used by us for diagnostic work-up for various types of immunodeficiencies with reference values based on more than 100 healthy controls. Diagnosis of primary immunodeficiencies (PID) is difficult. The patients are rare and represent a heterogeneous group with a variety of clinical signs and symptoms [8]. Usually complete blood count and total and specific immunoglobulin levels are determined in combination with analysis of the complement system and enumeration of lymphocyte subsets. However, this test panel in some cases is not enough since the clinical symptoms manifested in the PID [9,10] may depend on a lack of cellular function, not manifested in a low cell number.

In this study, we have evaluated the use of FASCIA for functional diagnostic evaluation of immunocompromised patients or patients with primary immunodeficiencies. In addition whole blood from healthy donors was incubated with immunosuppressive drugs using the FASCIA assay to determine how immunosuppressants affect proliferative responses *in vitro*.

2. Material and method

2.1. Antigens and pharmaceutical drugs

The mitogens and antigens were titrated for optimal responses and the following concentrations were used in the FASCIA experiments: 10 μg/mL phytohemagglutinin (PHA), 10 μg/mL concanavalin A (Con A), 5 μg/mL pokeweed mitogen (PWM), 100 ng/mL of both Staphylococcal enterotoxin A and B (SEA + SEB) (all from Sigma Aldrich, MO, USA), 10 μg/mL tuberculin purified protein derivative (PPD), 4 IU/mL tetanus toxin (both from Statens Serum Institute, Copenhagen, DK) and 20 μg/mL *Candida albicans* (Greer Laboratories Inc., NC, USA). Influenza vaccine "Fluarix", Varicella zoster virus vaccine "Varilrix" (both from GlaxoSmithKline AB, Middlesex, UK) and Pneumococcal vaccine "Prevenar 13", (Wyeth, NY, USA) were all diluted 1:100.

The concentrations of the immunosuppressive drugs used in the FASCIA experiments were chosen to correspond to clinically relevant doses. The following concentrations were used: 100 ng/mL rapamycin or sirolimus (Sigma), 100 ng/mL mycophenolate mofetil (MMF) "Cellcept" (Roche AB, Sweden), 0.1 μg/mL dexamethasone (Unimedic, Matfors, Sweden), and 10 ng/mL tacrolimus "Prograf" (Astellas Pharma, Malmö, Sweden).

2.2. Study population

Heparinized decoded whole blood from anonymized voluntary blood donors, collected at the Department of Clinical Immunology and Transfusion Medicine at the Karolinska University Hospital, is routinely used as positive control material for functional assays at the cellular immunology laboratory. In order to validate the FASCIA assay against golden standard assay [^{3}H]-thymidine incorporation after mitogen stimulation laboratory data from four decoded SCID patients are shown.

2.3. FASCIA

2.3.1. Normal

Heparinized whole blood (50 μL/tube) was diluted 1:10 in FASCIA-medium, containing RPMI 1640, supplemented with L-glutamine, 100 IU/mL penicillin, 100 IU/mL streptomycin (all from Gibco, Paisley, UK) as well as 2 mM L-glutamine

(Invitrogen, MD, USA). The blood/medium mixture was stimulated with PWM, PHA, Con A, SEA + SEB, PPD, Candida, tetanus toxoid, pneumococcal, influenza or varicella zoster vaccine in sterile polypropylene Falcon 12 × 75 mm FACS-tubes with caps (BD Biosciences, CA, USA) to a final volume of 500 μL. Thus each FASCIA tube contained 50 μL of whole blood and 450 μL culture medium including stimuli. The FASCIA-tubes were incubated for 7 days in 37 °C, 5% CO_2 and 95% humidity. At day 7, 300 μL of the cell supernatant was removed and stored in −80 ° C until determination of cytokine/chemokine content was performed. Cells were stained with CD3-FITC/CD4-PE Simultest mix (BD Biosciences). In addition, the "medium" and "PWM" tubes were stained with CD19-PC7 (Beckman Coulter, Marseille, France). The tubes were incubated 10 min at room temperature (RT) in the dark, erythrocytes were lysed with 1× IOTest lysing solution (Beckman Coulter) and the cell pellets were resuspended in 450 μL PBS. Blast numbers were acquired during 80 s with a FC500 or Navios flow cytometer (Beckman Coulter, CA, USA). In order to obtain an absolute number of proliferative responses per μL whole blood, a Trucount (BD Biosciences) tube was used. The number of proliferating cells was calculated using the following equations:

$$\text{Volume during 80 s } (V_{80s}): (\text{Acquired beads during 80 s} \times 450)/\text{Total number of beads} \quad (1)$$

$$\text{Number of cells}/\mu\text{L blood}: 10 \times ((\text{stimuli-medium})/V_{80s}) \quad (2)$$

A detailed protocol for the FASCIA procedure is available at the COST action BM0907 ENTIRE website.

2.3.2. Titration of whole blood

To establish the lower limit of cells that could be used in the FASCIA whole blood collected in heparin tubes was titrated using 0.5, 5, 25, 50 (normal FASCIA) and 100 μL of blood and mixed with FASCIA-medium with or without PWM, Con A or Candida according to the normal FASCIA-protocol. The absolute T-cell count ($CD3^+$), in the titrated blood volumes, was measured using anti-human CD3-PC7 (Beckman Coulter), CD45-FITC and Trucount tubes (BD Biosciences). The cells were analyzed on the Navios flow cytometer (Beckman Coulter) and presented as number of proliferating $CD3^+$ cells.

2.3.3. Proliferative response after 2, 5, 7 or 9 days of incubation

The FASCIA was performed using whole blood from three healthy controls. The cells were stimulated with or without PWM, Con A or Candida and incubated for 2, 5, 7 (normal FASCIA) or 9 days. After incubation the cells were analyzed according to the normal FASCIA-protocol and presented as number of proliferating cells/μL whole blood.

