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A new simple whole blood flow cytometry-based method for simultaneous identification of activated cells and quantitative evaluation of cytokines released during activation

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The multiple cellular and soluble elements of the immune system respond in a coordinated way, orchestrated by cytokines, to preserve the integrity of the organism. In this study, we describe a new and unique whole blood method that, with minimal sample manipulation, allows an overall evaluation of immune responses by simultaneously measuring cell activation and cytokine secretion. The identification of cells actively secreting cytokines is based on the stabilization of tumor necrosis factor α (TNFα) at the cell surface through the use of a specific inhibitor of the TNFα-converting enzyme. This inhibitor does not affect the release of cytokines other than TNFα and makes it possible to assess, in the same measurement, the phenotype of TNFα^+-secreting cells and quantify multiple secreted cytokines by using a specific and highly sensitive flow cytometry-based bead immunoassay. Upon stimulation of normal peripheral blood samples with either phorbol 12-myristate 13 acetate (PMA) plus ionomycin or lipopolysaccharide (LPS), both the number of TNFα^+ cells and the amount of secreted cytokines progressively increased, the former becoming detectable first. After stimulation for 3 h with PMA plus ionomycin, cellular responses were associated with surface TNFα expression on the majority of CD3$^+$ T cells and secretion of Th1-associated cytokines: interferon γ, interleukin (IL)-2, and to a lesser extent IL4. In turn, stimulation with LPS induced a response mainly by inflammatory cells. After 4 h of LPS-stimulation, the majority of CD14$^+$ monocytes showed surface TNFα expression; in parallel, high amounts of soluble IL1β, IL6, and IL8 became detectable. Likewise, stimulation of blood samples with cytomegalovirus (CMV) lysates induced viral-specific immune responses detectable in seropositive but not seronegative volunteers; such responses were associated with the detection of increased numbers of TNFα^+ monocytes, TNFα^+/CD8$^+$ T cells and TNFα^+/CD8$^-$ T lymphocytes in association with an increased secretion of IFNγ, IL6 and TNFα.
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Owing to the diversity of the cellular elements of the immune system, their wide distribution throughout the body and their mobility, immune responses require a complex coordinated network between different cell populations and soluble signalling elements.[1] Secretion of cytokines and chemokines together with the expression of their receptors on the cellular membrane or into soluble forms, direct the immune response in a coordinated manner, by inducing various reactions, including activation and/or inhibition of specific populations of naïve, memory and effector cells.[2]

For many years, it is believed that cytokines play a central role in the homeostasis of the immune system and the coordination of immune responses. In fact, different patterns of cytokine secretion have been identified which induce specific types of

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responses.[3] In recent decades, an increasingly high number of methods aimed at monitoring immune responses have been developed. At present, highly-sensitive methods are available that allow the identification of cytokine secretion at the messenger RNA (mRNA) level either using 'bulk' Northern and PCR techniques[4] or single-cell based, *in situ* hybridization approaches.[5] Despite this, direct evaluation of protein secretion is usually preferred,[6–11] because detection of specific mRNA does not assure secretion of the protein due to the translational regulation to which the synthesis of several cytokines is subjected.[12] Protein production and secretion can be studied by quantitative immunospecific methods such as enzyme-linked immunosorbent assay (ELISA) and Western blot.[4] Despite the high sensitivity and precision of these methods, none of them enables the simultaneous quantification of the cytokines produced or the identification and characterization of the secreting cells. In order to link cytokine secretion to the specific cell populations that produce them, new alternative approaches have been developed for identifying cytokine production at the single-cell level. Currently, the most representative techniques include the enzyme-linked immunosorbent spot assay (ELISPOT)[4] and flow cytometry procedures for the detection of either intracellular[13–15] or secreted cytokines captured and retained on the cell surface in an affinity matrix based on the use of bispecific (ie anti-CD45/anti-cytokine) antibody molecules.[16] Although these methods enable determination of which cells are actually producing a specific cytokine, they typically require extensive sample manipulation including inhibition of protein secretion or the prevention of cytokine diffusion into the extracellular medium, thereby altering the natural cytokine network. In addition, these latter approaches do not allow for the simultaneous quantitative evaluation of the overall production of one or more cytokines.

In the present study, we describe a new and simple flow cytometry whole blood method that allows simultaneous identification of specific populations of cytokine-producing cells and quantification of the multiple cytokines secreted by these cells into the extracellular medium, in which sample manipulation is minimal. The approach described allows rapid evaluation of immunological responses against nonspecific and specific antigens both at the cellular and soluble levels.

Materials and methods

Samples

Heparin anticoagulated peripheral blood (PB) samples from a total of 45 adult volunteers were obtained after informed consent according to the guidelines of the Ethics Committee of the University Hospital of Salamanca (Salamanca, Spain). In all experiments, PB samples were prepared and cultured *in vitro* within a maximum period of 2 h, after they were collected. Of these 45 samples, 12 were used for the evaluation of cytomegalovirus (CMV)-specific immune responses. From them, seven corresponded to normal healthy CMV-seropositive and five to CMV-seronegative donors. CMV-serostatus was determined by an immunoglobulin G (IgG) and immunoglobulin M (IgM) ELISA (VIDAS®CMV IgG and VIDAS®CMV IgM, Biomérieux, Marcy ÍEtoile, France). For stimulation purposes, PB samples were diluted 1:5 (vol:vol) in RPMI 1640 (Biowhittaker, Walkersville, MD, USA) containing 1% (w/v) L-glutamine (Sigma, St. Louis, MO, USA), except for the 12 samples used for the evaluation of CMV-specific immune responses, which were diluted 1:2 (vol:vol).

In Vitro Stimulation of Cytokine Production by PB Monocytes and T Lymphocytes

RPMI 1640-diluted whole blood samples were aliquoted into tubes containing 250 μl of the diluted sample either in the presence or the absence of 20 μmol/l of the BB3103 tumor necrosis factor (TNF)-α-converting enzyme (TACE) inhibitor (kindly provided by British Biotechnology, Oxford, England). For stimulation purposes 0.1 μg/ml lipopolysaccharide (LPS) (SIGMA) or 0.025 μg/ml of phorbol 12-myristate 13 acetate (PMA) (SIGMA) plus 1 μg/ml of ionomycin (SIGMA) or 5 μg/ml of CMV whole lysate (Advanced Biotechnologies Inc., Columbia, MD, USA) were added, to induce cytokine secretion by PB monocytes and T lymphocytes.[18–20] Additionally, in those samples ($n = 12$) used for the CMV-lysate stimulatory assays, purified costimulatory monoclonal antibodies (MAb) directed against CD28 and CD49d (Becton/Dickinson Biosciences (BDB), San Jose, CA, USA) were added at a final concentration of 1 μg/ml for optimal stimulation of T cells with various activation thresholds.[17] The tubes were placed in a sterile environment, at 37°C in a 5% CO_2 humidified atmosphere and cultured for variable periods of time. LPS-stimulated blood samples were harvested after 30 min, 1, 2, 3, 4, 5 and 6 h of incubation, PMA plus ionomycin-stimulated samples were harvested after 30 min, 1, 2, 3 and 4 h and CMV-stimulated samples were collected after 6 h of incubation,[20] as described below in the Results section. In all experiments, an unstimulated sample aliquot was always processed in parallel, as a negative control.

