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T helper cell polarisation as a measure of the maturation of the immune response

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***Background*: T helper cell polarisation is important under chronic immune stimulatory conditions and drives the type of the evolving immune response. Mice treated with superantigens *in vivo* display strong effects on T_h subset differentiation. The aim of the study was to detect the intrinsic capacity of T cells to polarise under various *ex vivo* conditions.**

***Methods*: Purified $CD4^+$ T cells obtained from superantigen-treated mice were cultured under T_h polarising conditions *in vitro*. By combining intracellular cytokine staining and subsequent flow cytometric analysis with quantitative cytokine measurements in culture supernatants by enzyme-linked immunosorbent assay (ELISA), the differential T_h polarising capacity of the treatment can be detected in a qualitative and quantitative manner.**

***Results and conclusions*: BALB/c mice were shown to be biased to develop strong T_h2 polarised immune responses using T_h0 stimulation of purified $CD4^+$ T cells from phosphate-buffered saline-treated mice. Nevertheless, our analysis methodology convincingly showed that even in these mice, Toxic Shock Syndrome Toxin-1 treatment *in vivo* resulted in a significantly stronger T_h1 polarising effect than control treatment. Our results indicate that populations of T_h cells can be assessed individually for their differential T_h1 or T_h2 maturation capacity *in vivo* by analysing robust *in vitro* polarisation cultures combined with intracellular cytokine staining and ELISA.**

Key words: T_h cell, cytokines, Polarisation, Magnetic-activated cell sorting, Intracellular cytokine staining, Fluorescent-activated cell sorter

Introduction

Polarisation of T helper cells is important in disease models associated with chronic immune stimulation, where the outcome depends on the type of immune response induced and the relative amount of cytokines released. Many diseases such as leprosy, allergy, multiple sclerosis and responses to immunotoxic agents have pathology associated with aberrant T_h1 and T_h2 polarisation.[1–7] The importance of T-cell polarisation in a disease such as leprosy is immediately obvious since the infection by *Mycobacterium leprae* can be resolved by a strong cell-mediated T_h1 response, while continued infection is associated with a humoral T_h2 response.[3,5]

Gram-positive bacteria including *Staphylococcus aureus*, *Streptococcus pyogenes* and *Mycoplasma arthritidis* secrete a number of exotoxins. These bacteria are well known for their multi-drug resistance in hospitals and are able to induce severe disease. Certain strains of *S. aureus* produce the superantigen Toxic Shock Syndrome Toxin-1 (TSST-1), which is a pyrogenic toxin and is implicated as a causative factor in toxic shock syndrome. As a superantigen, TSST-1 binds MHC II molecules expressed on antigen-presenting cells (APC) outside the antigenic groove, and stimulate $CD4^+$ helper T cells (T_h) contingent upon engaging particular T-cell receptor Vβ elements. As a result superantigens act as glue, linking T cells of various antigenic specificity to MHC molecules on APC, thereby leading to mutual activation of these cells. In mouse model systems using the Staphylococcal-derived superantigen TSST-1, one of the hallmarks of superantigenic stimulation is the capacity to induce T-cell differentiation towards different T_h subsets, including T_h1 and T_h2 type cells.[8]

It is hard to determine polarisation of T helper subsets during an acute response since real distinct populations only arise after *chronic* exposure to a particular antigen. However, many *in vivo* and *in vitro* experiments have a limited duration that does not reflect more chronic effects. It is therefore paramount to try to determine in which direction the cell population is polarising. For this reason we performed two secondary *ex vivo* stimulations under

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polarising conditions in our system, in order to determine how far our stimulated cells can be pushed in each direction, T_h1 and T_h2. Moreover, unpolarised T_h0 cells can be pushed in any direction in culture by addition of exogenous polarising cytokines in concert with neutralising antibodies to polarising cytokines in the opposite direction, such as interleukin (IL)-4 and interferon (IFN)-γ. Resistance to this directional polarisation, or co-expression of IFN-γ or IL-4 in the supernatant under opposite polarising conditions, however, indicates a shift towards differentiated T_h cell populations. Naturally, these analyses need to be performed on highly purified populations of $CD4^+$ T cells obtained from circulation or isolated lymphoid organs.