2.3.4. With immunosuppressant drugs

Whole blood from 11 healthy controls were diluted in FASCIA-medium and stimulated with SEA + SEB or PWM according to the FASCIA protocol. In order to evaluate the effect of immunosuppressive drugs in FASCIA, cells from the same healthy donors were subjected to clinically relevant concentrations of dexamethasone, MMF, rapamycin or tacrolimus. The immunosuppressive drugs were added to the blood/medium mixture and incubated according to protocol for 7 days. The number of blast transformed SEA + SEB and PWM stimulated $CD4^+$, $CD3^+CD4^-$ (henceforth defined as $CD8^+$) and $CD19^+$ (only after PWM incubation) lymphocytes in the presence of immunosuppressive drugs were expressed as percent proliferation, relative to stimulated cells without immunosuppressive drugs in the medium (normal) or expressed as proliferating cells/μL of whole blood, as indicated in Fig. 3.

2.4. Intracellular staining of interleukin-17 (IL-17) and interferon γ (IFNγ)

Whole blood from four healthy donors was stimulated with PWM or SEA + SEB in combination with tacrolimus (10 ng/mL) or rapamycin (100 ng/mL) according to the FASCIA protocol. At day 7, Brefeldin A (Biolegend, San Jose, CA, USA) was added to the cultures during the final 5 h of incubation. The erythrocytes were lysed with 1× IO Test lysing solution (Beckman Coulter) and extra-cellular staining was performed with CD8-PE-CF594 (BD Biosciences), CD45-PC5, CD3-Pacific blue and CD4-Krome orange (all from Beckman Coulter). The cells were fixed and permeabilized with Fix- and Perm wash buffer (Biolegend), followed by intracellular staining with IL-17A-PE (Biolegend) and IFNγ-APC (BD Biosciences). The cells were analyzed on the Navios flow cytometer (Beckman Coulter).

2.5. [^{3}H]-thymidine incorporation assay

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized whole blood with Lymphoprep solution (Axis-Shield, Norway). The PBMCs were resuspended in RPMI 1640 (Gibco) and stimulated with PHA, Con A and PWM or left un-stimulated and cultured in round bottom 96 cell well plates, 100,000 cells per well in triplicates. After 72 hour incubation in 37 °C, 5% CO_2 and 95% humidity, cells were pulsed with 2 μCi [^{3}H]-thymidine for 8 h. Scintillation liquid was added and a Tomtec harvester (Tomtec Inc., CN, USA) was used to evaluate cell proliferation and results were obtained as counts per minute (cpm).

2.6. Multiplex cytokine detection

Cytokines and chemokines in EDTA plasma, serum or cell supernatants from un-stimulated cells (medium) or stimulated with PWM, SEA + SEB and Candida were measured with a 25-plex Milliplex human cytokine/chemokine kit. The multiplex procedure was performed according to the instructions provided by the manufacturer (Millipore Corporation, MO, USA). Sample analysis was performed on a Luminex 200 platform (Luminex, TX, USA) using Milliplex Analyst Software (Millipore).

2.7. Statistics

In order to compare the effects of the immunosuppressive drugs in the FASCIA the paired *t*-test was used. In all statistical analysis a p-value <0.05 was considered statistically significant. For the analysis Graph Pad Prism (GraphPad Software Inc., CA, USA), version 4 was used.

3. Results

3.1. Properties of the FASCIA assay

The basis for the FASCIA assay is the measurement of blast formation of proliferating lymphocytes. During division a lymphocyte increases in size [11], this can be detected as an increase in forward scatter with flow cytometry. Addition of monoclonal antibodies, conjugated to fluorophores, directed to T- and B-cell lineage markers in combination with known reference beads enables enumeration of proliferating T-and B-cells. The flow cytometry gating strategies are shown in Fig. 1A. Reference values from whole blood obtained from more than 100 healthy donors stimulated with a standardized mitogen and antigen panel are shown in Table 1. Since [^{3}H]-thymidine incorporation is considered the "golden standard" for diagnosis of severe immunodeficiency [12,13], PBMC and whole blood from five healthy donors were stimulated with PHA, PWM and Con A, according to their respective protocols. Fig. 1B shows the correlation between the number of T-lymphocyte blasts and cpm obtained from the mitogen stimulation assay. Due to the low incidence of severe immunodeficiencies the above experiment could not be performed on patients. However, we had clinical data records from four patients with severe combined immunodeficiency (SCID) [14], where both mitogen stimulation and FASCIA had been performed side by side for validation purposes of the FASCIA method at the time of diagnosis. The final SCID classification varies between patients but all of the patients had decreased absolute numbers of T-cells compared to age matched healthy controls. SCID patient 4 showed normal, patient 1 almost normal numbers of B-cells, whereas patients 2 and 3 had decreased number of B-cells compared to healthy controls. Only SCID patient 4 had decreased number of NK-cells compared to healthy controls (Table 2). The proliferative responses by SCID patient's lymphocytes to PHA, PWM and Con A for the two proliferation assays are shown in Table 2 and expressed as percent proliferation compared to a healthy control. The results are comparable between the assays, except for the proliferation to Con A for SCID patient 3, which was 73% relative healthy control in FASCIA compared to 9% with [^{3}H]-thymidine incorporation. Moreover, for SCID patient 1, the PWM response was 50% in the [^{3}H]-thymidine incorporation assay compared to 2% in FASCIA. The data probably shows proliferating B-cells (75% of lymphocytes for SCID patient 1, Table 2) in the [^{3}H]-thymidine incorporation assay, where a set number of 100,000 PBMCs per well are cultured. Since the FASCIA method is based on diluted whole blood no such enrichment of B-cells is seen.

In order to establish the most suitable day to read the assay, a kinetics experiment was performed. Whole blood from three healthy donors was stimulated with Con A, PWM and Candida antigen and incubated for 2, 5, 7 and 9 days. Read out was the number of blast transformed CD4$^+$ and CD8$^+$ T-cells as well as CD19$^+$ B-cells (analyzed PWM stimulation only). There was very little blast formation at day 2 regardless of cell type analyzed. Con A stimulation was most prominent at day 5, both for CD4+ and CD8+ T-cells, after which it decreased slightly over time. PWM and Candida stimulation gave rise to a lower number of blasts at day 5, followed by an increase in number of blasts day 7 to 9 (Fig. 1C). For clinical laboratory practice, seven day incubation was decided to be the most useful.