Quantification of Cytokines Secreted into the Culture Supernatants

For the quantitative evaluation of cytokine secretion into the culture supernatants, 50 μl of supernatant were collected at each of the time points indicated above. The Cytometric Bead Array immunoassay system (CBA™, BDB) was used to quantify either single cytokines—human interleukin (IL) 6 ($n = 5$),

interferon γ (IFNγ) ($n = 5$) and tumor necrosis factor α (TNFα) ($n = 6$)—or to assess simultaneously IFNγ, TNFα, IL2, IL4, IL5 and IL10 (Human Th1/Th2 CBA kit; BDB)—on PMA/ionomycin-stimulated samples ($n = 5$), TNFα, IL1β, IL6, IL8, IL10, IL12 (inflammatory CBA kit; BDB)—on LPS-induced blood cell activation ($n = 7$) or IFNγ, TNFα, IL2, IL4, IL6 and IL10 (Human Th1/Th2 CBA kitII, BDB) or CMV-lysate stimulatory assays ($n = 12$).

Briefly, 50 μl of the supernatant were incubated at room temperature for 2 h (Th1/Th2 CBA kit and individual cytokines) or 3 h (Inflammatory CBA kit) with 50 μl of anticytokine MAb-coated beads and with 50 μl of the appropriate phycoerythrin (PE)-conjugated anticytokine antibody detector. After this incubation period, samples were washed once (5 min at 200 g) in order to remove the excess of detector antibodies. Immediately afterwards, data acquisition was performed on a FACSCalibur flow cytometer (BDB) equipped with an argon ion laser and a red diode laser, using the CellQUEST™ software program (BDB); information was stored for at least 300 events corresponding to each bead population analyzed per sample. In all cases, light scatter parameters were collected using a four-decade logarithmic amplification mode, following the recommendation of the manufacturer. For data analysis, both the Paint-A-Gate and the Cytometric Bead Array (CBA) software programs (BDB) were used. During analysis, the mean fluorescence intensity (MFI) value for the PE-associated fluorescence was recorded for each population of cytokine capture-beads.

To allow quantification of the captured cytokines, standard curves were developed using cytokine standards. Standard curves were plotted (cytokine calibrator concentration *vs* mean PE fluorescence intensity) using a four-parameter logistic curve fitting model (BD CBA software). A total of 12 standards for each cytokine were used at concentrations ranging from 0 to 10 000 pg/ml. Concentration of the cytokine in the sample supernatant was considered to be directly proportional to the mean fluorescence intensity of the corresponding bead population measured in fluorescence channels (relative linear arbitrary units scaled from 0 to 10^4). The specific concentration of each cytokine analyzed was determined through the comparison of the MFI of unknown samples to the standard curve.

Simultaneous Identification of Cell Activation and Quantification of Secreted Cytokines

For the simultaneous identification of cytokine-producing cells and the quantification of secreted cytokines, a total of five PB samples, stimulated as described above with either LPS or PMA plus ionomycin plus 12 PB samples stimulated with CMV-whole lysate were used. Amounts of 50 μl of the stimulated samples were harvested at the time periods listed above and immediately incubated at

room temperature for 2 h (PMA plus ionomycin or CMV-stimulated samples) or 3 h (LPS-stimulated samples) with 50 μl of the appropriate cytokine-capture bead populations and the appropriate mixture of PE-conjugated detector antibodies. At 15 min prior to the end of this incubation period, cell surface stainings were performed by adding either 10 μl of anti-CD14-FITC (MϕP9, BDB), 3 μl of anti-TNFα-PE (MAb11, BDB) and 10 μl of anti-CD45-PC5 diluted 1/10 in phosphate-buffered saline (PBS) (J33 MAb, Immunotech, Marseille, France) to LPS-stimulated samples or 10 μl of anti-CD3-FITC (HIT3a, BDB) plus 3 μl of anti-TNFα-PE, 10 μl of anti-CD45-PC5 and 10 μl of anti-CD4-APC (SK3, BDB) or anti-CD8 APC (B9.11, Immunotech) to either PMA/ionomycin- or CMV-stimulated samples, respectively. Prior to data acquisition, the sample containing both the stained cells and the cytokine-capture bead mixture was centrifuged (200 g, 5 min) and resuspended in 300 μl of PBS.

Absolute counts for each of the different subsets of PB leukocytes were calculated after adding known numbers of antibody-coated beads to each tube by reverse-pipetting, according to well-established methods.[21]

In all LPS- or PMA plus ionomycin-stimulated samples, soluble cytokine levels were also measured on the culture supernatant containing the plasma of the same stimulated specimen using the same cytokine-capture beads according to the instructions of the manufacturer, as described above. In order to make comparable the results of soluble cytokine measurements in culture supernatants and RPMI-diluted whole blood samples, the latter was corrected according to the haematocrite.

Data acquisition was performed on a FACS-Calibur flow cytometer, collecting information about between 5×10^4 and 1.5×10^5 cells. Unstimulated samples were analyzed to verify the effect of the stimulation. Evaluation of cytokine production was based on percentage of positive cells, after subtracting the percentage of cells staining above the threshold for positivity in the negative control.

Cell Staining for Intracellular Cytokines

Intracytoplasmic detection of TNFα production by PB lymphocytes and monocytes was performed in those 12 PB used to evaluate CMV-specific immune responses. After culturing 250 μl of RPMI diluted (1:2, vol:vol) PB for 2 h in the presence of 5 μg/ml of CMV whole lysate and the anti-CD28 and anti-CD49d costimulatory MAbs, 10 μg/ml of brefeldin A (BFA; Sigma) was added to each tube. Then cells were incubated for 4 h more. Afterwards, 10 μl of anti-CD3-FITC, 10 μl of anti-CD45-PC5, and 3 μl of CD8-APC were added and the cells were incubated for 15 min at room temperature in darkness. Immediately after this incubation period, cells were fixed, permeabilized and stained with 3 μl of anti-

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TNFα-PE MAb, using the Fix & Perm reagent (Caltag Laboratories, San Francisco, CA, USA). Finally, cells were washed once in 2 ml of PBS (5 min at 540 g) and resuspended in 0.5 ml of PBS for data acquisition in a FACS-Calibur flow cytometer, as described above. Unstimulated samples were analyzed to verify the effect of the stimulation. Evaluation of cytokine production was based on percentage of positive cells, after subtracting the percentage of cells staining above the threshold for positivity in the negative control.