Several techniques can be used to try to determine the direction of polarisation. First, the quantitative reverse transcriptase-polymerase chain reaction can be used to determine the number and nature of cytokine mRNA transcripts of stimulated cells, and can even be performed on a single cell level.[9] This has certain disadvantages, since the actual production and release of cytokines cannot be directly measured and many of the cytokines of interest are subject to post-transcriptional regulatory mechanisms.[10,11]

The determination of cytokine production via direct protein detection in supernatant of cultured cells can be done by enzyme-linked immunosorbent assay (ELISA). ELISA determination is a valuable tool to measure the average, overall cytokine response accumulated over time of a heterogeneous population of stimulated cells. However, this method alone is unable to distinguish whether a mixed T_h0 cytokine pattern is due to the release of diverse cytokines from naive T_h0 cells, or rather the production of cytokines by heterogeneous subpopulations of T_h1 and T_h2 cells. Additionally, this method does not allow determination of which proportion of the cells is responsible for this skewing. To work around this limitation and to identify cells producing more than one cytokine, an intracellular cytokine staining (ICS) method is used. This method makes it possible to concurrently check the presence of different cytokines in each cell.[8,9] When combined with ELISA results an impression of the proportion of cytokine-producing cells, as well as the combined cytokine profile of the cell population can be assessed. This approach gives invaluable insight into the direction of T helper cell polarisation.

Materials and methods

Mice

Female BALB/c mice aged 8–10 weeks supplied by Charles River Biotech (St. Constant, QC, Canada) were kept under standard conditions in the Jack Bell Research Centre of UBC (Vancouver, Canada) under guidelines of the Canadian Council on Animal Care.

Splenocyte purification

Mice were sacrificed by cervical dislocation, and spleens removed and placed into single cell suspension, washed then resuspended in 10 ml of RPMI 1640 (Stem Cell Technology, Vancouver, Canada) and centrifuged at 800 *g* for 7 min. Red blood cells were lysed with Gey's balanced salt lysing solution containing 0.7% NH_4Cl for removal of red blood cells and washed once in RPMI and then in magnetic activated cell sorter (MACS) buffer.

$CD4^+$ T-cell purification

$CD4^+$ T cells were positively isolated according to the protocol provided by Milltyni Biotech (Auburn, California, USA), using MS^+ columns, and an octo-MACS magnet. Following purification cells were further washed with RPMI, after which they were resuspended in RPMI-1640 medium supplemented with 10% heat-inactivated foetal calf serum (Stem Cell Technology), l-glutamine (2 mM; Stem Cell Technology), penicillin (80 U/ml; Stem Cell Technology), streptomycin (80 μg/ml; Stem Cell Technology), polymyxin B (2 μg/ml; Sigma, St Louis, Missouri, USA), sodium pyruvate (0.8 mM) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (0.24 M HEPES; Sigma) at a concentration of 2×10^6 cells/ml.

T-cell polarisation

Ninety-six-well flat-bottomed plates were coated with 50 μl of 10 μg/ml anti-CD3 (clone 145 2C11; Pharmingen, Mississauga, Ontario, Canada) in sterile 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. The next day, plates were washed with medium. Subsequently, cells were cultured at 1×10^6 cells/ml under the following conditions: T_h0, 2 μg/ml of anti-CD28 (clone 37.51), and 10 U/ml (10 ng/ml) of IL-2 (all Pharmingen); T_h1, as T_h0 plus 10 μg/ml of anti-IL-4, 10 μg/ml of anti-IL-10, 10 ng/ml of recombinant mouse IL-12, and 5 ng/ml of recombinant mouse IFN-γ (all Pharmingen); and T_h2, as Th0 plus 5 μg/ml of anti-IFN-γ, 5 μg/ml of anti-IL-12, and 50 ng/ml of recombinant mouse IL-4 (all Pharmingen). Following 4 days of polarised stimulation, cells were washed twice with cold RPMI. Subsequently, cells were resuspended in fresh complete medium and transferred to a fresh anti-CD3-coated plated prepared as already described, with a final culture volume of 200 μl. Cells were stimulated under T_h0-like conditions in the presence of monensin (Pharmingen) for 5 h if ICS analysis was required or alternatively, cell supernatants were harvested 48 or 96 h after secondary stimulation in the absence of monensin treatment.