3.2. Cytokine detection in FASCIA cell supernatants

With an increasing interest for various biomarkers both in the pharmaceutical industry and the clinic, we decided to measure the normal cytokine responses for healthy donors in the FASCIA. FASCIA cell supernatants were collected day 7 from tubes stimulated with PWM (n = 17), Candida antigen (n = 19) or the super antigens SEA and SEB (n = 12). Cytokines and chemokines were analyzed with multiplex technology. The results, with subtracted values from respective medium controls are shown in Table 3. All tested cytokine/chemokines were detectable, with a large variation between donors. Table 3 also shows the corresponding cytokine/chemokines in plasma/serum from healthy donors (n = 6–122).

3.3. Precursor cell number affects the number of blasts in FASCIA

One of the benefits with the FASCIA assay is that no Ficoll separation prior to stimulation is necessary. The whole blood is diluted 1:10 in supplemented RPMI1640 medium followed by stimulation and incubation. However, a concern is that patients with immunodeficiencies that are tested in FASCIA often may have low lymphocyte numbers. To determine the effect of precursor cell number (CD3+ T-cells) in FASCIA outcome whole blood from 11 healthy donors were titrated from 0.5, 5, 25, 50 (normal FASCIA volume) to 100 μL and stimulated with Con A, PWM and Candida antigen according to the FASCIA protocol. Mean number and range of CD3$^+$ T-cells in the respective whole blood volumes were as follows (Fig. 2A): 0.5 μL; 808 (285–3053), 5 μL; 5795 (3872–12,137), 25 μL; 24,073 (14,274–40,426, 50 μL; 49,122 (28,548–80,852) and 100 μL; 98,543 (64,394–161,703). To evaluate critically low precursor cell number we used the clinically relevant 5th percentile of our reference values (Table 1) as cut-off values for a positive outcome in FASCIA. Proliferation below the 5th percentile was considered pathological. Based on our reference values the cut-off for a positive FASCIA response was the following: for Con A; CD4$^+$ 665 cells/μL, CD8$^+$ 137 cells/μL, for PWM; CD4$^+$ 411 cells/μL, CD8$^+$ 72 cells/μL and for Candida CD4$^+$ 7 cells/μL, CD8$^+$ 1 cell/μL. The antigen specific response elicited by Candida antigen gave rise to a lower proliferative response compared to the mitogens Con A and PWM (Table 1). CD4$^+$ and CD8$^+$ T-cells were divided into groups with cell numbers ranging from 0–15,000 up to 105,000–120,000 (Fig. 2B). For each group the positive FASCIA outcome, *i.e.* number of blasts above cut-off, was calculated and expressed in percent below or above cut-off. 100% above cut-off corresponds to all precursor cell number in the respective group reaching the cut-off limit. The response in the 0–15,000 group was in general poor with positive outcomes below 20%, except for Candida stimulated CD8+ cells with positive outcomes at 37%. For CD4+ T-cells the best results were seen in the groups with cell numbers ranging from 15,000 to 60,000 precursor cells, while more than 15,000 cells were sufficient to give a positive response for CD8+ T-cells (Fig. 2A). The most

suitable cell number for a positive FASCIA response corresponds to 25 to 50 μL of whole blood, with a minimally required lymphocyte count of approximately 0.5×10^9 cells/L, why a lymphocyte count is recommended prior in combination with the assay.

3.4. FASCIA in the presence of immunosuppressants affects proliferative responses

Immunosuppressive drugs are used in various clinical settings, *e.g.* treatment of malignant disease, prevention against graft