Recovery Experiments

Recovery experiments were performed in parallel on five different unstimulated RPMI-1640-diluted (1/5, vol:vol), IFNγ-free, normal PB samples and their corresponding plasmas. For that purpose, precisely defined amounts of IFNγ (1250 and 5000 pg/ml) were added in parallel to each blood and plasma paired samples. Measurement of the spiked cytokine from the two sample types (RPMI-diluted blood and plasma supernatant) was performed by incubating a mixture of a volume of 50 μl of IFNγ-spiked sample and 50 μl of a solution containing beads coated with an anti-IFNγ monoclonal antibody (kindly provided by BDB) under the same conditions as those described above for the quantitative evaluation of soluble cytokines in culture supernatants.

Statistical Methods

Median and mean values and their standard deviations, as well as range, were calculated for each variable under study using the SPSS software (version 10.0; SPSS, Chicago, IL, USA). The statistical significance of the differences observed between groups was assessed using either the nonparametric Friedman and Wilcoxon tests for paired samples or the nonparametric Mann–Whitney U-test for unpaired samples (SPSS 10.0 software); P-values <0.05 were considered to be associated with statistically significant differences.

Results

Simultaneous Analysis of TNFα⁺-Activated Cells and a Secreted Cytokine Captured on Antibody-Coated Beads

By setting a threshold on red fluorescence (FL3 parameter in the flow cytometer instrument used), a clear discrimination could be achieved in whole blood samples between beads and CD45⁺ leukocytes on one side, and CD45⁻ red cells and platelets on the other (Figure 1a). Bead particles and leukocytes showed different light scatter characteristics, which further allowed specific discrimination between them and simultaneous identification of beads and leukocytes as illustrated in Figure 1b. The

feasibility of analyzing further stainings separately on specific leukocyte subsets and cytokine-capture beads in either unstimulated or stimulated non-lysed-nonwashed whole blood samples is illustrated in Figure 1, in panels c-f. Stimulation of PB with either LPS, in the presence of IL6 capture beads (Figure 1d) or with PMA plus ionomycin, in the presence of IFNγ capture beads (Figure 1f), was associated with a significant increase in the PE-associated fluorescence of the capture beads due to secretion of IL6 (Figure 1d) and IFNγ (Figure 1f), respectively, as compared to nonstimulated paired samples treated under identical conditions (Figure 1c and e, respectively). Simultaneously, the subsets of leukocytes showed accumulation of TNFα on the cell surface membrane; these cells corresponded to CD14⁺ monocytes in LPS-stimulated samples (Figure 1d) and CD3⁺ T cells in PMA plus ionomycin-activated cell cultures (Figure 1f).

Kinetic Analysis of Immune Responses to LPS and PMA Plus Ionomycin through the Simultaneous Measurement of TNFα⁺-Cytokine-Secreting Cells and Quantification of Soluble Amounts of Secreted Cytokines

In order to evaluate the kinetics of the effect of the BB3103 inhibitor on TNFα secretion by PB leukocytes stimulated with either LPS or PMA plus ionomycin, sequential analysis of TNFα secretion in the presence or absence of BB3103 was performed. As shown in Table 1, the BB3103 compound showed an effectiveness of between 90 and 95% during the period of time analyzed (between 30 min and 6 h, after stimulation).

TNFα⁺ monocytes were already present at detectable numbers after 30 min of stimulation with LPS (Figure 2a), whereas secreted IL6 soluble levels could only be detected in the same sample aliquots after 2 h of culture (Figure 2b). Since TNFα⁺ monocytes were first detected, their relative number progressively increased till the last time-point analyzed (6 h) (Figure 2a); at the same time, soluble amounts of IL6 also increased progressively. As shown in Figure 2b, no significant differences were observed between the amount of soluble IL6 detected in whole blood and the corresponding plasma samples, at any time-point analyzed.

Kinetic analysis of the T-cell response in whole blood samples stimulated with PMA plus ionomycin was performed through the simultaneous enumeration of TNFα⁺ T cells and quantification of soluble amounts of secreted IFNγ. As shown in Figure 2c and d, TNFα⁺ T cells and soluble IFNγ levels were already detectable 30 min and 1 h after stimulation, respectively. Thereafter, both the percentage of TNFα⁺ T cells and the amount of soluble IFNγ progressively increased till the last time-point analyzed (4 h) (Figure 2c and d). In contrast to what occurred with the measurement of soluble amounts
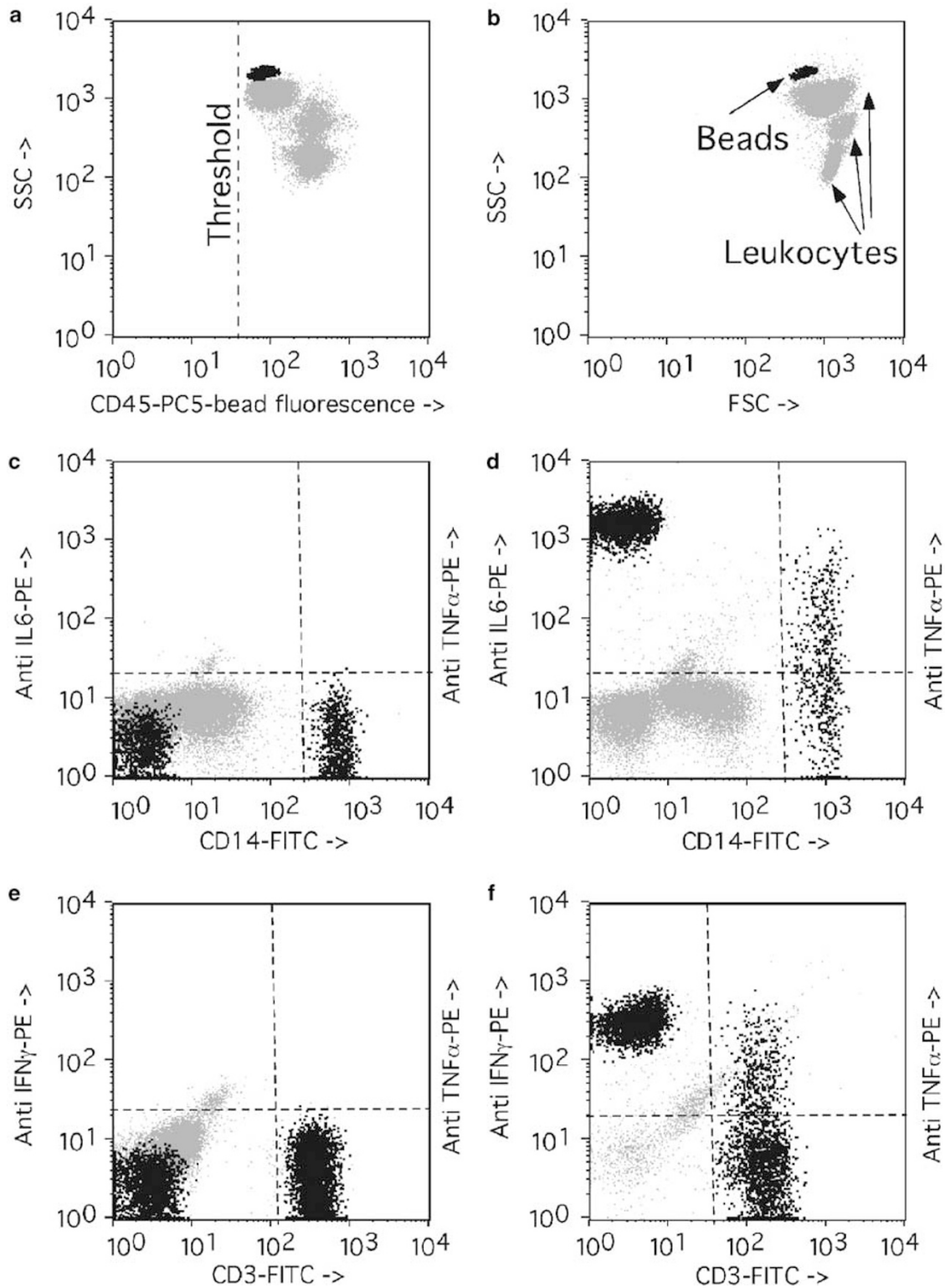
Figure 1 Representative bivariate dot plots of experiments in which assessment of TNFα expression on activated cells and quantitation of soluble cytokines captured on beads were simultaneously performed. Dot plot **a** shows how the threshold was set to distinguish events corresponding to either beads or CD45^{+} leukocytes from other events. In panel **b**, events corresponding to beads were specifically distinguished (black dots) from those representing CD45^{+} cells (gray dots) on the basis of their higher sideward (SSC) and forward (FSC) light scatter properties. Plots **c** and **d** show a simultaneous staining for IL6 capture beads (black dots in the left side of both plots) and TNFα on CD14^{+} monocytes (black dots in the right side of the two plots) from a peripheral blood sample mixed with the BB3103 compound and cultured for 4 h either in the absence (panel **c**) or in the presence of 0.1 μg/ml of LPS (panel **d**). In the lower two panels, simultaneous stainings for IFNγ on beads (black dots in the left side of both plots) and TNFα on CD3^{+} T cells (black dots in the right side of both plots) from a peripheral blood sample mixed with the BB3103 compound and cultured for 3 h either in the absence (panel **e**) or in the presence of PMA (0.025 μg/ml) plus ionomycin (1 μg/ml), are shown.