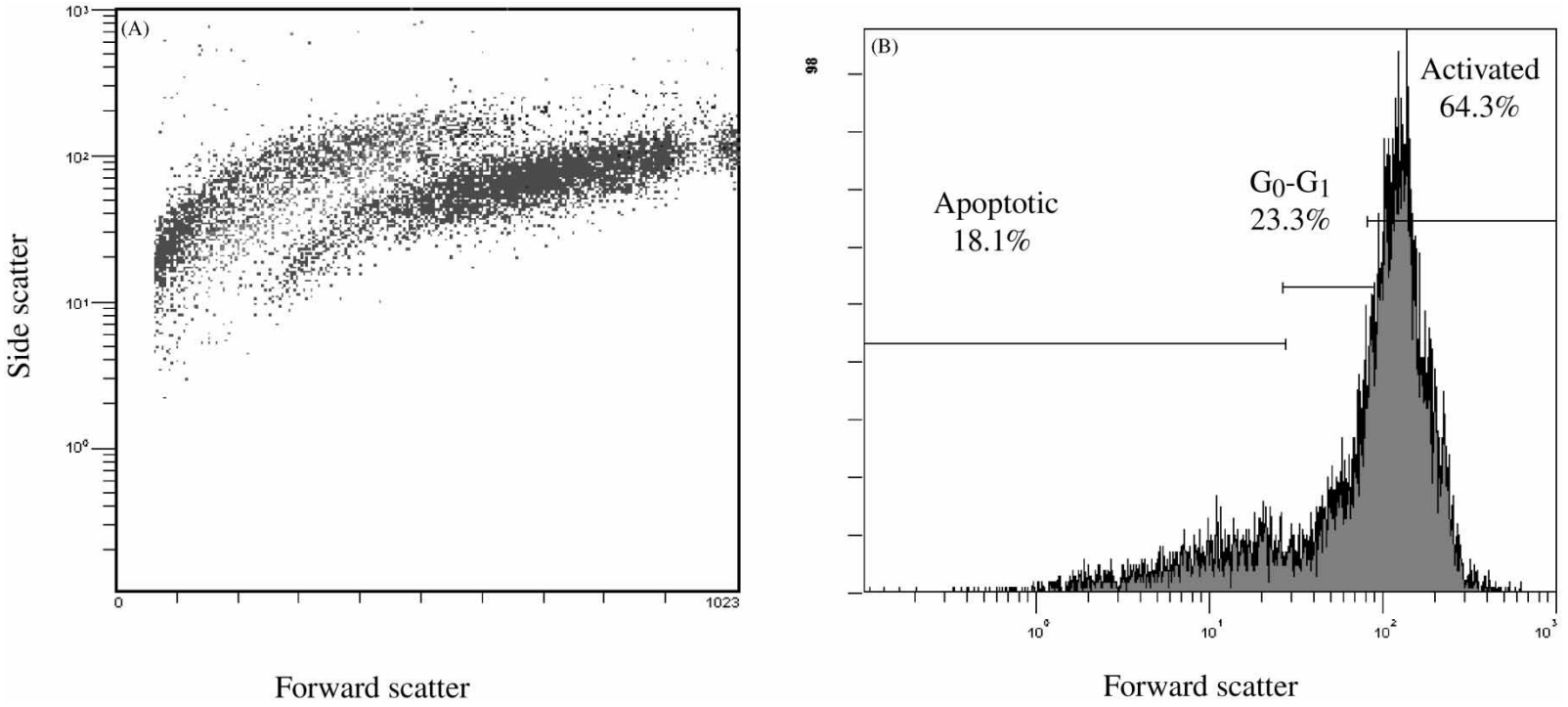

FIG. 1. Propidium iodide (PI) staining of stimulated, fixed and permeabilised cells to track cell cycle and to gate out apoptotic cells. PI, and DNA intercalating dye, serves both as a positive control for the permeabilisation of the samples with permwash, but also provides a way of marking cells in the G_0-G_1, G_2-S and most importantly, apoptotic pathway. Cells with less than one copy of their DNA are undergoing the later stages of apoptosis. Colored gating with the EXPO32 ADC software (Applied Cytometry Systems, Sacremento, California, USA) on the FL3 histogram (B) allows visualisation of the apoptotic cells so that they can be excluded from the live cell gate (A) used in later samples to minimise the fluorescent contamination of staining induced by highly auto-fluorescent dead cells.