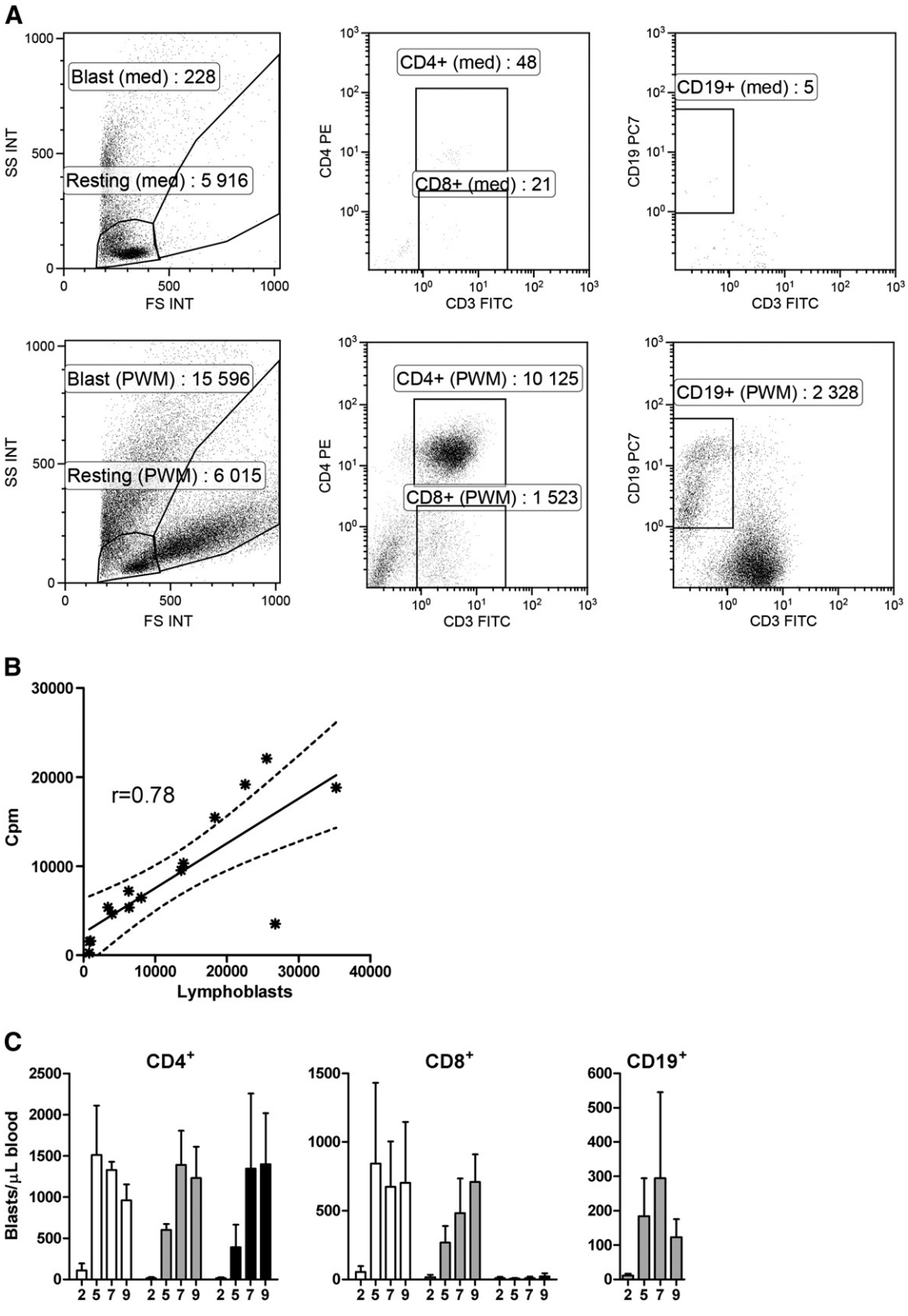

Table 1 Reference values from whole blood obtained from more than 100 healthy donors stimulated with a standardized mitogen and antigen panel.

Antigen	Cell fraction	*n*	Mean cells/μL	5th–95th percentile cells/μL
PWM	CD4	159	1483	411[a]–3019
	CD8	159	314	72[a]–691
	CD19	159	226	29[a]–738
PHA	CD4	159	1577	237–3562
	CD8	159	1241	75–3943
Con A	CD4	151	2141	665[a]–4553
	CD8	151	897	137[a]–2524
SEA + SEB	CD4	102	4148	507–8191
	CD8	102	1270	79–3088
PPD	CD4	140	482	4–2034
	CD8	140	11	0–52
Candida	CD4	131	653	7[a]–2436
	CD8	131	31	1[a]–60
Pneumococcal vaccine	CD4	146	34	0–147
	CD8	146	2	0–12
Tetanus toxoid	CD4	148	81	0–420
	CD8	148	2	0–10
Influenza vaccine	CD4	132	143	13–517
	CD8	132	33	0–56
Varicella zoster vaccine	CD4	103	51	0–170
	CD8	103	6	0–23

FASCIA reference values from healthy blood donors (n = 102–159). Results are expressed as mean number and 5th to 95th percentile of proliferating $CD4^+$, $CD8^+$ or $CD19^+$ (PWM only) per μL whole blood. The 5th percentile was used as a cut-off value to define a positive FASCIA outcome.

[a] 5th percentile; cut-off values used to define a positive FASCIA outcome.

Table 2 Proliferative responses by SCID patient's lymphocytes to PHA, PWM and Con A for the two proliferation assays.

	SCID 1	SCID 2	SCID 3	SCID 4	5th–95th
T-cell (%)	7	10	61[a]	1	48–75
$\times 10^9$/L	0.05	0.1	0.43	<0.01	2.3–6.5
B-cell (%)	75	9	12	94	14–39
$\times 10^9$/L	0.59	0.1	0.08	0.77[a]	0.6–3
NK-cell (%)	12[a]	77	24	1	2–14
$\times 10^9$/L	0.1[a]	0.89[a]	0.16[a]	<0.01	0.1–1.3
PHA-T	9	0	31	0	
PHA-F	0	1	25	0	
Con A-T	0	0	9	1	
Con A-F	0	19	73	0	
PWM-T	50	2	34	0	
PWM-F	2	14	15	0	

T = [^{3}H]thymidine incorporation, F = FASCIA.

FASCIA and [^{3}H]-thymidine incorporation was performed on whole blood and PBMCs obtained from four SCID patients. Cells were stimulated with PWM, PHA and Con A according to the assays respective protocols. The table shows percent of lymphocytes and absolute count ($\times 10^9$/L) of T, B- and NK-cells for each patient with 5th to 95th reference values for age matched controls as well as percent proliferation in FASCIA and [^{3}H]-thymidine incorporation compared to healthy control for each patient.

[a] Indicates values within the 5th to 95th percentile.

rejection and relief of symptoms in autoimmune and other chronic inflammatory diseases. Most immunosuppressive drugs act non-selectively [15], potentially increasing the risk of infections. To test the effect of immunosuppressive drugs *in vitro*, we used the FASCIA method and added clinically relevant doses of dexamethasone (0.1 μM), MMF (100 ng/mL), rapamycin (100 ng/mL) or tacrolimus (10 ng/mL). Whole blood, donated from 10 healthy controls, was stimulated with PWM or the super-antigens SEA and SEB in combination with or without the respective immunosuppressive drugs according to the FASCIA protocol. The number of $CD4^+$, $CD8^+$ and $CD19^+$ (PWM stimulation only) blasts obtained from cultures without the immunosuppressive drugs was compared to the number of blasts from the same donor obtained in the presence of the respective drug. Rapamycin and MMF decreased proliferation for $CD4^+$, $CD8^+$ and $CD19^+$ cells. Generally the major effects were observed in the $CD4^+$ T-cell population. Rapamycin decreased the mean number of $CD4^+$ proliferating T-cells/μL from 1359 to 267 cells/μL for PWM and from 2418 to 507 cells/μL for SEA + SEB stimulation. MMF decreased the $CD4^+$ proliferating T-cells from 1359 to 113 for PWM and from 2399 to 179 for SEA + SEB stimulation. Dexamethasone decreased proliferation of SEA + SEB stimulated $CD4^+$ T-cells (the decrease in $CD8^+$ blasts did not reach significance), while dexamethasone in combination with PWM stimulation decreased the proliferative response for $CD8^+$ and $CD19^+$ cells. However, $CD4^+$ cells stimulated with PWM in the presence of dexamethasone did not decrease in proliferation compared to controls. The mean number of proliferating cells/μL was 1358 without and 1470 with dexamethasone. This is a consequence of some individuals demonstrating an enhanced proliferative effect of PWM on $CD4^+$ T cells in the presence of dexamethasone, although the effect in most cases was suppressive. The reason for this variation in reactivity is unknown. Moreover, the