Table 1 Kinetic analysis of the effect of the BB3103 TACE inhibitor on TNFα secretion by PB leukocytes stimulated with either LPS or PMA plus ionomycin

Time (h)	Soluble TNFα levels in LPS-stimulated PB			Soluble TNFα levels in PMA+ionomycin-stimulated PB		
	Without BB3103 (pg/ml)	With BB3103 (pg/ml)	% Inhibition	Without BB3103 (pg/ml)	With BB3103 (pg/ml)	% Inhibition
2	NA	NA	NA	4770 ± 2223	320 ± 145	92 ± 2
3	1289 ± 534	54 ± 31	96 ± 3	8260 ± 2631	756 ± 261	89 ± 10
4	4315 ± 1383	273 ± 112	93 ± 4	10 909 ± 3475	1247 ± 770	89 ± 4
5	6250 ± 2154	538 ± 151	91 ± 3*	NA	NA	NA
6	7553 ± 2171	722 ± 181	90 ± 2*	NA	NA	NA
P-value	0.0001	0.0001	0.03	0.006	0.03	0.51

Results are expressed as mean ± standard deviation values. NA: not analyzed.
*Statistically significant differences ($P < 0.05$) found in comparison with the percent inhibition observed at 3 h.
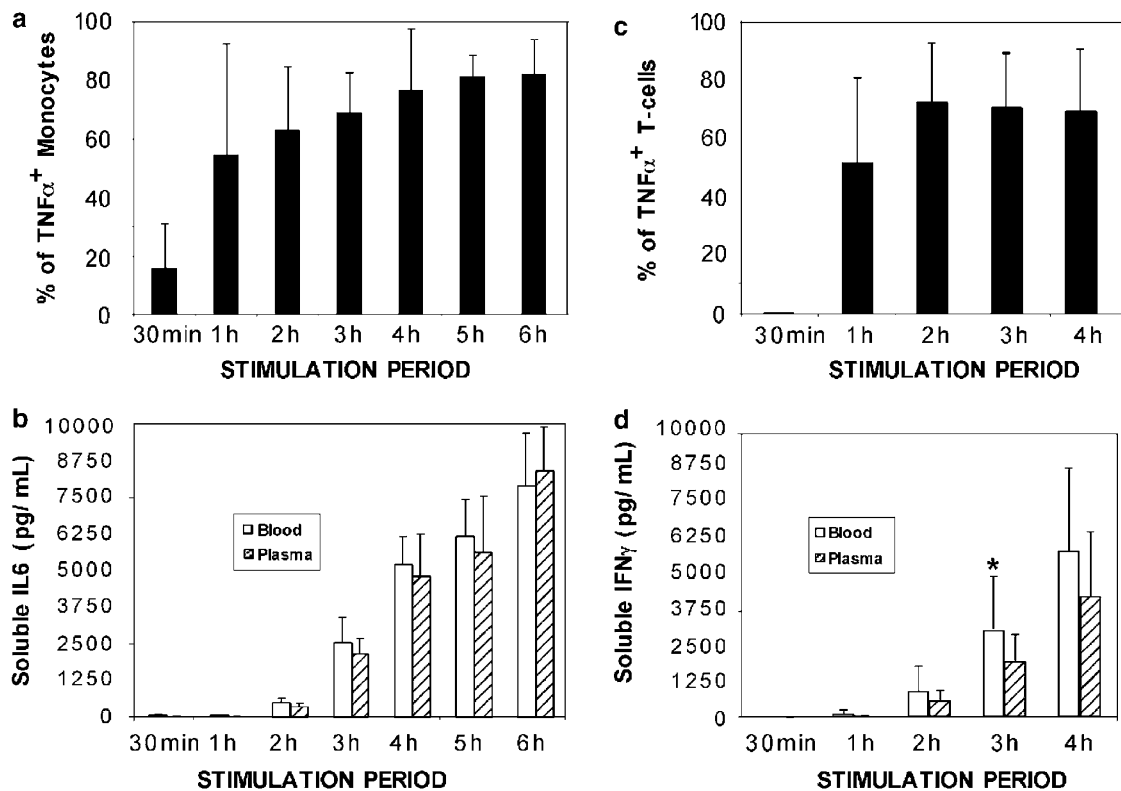