ICS staining

Polarised T cells were pelleted in 96-well round-bottomed plates and stained for surface molecules in a 3% foetal calf serum–phosphate-buffered saline (PBS) (fluorescent activated cell sorting (FACS)) buffer. Following this, cells were fixed and permeabilised using an intracellular staining kit (Pharmingen) according to the manufacturer's instructions. It's worthwhile to note that fixed, non-permeabilised cells can be stored in FACS buffer at 4°C for at least 1 week before ICS is performed. Cells were stained for intracellular cytokines with anti-IL-4-PE (clone 11B11) and anti-IFN-γ-FITC (clone XMG1.2) at a 1/50 dilution (Pharmingen). Unlabelled antibody of the same clones is utilised at 10 times excess to set negative gates to control for non-specific staining. Cells were resuspended in FACS buffer and then analysed on a BD FACS Calibur (Becton Dickinson Biosciences, Mississauga, Ontario, Canada). A total of 10,000 events in the live cell gate (dead cells excluded by propidium iodine staining) were counted and retained for each sample. Gates were set so that only ~1% of cells are positive for the cytokine of interest in the labelled/unlabelled negative control, to account for auto-fluorescence and non-specific staining.

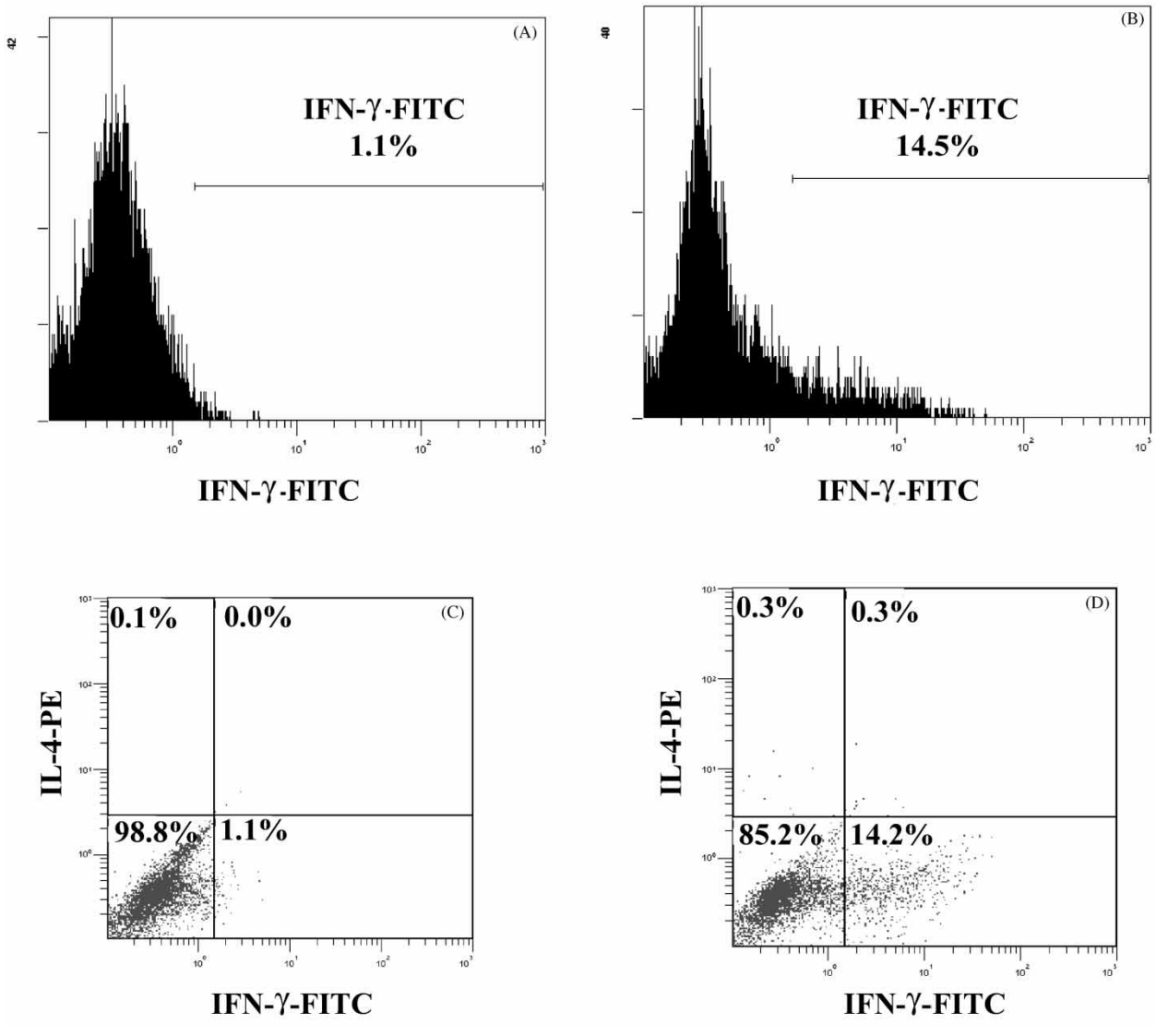

FIG. 2. Creation of a positive cell gate for IFN-γ staining. Due to shift in the forward side scatter characteristics of activated cells, it is essential that identically stimulated cells are used as a negative control for cytokine specific staining, and are saturated with 10 x excess unlabelled antibody (of the same isotype) prior to and during staining with the fluorochrome conjugate. Using this unlabeled negative control sample, cell gates are drawn on both the histogram (A) and the quadrant markers (B) such that only ~1.0% of the cells are measured as being positive for the cytokine gate. An identically stained sample, without the addition of the 10 x excess of unlabelled antibody, is then run to determine whether the positive control is in fact positive, and to establish FACS compensation settings (C and D). During later analysis, the 'background' per cent of cells gated positive in the unlabelled negative control is subtracted from the value of the samples measured as background.

Results

Live cell gating

Propidium iodine staining of fixed and permeabilised cells, followed by subsequent gating of cells based on cell cycle and DNA content, provides both a useful live cell inclusion gate for intracellular staining analysis while providing a good positive control for cell permeabilisation during the staining process. Dead cells have obvious changes in forward side scatter, as they are smaller (less FS) and more granular (more SS) (Fig. 1). A total of 10,000 events in the live cell gate are counted and retained for each sample.