Figure 1 A: Flow cytometry gating strategies for enumeration of lymphoblasts in the FASCIA assay. Proliferating lymphoblasts are defined by size in forward scatter (left column) after seven days of incubation with or without immune-stimulatory antigens. The gated proliferating cells are further defined by lineage markers. The figure shows un-stimulated (Medium, upper row) or PWM-stimulated proliferation (lower row) by $CD4^+$, $CD8^+$ (middle column) or $CD19^+$ cells (right column) from a typical healthy donor. Each gate represents absolute cell count obtained during 80 s of acquisition. B: FASCIA and [^{3}H]-thymidine incorporation was performed on whole blood and PBMCs obtained from five healthy donors and stimulated with PHA, PWM and Con A. The figure shows correlation with 95 % confidence interval (dotted line) between incorporated [^{3}H]-thymidine expressed as counts per minute (cpm) and the number of lymphoblasts defined by forward scatter in FASCIA for all stimuli. C: FASCIA was performed on whole blood from healthy blood donors (n = 3). Cells were stimulated with Con A (white bars), PWM (grey bars) or Candida (black bars) and incubated 2, 5, 7 or 9 days. The $CD4^+$, $CD8^+$ and $CD19^+$ (PWM only) proliferation is expressed as blast/μL of whole blood. The error bars represent standard deviation.

Table 3 Cytokine/chemokines in plasma/serum from healthy donors.

	PWM *n* = 17		SEA + SEB *n* = 12		Candida *n* = 19		Plasma/serum	Plasma/serum
	Mean	5th–95th	Mean	5th–95th	Mean	5th–95th	Mean	5th–95th
Eotaxin	73	15–169	40	0–123	74	0–149	127	20–404
G-CSF	861	29–1974	87	0–334	1591	0–4951	21	0–63
GM-CSF	2227	326–5042	1908	538–4693	717	28–2943	21	0–93
IFNy	13,446	479–30,694	12,199	877–41,172	3720	1–13,148	32	0–95
IL-1α	498	73–946	147	41–341	160	3–343	41	0–202
IL-1β	1290	5–3570	294	8–1467	767	1–2331	5	0–23
IL-1Rα	106	58–145	70	50–91	60	25–87	70	3–211
IL-2	73	7–167	98	23–271	36	8–108	3	0–6
IL-4	136	9–258	87	19–197	119	2–265	3	0–9
IL-5	123	0–351	774	102–1970	193	0–518	1	0–3
IL-6	4863	172–9957	1168	98–4850	4550	11–10,433	9	0–35
IL-7	55	12–106	28	1–75	60	1–139	5	0–22
IL-8	13,005	0–38,380	13,260	3237–34,606	9743	0–22,222	10	0–26
IL-10	552	17–1148	194	18–351	190	1–585	10	0–30
IL-12(p40)	201	11–548	119	15–293	271	4–1058	32	0–128
IL-12(p70)	94	8–188	45	4–140	103	0–256	8	0–39
IL-13	337	5–842	914	137–2031	202	1–541	5	0–4
IL-17	580	55–1428	188	31–441	158	0–412	7	0–22
IP-10	10,999	1366–29,376	13,624	6700–30,310	4821	0–12,356	665	188–1675
MCP-1	12,149	1963–35,370	16,110	3790–45,648	11,492	2980–22,356	291	102–764
MIP-1α	5809	6–9654	3066	24–9817	5329	0–9674	33	0–78
MIP-1β	13,931	495–37,690	3150	75–12,642	12,431	54–40,079	32	11–57
TNFα	845	120–2008	599	64–1699	1179	8–3738	7	0–21
TNFβ	214	12–590	255	38–706	73	0–229	5	0–21
VEGF	193	149–236	166	134–198	164	58–253	183	50–437

Cytokine concentration in FASCIA cell supernatants obtained day seven from healthy donors (*n* = 12–19) after stimulation with PWM, SEA + SEB or Candida. The table shows results after subtraction of background values obtained from the un-stimulated "medium" culture supernatants expressed as pg/mL, with 5th to 95th percentile for the respective stimuli and cytokine/chemokine concentrations. The corresponding reference values measured in EDTA plasma or serum samples from healthy controls (*n* = 6–122) are also shown. Results are expressed as pg/mL, with 5th to 95th percentile for the respective cytokine/chemokine concentrations in plasma or serum.

effect of tacrolimus was not decisive. In combination with PWM stimulation proliferation decreased for $CD4^+$ and $CD19^+$ cells, but not for $CD8^+$ cells. There was no difference between cells stimulated with SEA + SEB without tacrolimus, compared those cultured with tacrolimus (Fig. 3). To continue, we performed intracellular staining of IL-17 and IFNγ in FASCIA in combination with tacrolimus and rapamycin after seven days of incubation. Whole blood obtained from four healthy donors was stimulated with PWM or SEA + SEB in combination with or without tacrolimus or rapamycin. The median percent IL-17-producing $CD4^+$ and IFNγ-producing $CD4^+$ and $CD8^+$ T-cells decreased in FASCIA cultures with both tacrolimus and rapamycin regardless of stimuli, compared to control cultures without the immunomodulatory compounds. There was a slight increase in the production of IFNγ by $CD4^+$ T-cells, stimulated with SEA + SEB in combination with tacrolimus (not significant), compared to control cultures without tacrolimus. This was not seen for $CD8^+$ T-cells or in cultures with rapamycin (data not shown).