Figure 2 Kinetic analysis of both CD14^{+} monocyte and CD3^{+} T-cell responses after stimulation of PB samples ($n = 5$) with LPS (panels **a** and **b**) or PMA plus ionomycin (panels **c** and **d**) for different time periods. In panels **b** and **d** the amount of secreted IL-6 (pg/ml) and IFNγ (pg/ml) in paired blood and plasma samples, are shown, respectively. Panels **a** and **c** show the percentage of TNFα-secreting monocytes after stimulation with LPS (panel **a**) and TNFα-secreting T lymphocytes after PMA plus Ionomycin stimulation (panel **c**) at different times in culture. *Statistically significant differences were found between IFNγ levels in blood and plasma after 3 h of incubation with PMA plus ionomycin ($P < 0.05$).

of IL6 after stimulation with LPS, quantification of soluble IFNγ from paired blood and plasma samples after stimulation with PMA plus ionomycin provided different results. Accordingly, blood levels of soluble IFNγ were constantly higher than the plasmatic ones (Figure 2d), differences reaching statistical significance at 3 h after stimulation. In order to compare the precision of the measurements performed in both types of samples, recovery experiments in which known amounts of IFNγ were spiked into IFNγ-free paired blood and plasma samples, were performed. As shown in Table 2,

blood measurements showed both a better recovery and a better precision than those performed on the plasma samples.

Simultaneous Analysis of the Activation of Multiple Cell Subsets and Quantification of Secretion of Multiple Soluble Cytokines in Response to LPS and PMA Plus Ionomycin

Based on the results of the kinetic studies described above, for those experiments aimed at the simulta-

neous detection of TNFα-secreting cells and the quantification of multiple soluble cytokines produced, the time-points for the evaluation of responses to LPS and PMA plus ionomycin were set at 4 and 3 h after stimulation, respectively. Accordingly, in the same sample tube, we could simultaneously identify LPS-responding CD14$^+$ monocytes and quantify the levels of several different inflammatory cytokines produced in the extracellular medium. In a similar manner, we could also identify TNFα$^+$ cells in response to PMA plus ionomycin within different subsets of T cells (CD3+CD4− or CD3+CD4+) and, at the same time, quantify the soluble levels of different cytokines they released, through the use of a mixture of different populations of cytokine-specific capture-beads (Figure 3a and b).

Table 2 Recovery of spiked IFNγ into paired whole blood and plasma samples ($n = 5$)

	Amount of IFNγ spiked into the sample	
	1250 (pg/ml)	5000 (pg/ml)
Recovered IFNγ (pg/ml) (% recovered)		
Blood	1206±99 (96±8)	4859±315 (97±6)
Plasma	905±148 (72±12)	3284±626 (66±13)
P-value	0.04	0.04

Results are expressed as the mean amount of IFNγ±standard deviation (the percentage of spiked cytokine recovered is shown in brackets).
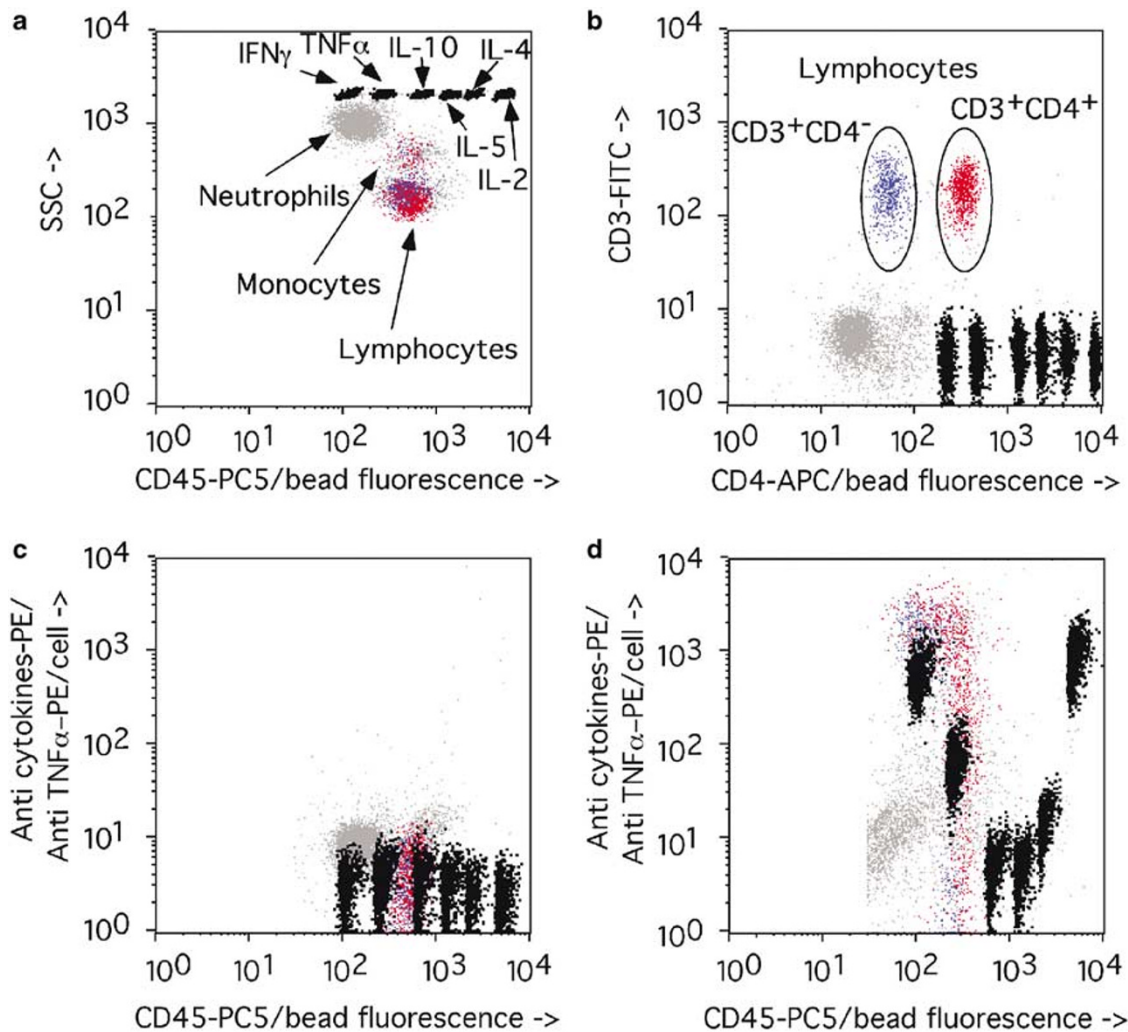