ICS-positive gates

The setting of stringent, valid positive gates for ICS is essential, and requires the use of 10 times excess unlabelled anti-cytokine antibody used concurrently with labelled antibody in a known positive control. These criteria are essential, as the use of unstimulated controls alone does not adequately account for changes in autofluorescence seen following activation due to the inevitable increases in forward/side scatter. Due to cell fixation and permeabilisation, there is an inherent amount of non-specific staining that must be accounted for. Typically, the gates are set so that only $\sim 1\%$ of cells are positive for the cytokine of interest in the labelled/unlabelled negative control (Fig. 2). This background was subtracted from the experimental readings during analysis.

Cytokine production as detected by ELISA

Absolute production

T_h1 polarisation of purified $CD4^+$ T cells from mice treated *in vivo* with PBS resulted in significantly higher IFN-γ production compared with T_h2 polarised or unpolarised cells (Fig. 3). Production of IFN-γ by $CD4^+$ T cells isolated from mice treated repeatedly with TSST-1 was considerably elevated ($n = 10$). Production of IL-4 from polarised $CD4^+$ T cells was also increased in mice treated *in vivo* with TSST-1 (Fig. 4). Moreover, T_h1 polarisation resulted in significantly lower IL-4 production than T_h0 or T_h2 polarisation. T_h cells from mice treated repeatedly with TSST-1 produce more IFN-γ and IL-4 than cells from PBS-treated mice; however, there is more relative polarisation in the T_h2 direction under all stimulatory conditions including T_h0, T_h1 and T_h2.

Relative production

Absolute IFN-γ production is made relative by dividing production values by the mean IFN-γ concentration following T_h1 polarisation of $CD4^+$ T cells from *in vivo* PBS-treated mice, and then expressed as a percentage. Relative T_h2 polarisation is assessed by dividing IL-4 production values by the mean IL-4 concentration following T_h2 polarisation of $CD4^+$ T cells from *in vivo* PBS-treated mice, and then expressed as a percentage. Relative cytokine production is assessed by equating cytokine levels detected by ELISA with T-cell polarisation. The average maximal production of IFN-γ by $CD4^+$ T cells isolated from PBS-treated mice stimulated under T_h1 conditions was determined to be the 100% response, and other IFN-γ levels were then expressed as a percentage of this maximal production of the positive control. Similarly, the production of IL-4 relative to that by T_h2-stimulated $CD4^+$ T cells from PBS-treated mice was calculated. Results were graphed to indicate the shifts in the cytokine production of the different T-cell phenotypes (Fig. 5).