3.5. A functional assay to monitor changes in proliferation over time

To test if the FASCIA assay can be used as a monitoring tool, three healthy donors were bled under similar circumstances during three consecutive weeks. The whole blood was diluted and stimulated with PWM, Con A and Candida according to the FASCIA protocol. The proliferation (in cells/μL) was stable over time for most cell types regardless of stimulation and donor origin. The responses were also similar between donors. The coefficient of variation (CV) was from 14 to 41% for $CD4^+$ T-cells, 9 to 62 % for $CD8^+$ T-cells and for $CD19^+$ B-cells between 4 and 64 % (Supplementary Fig. 1).

4. Discussion

Today, the golden standard to evaluate lymphocyte function in immunocompromised patients is to use PBMCs stimulated with mitogens, followed by incorporation of radioactive thymidine. The disadvantages with the assay are many. First, to obtain enough PBMCs, at least 4 mL of whole blood is needed; a large volume when obtained from a small child. Second, the preparation of PBMCs is time consuming and requires cell enumeration and third, handling of radioactive material can be potentially hazardous to health and also requires special facilities suitable for work with radioactive compounds and scintillation liquids [16]. Gaines et al. have previously shown a correlation between the flow cytometry based "FASCIA" assay and mitogen stimulation of PBMC pulsed with [^{3}H]-thymidine in a research setting [7]. Here we show that FASCIA is also a suitable assay for the clinical laboratory. FASCIA is an assay

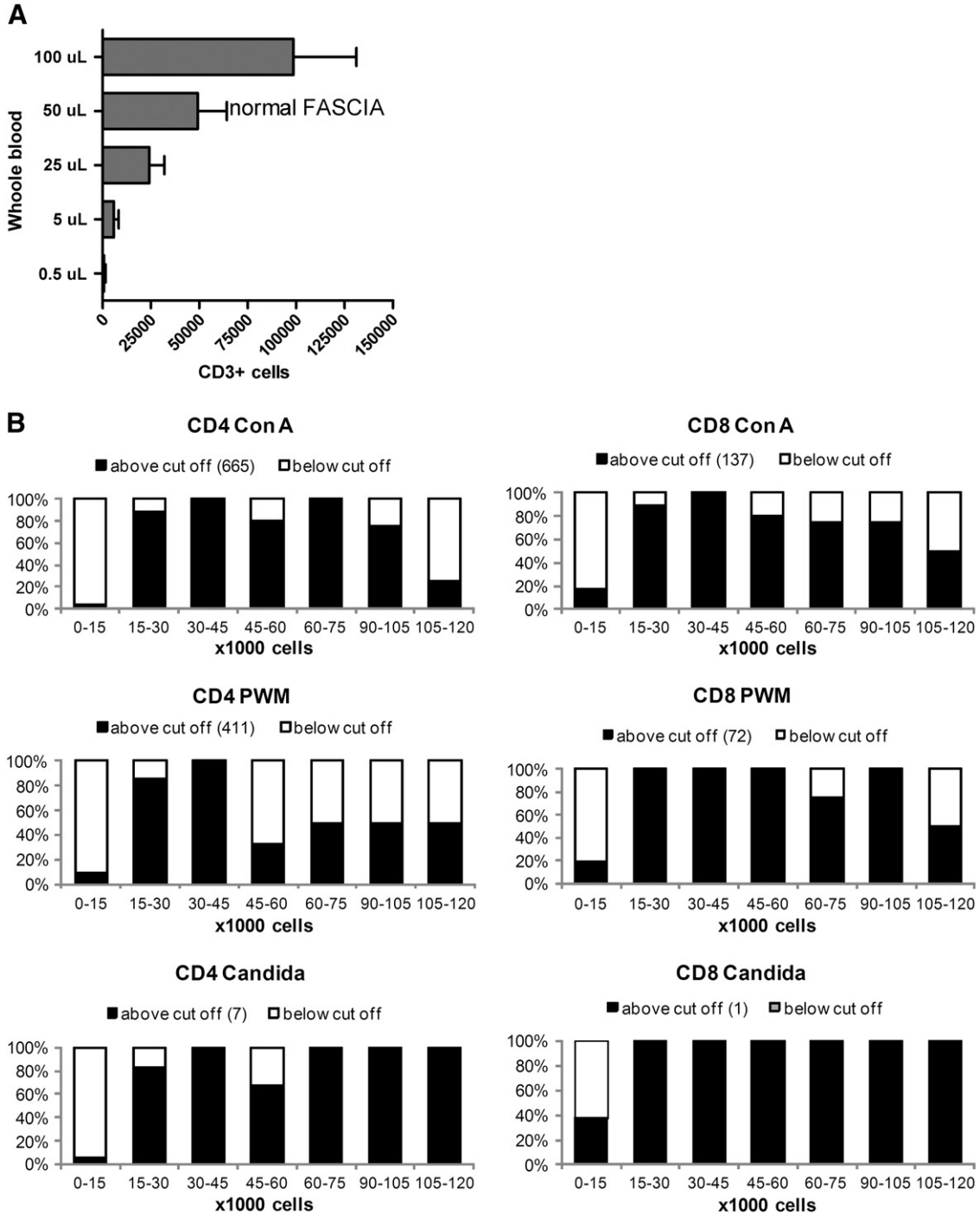

Figure 2 A: Absolute count of CD3+ T-cells was enumerated in titrated whole blood, volumes 0.5, 5, 25, 50 or 100 μL from healthy donors (n = 11) using BD Trucount tubes. In a normal FASCIA 50 μL of whole blood is used. The error bars represent standard deviation. B: To evaluate critically low precursor cell number we used the clinically relevant 5th percentile of our reference values as cut-off values for a positive outcome in FASCIA. Based of reference values the cut-off was the following: for Con A; $CD4^+$ 665 cells/μL, $CD8^+$ 137, for PWM; $CD4^+$ 411 cells/μL, $CD8^+$ 72 and for Candida $CD4^+$ 7 cells/μL, $CD8^+$ 1. Whole blood from 11 healthy donors were titrated from 0.5, 5, 25, 50 (normal FASCIA volume) to 100 μL and stimulated with Con A, PWM or Candida antigen according to the FASCIA protocol. Based on absolute count of $CD3^+$ T-cells in the titrated whole blood, the cells were divided into seven groups and a positive or negative FASCIA outcome was determined using the 5th percentile cut-off values. The figure shows percent proliferation, dependent of absolute count, above (black) or below (white) cut-off for $CD4^+$ or $CD8^+$ T-cells.