Figure 3 Representative bivariate dot plots of an experiment in which assessment of TNFα expression on activated T cells and quantitation of multiple soluble cytokines (IFNγ, TNFα, IL2, IL4, IL5 and IL10) captured on cytokine specific populations of beads, were simultaneously performed. Panel **a** illustrates how the different bead populations were identified based on their intrinsically distinct red fluorescence (black dots). Panel **b** shows how the two major CD3$^+$/CD4$^+$ (red dots) and CD3$^+$/CD4$^-$ (blue dots) T cell subsets were identified. In the lower two panels, the amount of secreted cytokines captured in their specific cytokine-capture bead population (black dots) and the amount of TNFα expressed on the surface of both CD3$^+$/CD4$^+$ (red dots) and CD3$^+$/CD4$^-$ T cells are shown for the same PB sample cultured for 3 h in the absence (panel **c**) or the presence of PMA (0.025 μg/ml) plus ionomycin (1 μg/ml) (panel **d**). Events corresponding to CD3$^-$ PB non-T-cell leukocytes are depicted as gray events in all four dot plots.

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LPS stimulation of blood cells induced TNFα expression on cytokine-secreting monocytes (77±21%) together with production into the extracellular medium of similar amounts of IL1β (4247±2368 pg/ml), IL6 (3390±762 pg/ml), IL8 (3664±1241 pg/ml) (Figure 4a), as well as TNFα (273±112 pg/ml). As shown in Figure 3, after 3 h of stimulation with PMA plus ionomycin, both CD4$^+$/TNFα$^+$ and CD4$^-$/TNFα$^+$ T cells could be detected, representing 82±11 and 82±5% of all CD4$^+$ and CD4$^-$ T cells. Such response was associated with production in the culture supernatant of detectable levels of Th1 cytokines (IFNγ: 3747±1727 pg/ml; TNFα: 223±127 pg/ml; and IL2: 1163±381 pg/ml) as well as low levels of IL4 (50±22 pg/ml) in the absence of other Th2 cytokines (undetectable amounts of IL-5 and IL-10) (Figure 4b).

Simultaneous Analysis of the Activation of Multiple Cell Subsets and Quantification of Secretion of Multiple Soluble Cytokines in Response to CMV

After stimulation with a CMV lysate, both TNFα$^+$ monocytes and TNFα$^+$ T cells became detectable. The percentage of both TNFα$^+$ monocytes and TNFα$^+$ T cells was similar once TNFα was detected at the cytoplasmic level after inhibiting protein secretion with brefeldin A or it was measured at the cell surface with the use of the TACE inhibitor:
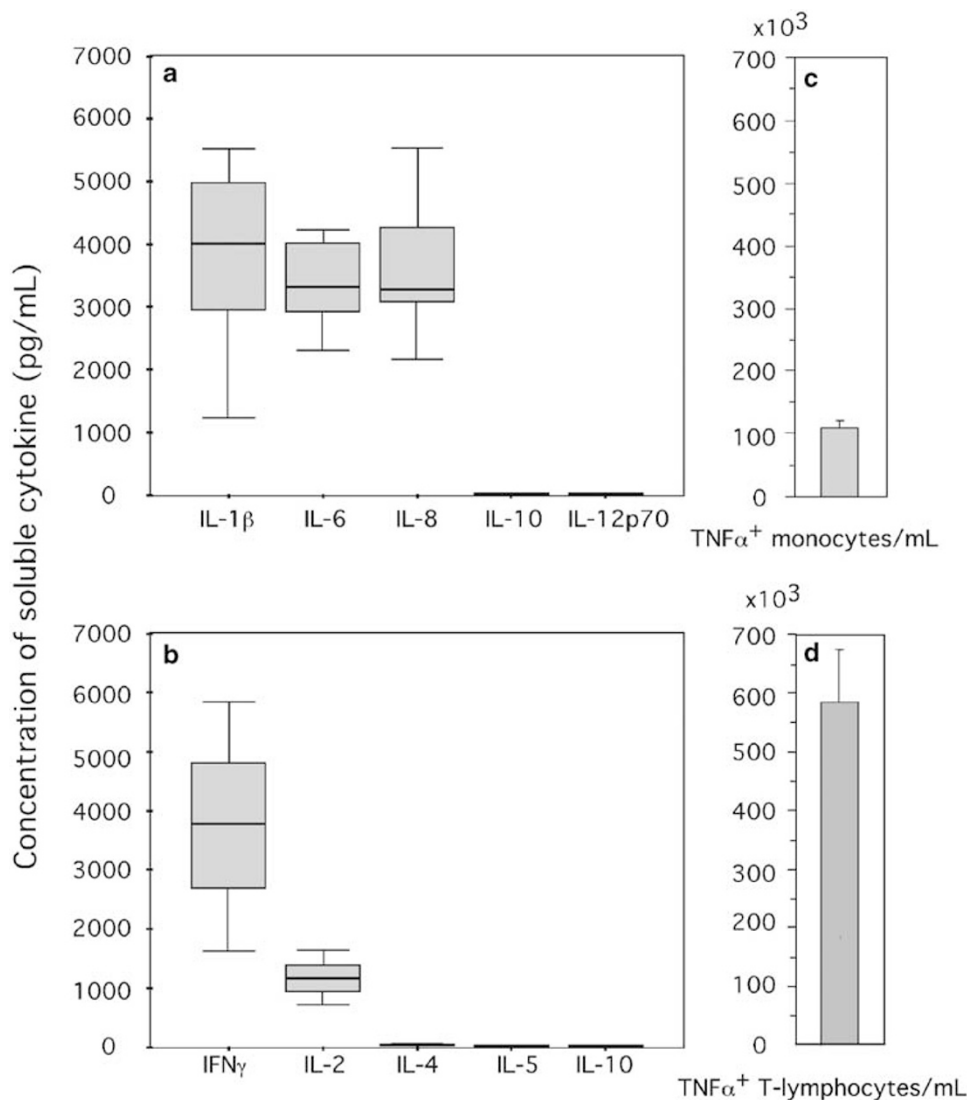