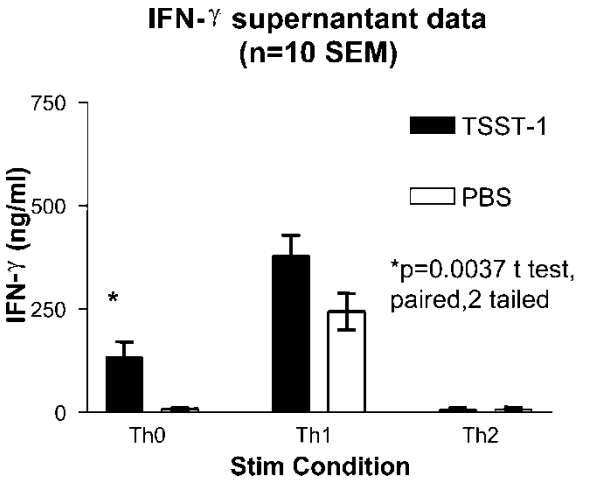

FIG. 3. Absolute IFN-γ production by T_h cells isolated from repeatedly *in vivo* PBS or TSST-1 treated mice as detected by ELISA (detection limit, 7.5 pg/ml) under different *in vitro* polarising conditions, including T_h0, T_h1 and T_h2 conditions.

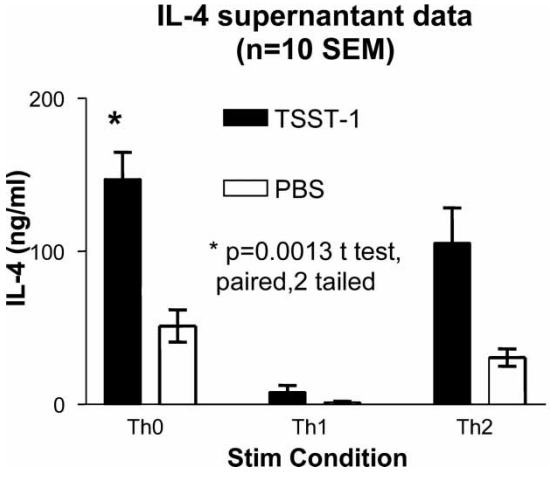

FIG. 4. Absolute IL-4 production by T_h cells isolated from repeatedly *in vivo* PBS or TSST-1 treated mice as detected by ELISA (detection limit, 7.5 pg/ml) under different *in vitro* polarising conditions, including T_h0, T_h1 and T_h2 conditions.