based on whole blood, diluted ten times with standard culture media, stimulated with mitogen or antigen specific stimuli followed by seven days of incubation. The read-out is lymphocyte blast formation in combination with monoclonal antibodies directed to T- and B-cell lineage markers. Thus, compared to the golden standard assay, FASCIA is comparably

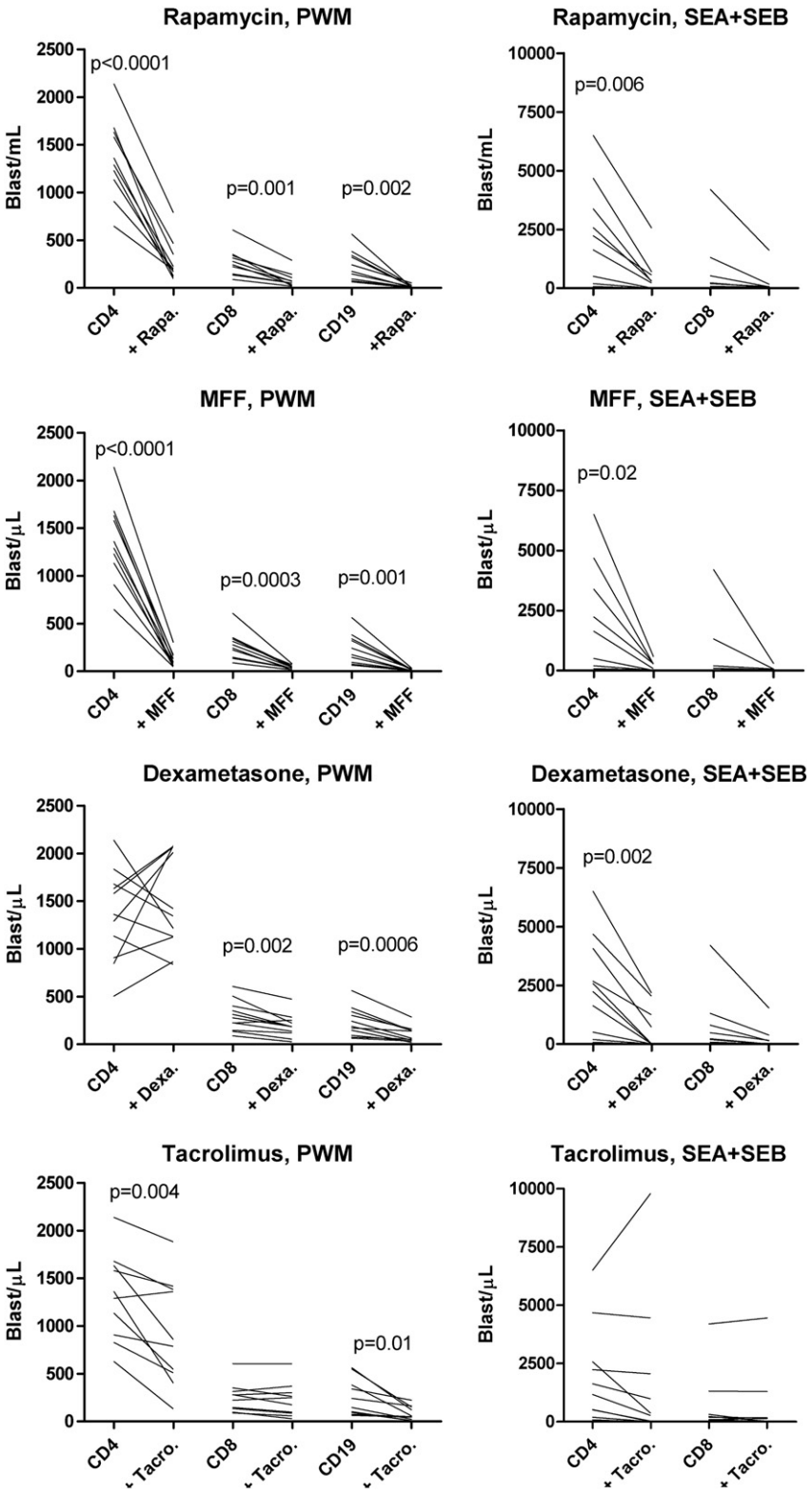

Figure 3 Whole blood from healthy donors (n = 11) was stimulated with PWM or SEA + SEB with or without 100 ng/mL rapamycin, 100 ng/mL MMF, 0.1 μg/mL dexamethasone or 10 ng/mL tacrolimus according to the FASCIA protocol. Proliferation is expressed lymphoblasts/μL whole blood for $CD4^+$, $CD8^+$ and $CD19^+$ (PWM only) cells. Each donor is represented by an individual line connecting the reactivity to a stimulant in the presence and absence of the immunosuppressive drug. A p-value above 0.05 was considered positive.

fast, safe and simple. In patient samples from four children under investigation for SCID, we compared proliferation by PBMCs in the [^{3}H]-thymidine incorporation assay with blast cell transformation in FASCIA. The two assays rendered similar results. However, one SCID patient showed proliferation to PWM in the [^{3}H]-thymidine incorporation assay but not in

FASCIA, whereas the proliferative response to PHA and Con A was non-existent in both assays. PWM stimulate B-cells poly-clonally [17] and PBMCs from a SCID patient with a T-B+ phenotype contains a high percentage of B-cells in the lymphocyte compartment [14]. This may explain the difference in proliferative response against PWM between the two assays, since in the [^{3}H]-thymidine incorporation assay the cell number per well is fixed which is not the case in the FASCIA. Moreover, *in vitro* responsiveness to antigens may vary over time and differ depending on patient age, disease and immunization history [13]. Depending on the underlying mutation, cells from patients with SCID can proliferate to some mitogens, but not others. Lymphocytes from ZAP-70 deficient patients will not proliferate when stimulated with PHA, due to defective calcium mobilization, but proliferate normally when stimulated with Ionomycin in combination with Phorbol myristate acetate [14].