Figure 4 Total amount of soluble cytokines secreted by PB cells ($n = 5$) stimulated with either 0.1 μg/ml of LPS (panel **a**) or 0.025 μg/ml of PMA plus 1 μg/ml of ionomycin (panel **b**) after 4 and 3 h of culture, respectively. Panels **c** and **d** show the number of TNFα$^+$ monocytes and TNFα$^+$ T cells present in the same samples after stimulation with LPS or PMA plus ionomycin, respectively. Boxes extend from the 25th to the 75th percentiles; the line in the middle and the vertical lines represent median values and 95% confidence intervals, respectively.
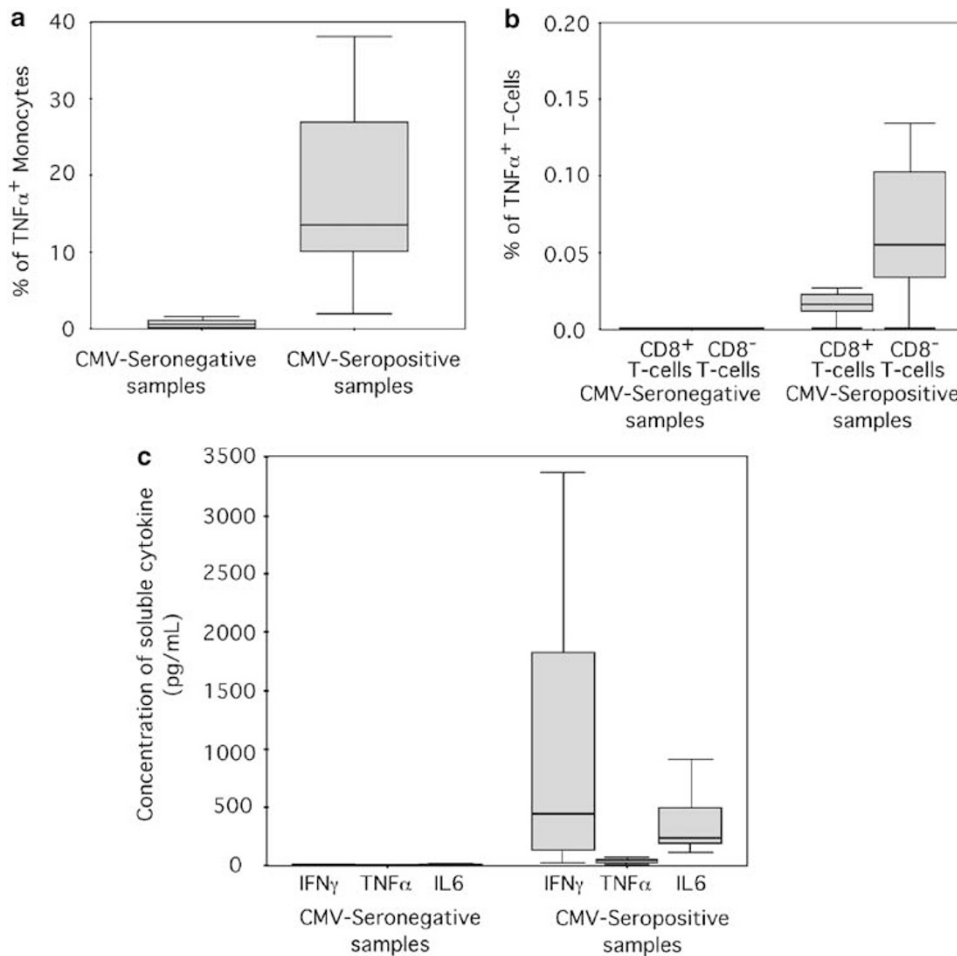
Figure 5 Comparative analysis of the PB monocyte and T-cell responses after 6 h of stimulation with CMV lysate in the presence of the BB3103 TACE inhibitor, in CMV-seropositive ($n=7$) and CMV-seronegative ($n=5$) volunteers. The two upper panels show the percentage of TNFα$^+$ monocytes (panel **a**) and of TNFα$^+$/CD8$^+$ and TNFα$^+$/CD8$^-$ T cells (panel **b**) from total monocytes and T cells, respectively. The lower panel (panel **c**) shows the soluble concentrations of secreted cytokines by TNFα$^+$-cytokine secreting monocytes and T cells. Boxes extend from the 25th to the 75th percentiles; the line in the middle and the vertical lines represent median values and 95% confidence intervals, respectively.

median number of TNFα$^+$ monocytes of 2.4 vs 11% (range: 1.5–3.4 vs 1.8–18%, respectively; $P=0.5$); of TNFα$^+$CD8$^+$ T cells, 0.01 vs 0.01%; (range 0–0.03 vs 0–0.02%, respectively; $P=0.14$), and of TNFα$^+$CD8$^-$ T cells of 0.03 vs 0.03%; range (0–0.3 vs 0–0.05%, respectively; $P=0.27$). As shown in Figure 5, after 6 h of stimulation with CMV lysate, higher percentages of TNFα$^+$ monocytes were observed in CMV-seropositive donors as compared with CMV-seronegative individuals (18 ± 14 vs 0.6 ± 0.6%, $P=0.004$). Likewise, the percentages of both TNFα$^+$CD8$^-$ (0.14 ± 0.22 vs 0 ± 0%, $P=0.009$) and TNFα$^+$CD8$^+$ (0.03 ± 0.04 vs 0 ± 0%, $P=0.009$) T-lymphocytes was also higher in CMV-seropositive samples. Additionally, as might be expected, the soluble concentrations of secreted IFNγ (1105 ± 1296 vs 3.3 ± 5.6 pg/ml; $P=0.007$), TNFα (42 ± 39 vs 0 ± 0 pg/ml; $P=0.003$), IL-6 (371 ± 319 vs 24 ± 48 pg/ml; $P=0.007$) were equally higher in CMV-seropositive as compared to CMV-seronegative

samples. In contrast, virtually no secretion of IL10 and IL4 was detected in both groups of individuals.

Discussion

In recent years, important advances have been achieved in the understanding of the immune system and its involvement in the development, progression and control of an increasingly high number of disease conditions.[22,23] In addition, new preventive and curative therapies have been developed and assayed, which are based on the modulation of the status of the immune system and of immune responses,[24] including vaccination trials against infectious agents (ie HIV) or tumors (ie melanomas).[25,26] Owing to this, an increasingly high demand exists for immune monitoring approaches. Such immune monitoring procedures should ideally be able to detect specific qualitative and quantitative

changes at both the cellular and soluble levels, in the absence of extensive *ex vivo* or *in vitro* sample manipulation.

In the present study, we describe a new and simple flow cytometry-based method for the overall evaluation of immune responses at both the cellular and soluble levels. The reported technique is specifically aimed at the simultaneous immunophenotypic identification and enumeration of cytokine-secreting cells and the quantification of multiple cytokines secreted by these cells into the extracellular medium. As shown, this method could be applied to the direct analysis of immune responses in whole blood specimens reducing sample manipulation and fractioning to a minimum.