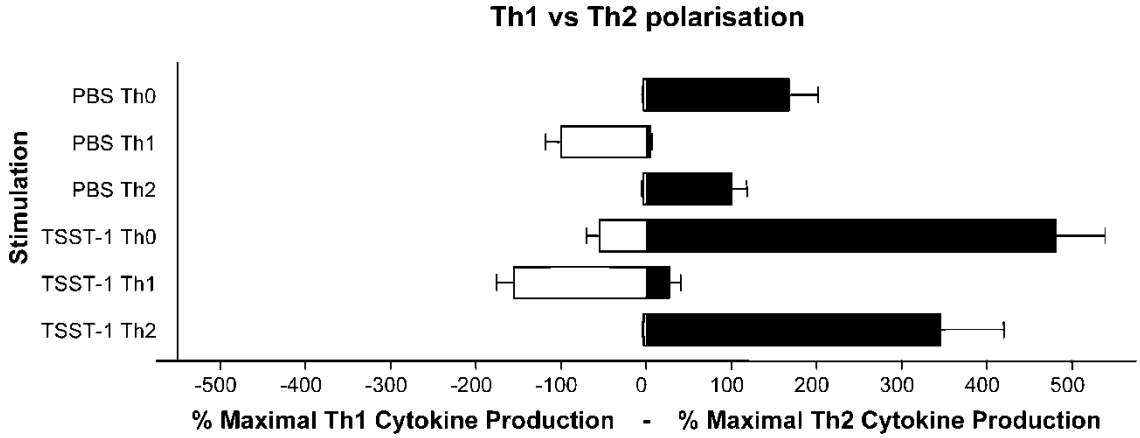

FIG. 5. Relative cytokine production, T_h1 versus T_h2. IFN-γ production is made relative by first dividing production values by the mean IFN-γ concentration following T_h1 polarisation of $CD4^+$ T cells from *in vivo* PBS-treated mice, and then expressing this as a percentage. Relative T_h2 polarisation is assessed by dividing IL-4 production values by the mean IL-4 concentration following T_h2 polarisation of $CD4^+$ T cells from *in vivo* PBS-treated mice, and then expressing this as a percentage. T_h0 stimulation of $CD4^+$ T cells from PBS-treated mice clearly shows the BALB/c strains bias towards a strong T_h2 response. T_h1 and T_h2 stimulation of the $CD4^+$ T cells from the PBS-treated mice demonstrates the relative 100% maximal production of IFN-γ and IL-4, respectively. $CD4^+$ T cells from mice treated repeatedly with TSST-1 have both an enhanced T_h1 and T_h2 response under non-polarising T_h0 stimulation conditions, compared with T_h cells from mice treated with PBS. There is a 480% of maximal increase in IL-4 production, while the production of IFN-γ is low at 58% of maximal. T_h1 polarisation of the $CD4^+$ T cells derived from the TSST-1 repeatedly treated mice demonstrates that, despite strong T_h1 polarisation, there is a difference in IL-4 production under this stimulatory condition, indicating that despite strong T_h1 polarisation cells are still actively producing IL-4, indicating a shift to the T_h2 phenotype.

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Intracellular cytokine staining

Under non-polarised conditions, ICS staining of $CD4^+$ cells from the PBS-treated BALB/c mice appeared naturally T_h2 biased, as 5.0% of $CD4^+$ T cells produced IL-4, while IFN-γ production was not detectable (Fig. 6A). However, the BALB/c mice were clearly capable of mounting a strong T_h1 response, as 14.2% of T_h1-polarised $CD4^+$ T-cells were positive for IFN-γ (Fig. 6B). T_h2 polarisation of the PBS-treated mice resulted in 4.5% IL-4-positive $CD4^+$ T cells, a slight decrease from that detected in the T_h0 stimulated group (Fig. 6C).

Discussion

While the conditions in our system clearly induced T_h1 and T_h2 polarised $CD4^+$ populations, there is room for further optimisation. While we used both IL-12 and IFN-γ to induce T_h1 polarisation, it has been reported that IL-12 alone, may be adequate due to its potency to induce IFN-γ production in $CD4^+$ T cells.[10] In addition, the requirement of neutralising antibodies against various polarising cytokines depends on the purity of the $CD4^+$ population being studied. For example, the addition of anti-IL-12, which is not produced by T cells, but is potentially produced by contaminating monocytes, can be ignored if $CD4^+$ cell purity is over 90%. In fact, the addition of neutralising antibodies to IL-4 alone has been shown in some systems to be sufficient for inducing T_h1 polarisation.[11] Other researchers commonly use a secondary pulse with PMA[1,12,13] or PHA.[1,13] While this type of secondary stimulation may be less expensive than treatment with anti-CD3, anti-CD28 and IL-2, we feel it is less relevant physiologically. In addition, the method as outlined is easily adaptable to the use of irradiated antigen-pulsed APCs in place of the anti-CD3 and anti-CD28 antibodies.

Utilisation of a T-cell polarisation pulse, followed by comparison of relative cytokine production by intracellular cytokine staining under polarised conditions allows *ex vivo* assessment of the T-cell polarisation state *in vivo*. Since strong and clear T-cell polarisation typically takes place in chronic disease states or exposure to (potentially toxic) agents, this technique provides an assessment of the current