The reference values shown here are all from adult healthy female and male blood donors, obtaining blood samples from healthy children is ethically challenging. The use of a large panel of mitogens as well as bacterial, fungal and viral antigen specific stimuli, *e.g.* PWM, PHA, Con A, SEA + SEB, PPD, Candida, tetanus toxoid, pneumococcal, influenza or varicella zoster vaccine allows a representative estimation of the lymphocyte function of an individual. We show, when blood samples from healthy donors were obtained and stimulated during three consecutive weeks, that the assay can be used as a monitoring tool to follow immunological changes over time, *e.g.* to follow cellular response after vaccination or post hematopoietic stem cell transplantation. FASCIA requires a small blood volume with practically endless possibilities for antigen specific stimuli. Every laboratory can use their protein antigen of choice to induce proliferation. When clinically applicable, we stimulate cells with peptides specific for Cytomegalo virus, Herpes simplex or Epstein Barr virus (not shown). Moreover, the use of flow cytometry enables detailed immunophenotyping of the proliferating cells, *e.g.* the use of lineage markers in combination with activation- and/or differentiation markers, such as CD69 [18] or CD45RA-expression [19]. The cytokine profiles of the cell supernatants after incubation can provide further information regarding lymphocyte function. We demonstrate that stimulation with PWM, Candida or a combination of super-antigens: SEA and SEB induce measurable concentrations of up to 25 cytokines and chemokines after 7 days of incubation.

One of the benefits with FASCIA, the direct dilution of whole blood without enumeration of cells may also be a drawback. Many PIDs are associated with a decreased number of leukocytes, compared to healthy controls [9], which possibly could affect FASCIA outcome. To evaluate critically low T-cell precursor number in each FASCIA tube we used the clinically relevant 5th percentile of our reference values as the cut-off. T-cell numbers that elicited proliferation higher than the 5th percentile were considered positive. The best proliferation was seen in tubes with T-cell numbers raging from 15,000 to 60,000 cells per tube. Thus, if the total cell number, in the respective FASCIA tubes, is below 15,000 $CD4^+$ or $CD8^+$ T-cells, the FASCIA results should be interpreted in combination with other laboratory findings as well as clinical symptoms. This corresponds to a minimal lymphocyte count of 0.5×10^9/L, if 50 μL of whole blood is used in the FASCIA.

Immunosuppressive drugs, such as MFF, rapamycin, tacrolimus or dexamethasone are used to prevent graft rejection, in the treatment of malignant and chronic inflammatory diseases [15]. The mechanism of action varies between the different drugs and they affect many different cell types [20–22]. In order to evaluate the effect of immunosuppressive drugs on T- and B-cell proliferation in FASCIA, we stimulated whole blood from healthy controls with PWM or a combination of SEA and SEB with or without clinically relevant doses of MFF, rapamycin, tacrolimus or dexamethasone. In tubes containing rapamycin and MMF T- and B-cell proliferation decreased compared to control tubes without the immunosuppressants. The effect of tacrolimus and dexamethasone was not as clear. Dexamethasone decreased proliferation compared to control in combination with SEA + SEB and PWM, except for $CD4^+$ cells stimulated with PWM where proliferation increased for six healthy controls and decreased for five healthy controls The reason for this is unknown but the discrepancy in proliferative responses between the donors might be explained by individual differences in dexamethasone sensitivity between T-cell subsets in the FASCIA culture tubes [23,24]. There was no difference in proliferation between cells stimulated with SEA + SEB in combination with or without tacrolimus. However, when tacrolimus was combined with PWM, proliferation decreased for $CD4^+$ and $CD19^+$ cells, but not $CD8^+$ cells. It has recently been shown that IL-17 production by $CD4^+$ cells stimulated with aCD3/aCD28 *ex vivo* was decreased by rapamycin but not by tacrolimus. In order to evaluate IL-17-producing T-cells in FASCIA, we performed intracellular staining of $CD4^+$ cells in cultures stimulated with PWM or SEA + SEB in combination with or without tacrolimus or rapamycin. Our data indicate that tacrolimus and rapamycin equally decrease cytokine production by $CD4^+$ T-cells both in PWM and SEA + SEB-stimulated cultures, compared to cultures without immunosuppressants. The inconclusive results between the chemically similar mTOR inhibitor rapamycin and calcineurin inhibitor tacrolimus may be explained by tacrolimus' shorter half-life or possibly by the ability of rapamycin to induce non-proliferating, suppressive T-cells [25,26].

Immunosuppressants are powerful drugs, affecting both cell function and proliferation and used in many clinical settings. It is therefore of major importance to evaluate laboratory data with this in mind. Proliferation by T- and B-cells from healthy donors in the FASCIA assay was decreased by most immunosuppressant drugs, indicating that cellular function in a patient, investigated for an immunodeficiency should, if possible, be performed in the absence of immunosuppressive drugs. Alternatively, future studies may reveal FASCIA useful for *in vitro* prediction of individual responses to immunosuppressive therapy.

Here we demonstrate that FASCIA, a flow cytometry-based functional assay for T- and B-cells, is a valuable tool for the clinical laboratory to investigate polyclonal as well as antigen specific blast formation in immunocompromised patients. During the last three years, the assay has been used as a routine diagnostic test at the laboratory for clinical immunology the Karolinska university hospital. Reference intervals of healthy, adult controls for reactivity upon mitogen stimulation and against common microbial antigens have been established. Today, the FASCIA has replaced [^{3}H]-thymidine incorporation as the standard assay for evaluation of lymphocyte reactivity; it is fast, simple, requires a small amount of whole blood and holds almost endless possibilities for development.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clim.2014.05.010.

Conflict of interest

The author(s) declare that there are no conflicts of interest.

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