In order to identify cytokine-secreting cells in the absence of extensive sample manipulation, the use of specific markers expressed at the cell surface level is preferred over intracellular markers. Once produced, cytokines are rapidly released into the extracellular medium, which prevents their detection both intracellularly and on the cytoplasmic membrane, unless stabilization procedures are employed. Most commonly, inhibitors of intracellular transport such as brefeldin A and monensin are used to induce intracellular accumulation of cytokines until immunophenotypically detectable amounts are obtained.[13–15] However, such approaches require extensive sample manipulation for the detection of the synthesized cytokines, including cell fixation and permeabilization procedures. In the 1990s, Manz *et al*[16] described a method based on the use of bispecific (CD45/cytokine) monoclonal antibodies, which allowed capture of specific cytokines on the cell surface as soon as they had been produced. However, such approach requires extensive dilution of the sample in order to avoid proximity between cells that could give rise to false positive results due to the capture of cytokines by CD45^{+} cells adjacent to the secreting cells. More recently, we have shown that *in vitro* stimulation of cytokine secretion in the presence of highly specific inhibitors of TACE, was associated with a progressive accumulation of detectable amounts of TNFα on the cell surface at the cytoplasmic membrane; interestingly, the amount of TNFα stabilized at the cell surface was similar to that accumulated at the intracellular level once inhibitors of intracellular transport (ie brefeldin A) were used in parallel for both LPS- and PMA plus ionomycin-stimulated cells.[27] An additional advantage of this approach is that it does not directly interfere with other cell functions such as antigen presentation. In the present study, we confirm and extend these observations by showing that the BB3103 compound inhibits between 90 and 95% of all TNFα secreted by either LPS-stimulated PB monocytes or PMA plus ionomycin-activated CD3^{+} T cells. Such inhibitory effect remains relatively stable for up to several hours; in addition, it allowed detection of small numbers of TNFα-secreting cells in the context of

antigen-specific immune responses with a similar efficiency than conventional flow cytometry assays used for the detection of secreted cytokines at the intracytoplasmic level, as proven for the CMV-stimulatory model assayed here.

TNFα is one of the most ubiquitous and earliest cytokines to be secreted.[28] In addition, most cytokine-secreting cells also produce TNFα[13,29–33] that makes it a good candidate as a universal marker to be used for the identification of cytokine-secreting cells. Up till now, the most commonly used cell surface marker to detect recently activated lymphocytes and other immune cells has been CD69.[34–36] Although no direct comparison between the two markers has been performed, data from the literature indicate that CD69 could be far less specific than TNFα, because a significant proportion of CD69^{+} cells do not produce cytokines.[37]

Although TNFα secretion pathways have been reported that are independent of TACE,[38] most TNFα molecules are produced as cytoplasmic membrane proteins that are rapidly cleaved into a soluble form by TACE.[39–41] The use of specific TACE inhibitors has been associated with progressive accumulation of TNFα on the surface of the cytoplasmic membrane of TNFα producing cells, allowing for their specific and unequivocal identification.[27] Such TACE inhibitor compounds do not affect secretion of cytokines other than TNFα into the extracellular medium, thus allowing cells to respond under conditions that mimic those occurring *in vivo*. Interestingly, it should be noted that despite the fact that up to 5–10% of all TNFα was still secreted in a soluble form, no unexpected reactivity for TNFα was observed on cells that would not respond to the stimuli used.

A major goal of our study was to show the feasibility of simultaneously measuring TNFα^{+} cells and quantify the amount of specific cytokines secreted by these cells. For this purpose, we used commercially available fluorescent beads coupled with cytokine-specific monoclonal antibodies. These cytokine-capture beads have been optimized to be used in cell-free body fluids such as the plasma and they have proven to be comparable to conventional ELISA assays[42] with the advantage of allowing the simultaneous quantification of multiple cytokines in small sample volumes. In the present study, we clearly show that these cytokine-capture beads could also be used to precisely quantify cytokines in whole blood-stimulated samples, at the same time the number and phenotype of TNFα^{+} cells is evaluated. As shown in the present study, distinction between beads and cells could easily be made due to their uniquely different light scatter properties. At the same time, we could measure cellular TNFα and one or more soluble cytokines. Interestingly, despite the relatively high amounts of several soluble cytokines accumulated at the extracellular level, no reactivity for these cytokines was detected on cells. Sequential analysis of cytokine responses to either PMA/ionomycin or LPS stimulation

showed that, at least in these two models, surface TNFα was clearly detected prior to soluble IL6 and IFNγ, respectively. These results would support previous findings indicating that TNFα is the first cytokine to be produced by stimulated T cells[28] and extend this concept also to the monocytes, at the same time they point out a potential advantage of the cellular assays.

The final step of our study was to prove the feasibility of quantifying multiple cytokines simultaneously with the detection of the activated cytokine-secreting cells in a single tube assay. Obviously, when more than one cytokine are simultaneously evaluated, no single time-point may be optimal for all cytokines assayed.[28] Therefore, in the present study stimulation periods of 3 and 4 h were chosen for PMA/ionomycin- and LPS-activated samples as the most sensitive time-points for detecting TNFα-secreting T cells and TNFα-secreting monocytes, respectively. At these time-points, variable levels of different cytokines were detected which could be linked to the number of TNFα$^+$ cells simultaneously evaluated. Since the beads used can be coated with monoclonal antibody reagents of any specificity, in this assay quantification of soluble cytokines could also be potentially complemented or replaced by the measurement of other soluble proteins secreted by the activated cells (eg granzyme B). This possibility is particularly important because TNFα is also produced by nonhematopoietic cells[43] such as astrocytes, endothelial cells or smooth muscle cells, which secrete other mediators different from the cytokines analyzed here. The use of the bead array technology in which combinations of beads of different sizes and fluorescence properties can be mixed, makes it possible to increase the number of soluble proteins measured up to more than 100.[44]

Although we showed that this method could be used for the evaluation of immunological responses by peripheral blood cells against generic stimuli such as LPS and PMA/ionomycin, a major challenge would be to prove that it could also be applied to the study of T-cell-mediated antigen-specific immune responses. In line with this, in the present study based on an assay model for evaluating anti-CMV-specific immune responses, we proved that this method could also be used to distinguish precisely CMV-seropositive from CMV-seronegative individuals. In addition to the quantitative evaluation of the amount of different soluble T-helper (eg IFNγ) and inflammatory (eg IL6) cytokines, that could also be obtained though conventional multiple ELISA assays, we were able to identify the monocytes and T-cell subsets responsible for CMV-induced cytokine secretion. The possibility of using TNFα expression on the cell surface for the identification of cytokine-secreting cells is of particular interest, because it allows not only the identification of cytokine-secreting cells but also provides a unique opportunity to further investigate the phenotypic

and functional characteristics of CMV-specific T cells through the use of 5 or more color flow cytometry assays or the specific purification of each of the TNFα$^+$ cell subsets. Further studies, are necessary to prove its utility in the evaluation of other antigen-specific immune responses, particularly those aimed at monitoring vaccination against infectious agents or tumors.

In summary, in the present study we describe a new and simple flow-cytometry-based immunological method that could be used to monitor immune responses in whole blood samples under different physiological and pathological conditions, through the simultaneous identification and quantitation of several populations of cytokine-secreting cells and multiple cytokines secreted into the extracellular compartments by these cells.

Acknowledgements

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