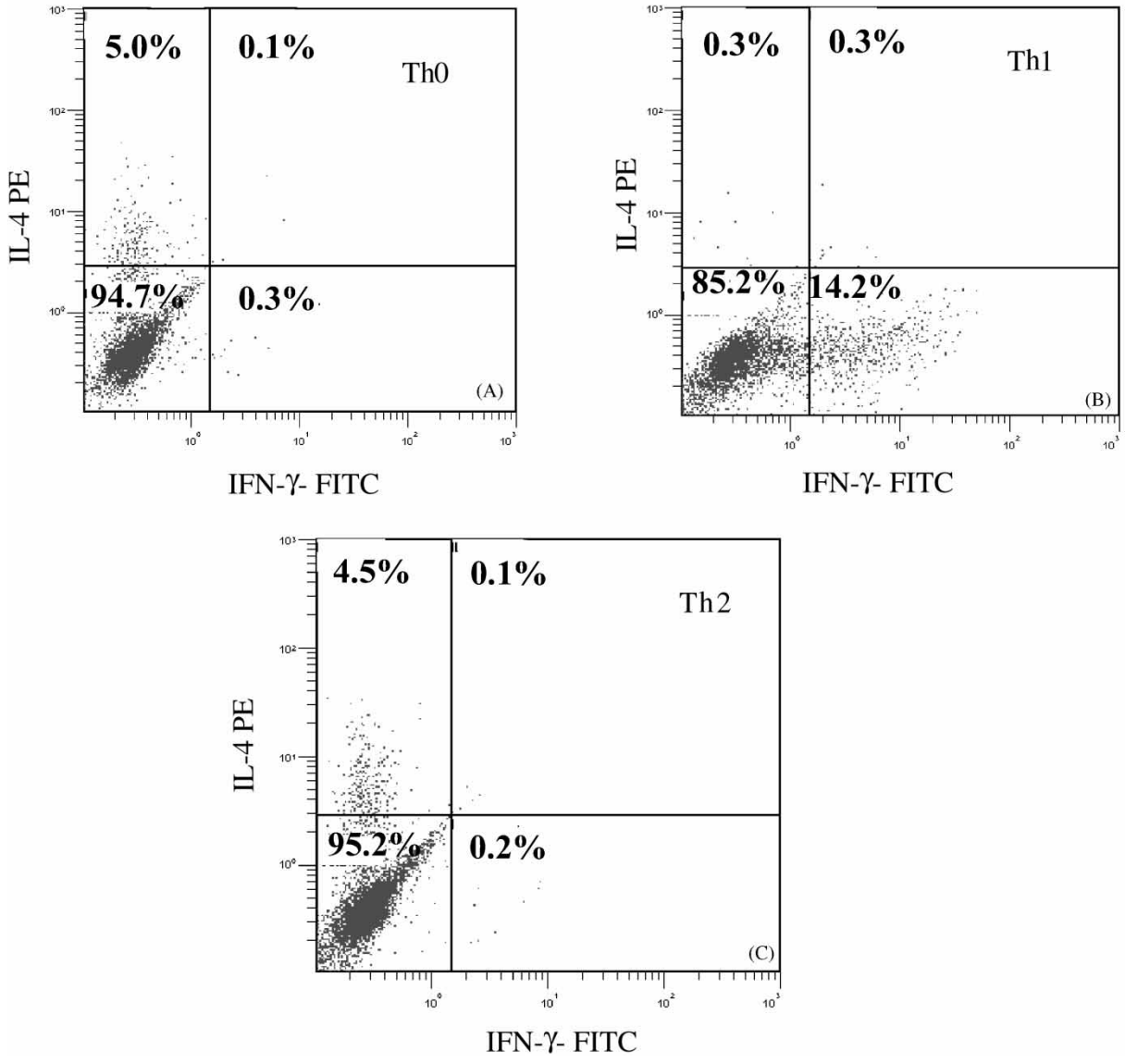

FIG. 6. IL-4 and IFN-γ dual staining of T_h0, T_h1 and T_h2 polarised $CD4^+$ T cells from repeatedly PBS-treated mice. T_h0 stimulation indicates that 5.0% of the cells were IL-4-positive, while IFN-γ-positive cells were of very low number (0.3%). Under T_h1 stimulatory conditions, however, this IL-4-positive population disappears. A total of 14.2% of T_h cells under these conditions are IFN-γ producers. T_h2 polarisation, however sees a return to low number of IFN-γ-positive cells, with IL-4-producing cells detected at 4.5%.

direction of polarisation the T cells are heading towards during acute responses, on a population basis via relative cytokine production, and on a cell-by-cell basis via ICS. Several caveats must be kept in mind when interpreting data under such conditions, however. Most importantly, the comparison of cytokine production by ELISA must be made on a relative scale, as outlined since the potency of the key T_h1 and T_h2 cytokines, IFN-γ and IL-4 respectively, may not necessarily be equal. Similar relative comparisons have been made during micro-array analysis of T_h1 and T_h2 gene transcripts.[14]

Additionally, all polarisations must be compared with the results obtained from $CD4^+$ T cells isolated from a PBS-treated mouse polarised under identical conditions. The rationale for this is apparent when looking at the data demonstrated here, since BALB/c mice are naturally T_h2 biased in their cytokine profile, while other strains, such as SJL mice, are naturally T_h1 biased.[15]

The addition of ICS to this method most importantly allows for further assessment of the T helper cell population in experimental groups, to determine whether a mixed cytokine profile detected via ELISA and comparison of relative cytokine production truly represents an undifferentiated T_h0 response of naïve cells, or rather is indicative of a heterogeneous population of simultaneously differentiating T_h1 and T_h2 cells. Additionally, it can allow for further characterisation of the cytokine producing subsets.[12,13]

While proportion is the only variable measured here, if this technique is combined with cell surface

marker staining (prior to fixation and permeabilisation) or staining for proliferation further important data can be garnered from this robust method.

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