

## Thalidomide and its analogues have distinct and opposing effects on TNF- $\alpha$ and TNFR2 during co-stimulation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells

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### SUMMARY

Thalidomide (Thd) is clinically useful in a number of conditions where its efficacy is probably related to its anti-TNF- $\alpha$  activity. More recently, Thd has also been shown to co-stimulate T cells and second generation co-stimulatory (IMiD<sup>TM</sup>) analogues are currently being assessed in the treatment of cancer patients. However, in contrast to their known suppressive effects during inflammatory stimuli, the effects of Thd/IMiDs on TNF- $\alpha$  and TNF receptors (TNFRs) during T cell co-stimulation are not known. We sought to determine the effect of Thd, two clinically relevant IMiDs (CC-4047, ACTIMID<sup>TM</sup> and CC-5013, REVIMID<sup>TM</sup>) and a non-stimulatory SelCID analogue (CC-3052) on TNF- $\alpha$  production and on the expression and shedding of TNFRs during co-stimulation. We found that co-stimulation of PBMC with Thd/IMiDs, but not CC-3052, prevented  $\alpha$ CD3-induced T cell surface expression of TNFR2 and thereby reduced soluble TNFR2 (sTNFR2) levels. However, there was no effect on total (surface/intracellular) TNFR2 protein expression, suggesting inhibition of trafficking to the cell membrane. The extent of co-stimulation by Thd/IMiDs (assessed by CD69/CD25 expression and IL-2/sIL-2R $\alpha$  production) was similar for CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and correlated with TNFR2 inhibition. Co-stimulation, but not the early inhibitory effect on TNFR2, was IL-2-dependent and led to increased TNF- $\alpha$  production by both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes. The clinical relevance of this observation was confirmed by the elevation of serum TNF- $\alpha$  during REVIMID<sup>TM</sup> treatment of patients with advanced cancer. Together, these results suggest a possible role for TNF-mediated events during co-stimulation and contrast with the TNF inhibitory effects of Thd and its analogues during inflammatory stimuli.

**Keywords** co-stimulation immunomodulation thalidomide T lymphocyte TNF

### INTRODUCTION

Thalidomide (Thd) is emerging as an important immunotherapeutic drug in a number of clinical situations [1–4]. It is well established as an anti-TNF- $\alpha$  agent in the context of monocyte/macrophage activation *in vitro* [5,6] and clinically during TNF- $\alpha$ -mediated inflammatory disease [6]. However, other immunomodulatory properties may explain the extensive range of its clinical activity. More recently, it has been shown to inhibit monocyte IL-12 production [7] and also to be able to induce the activation and proliferation of CD8<sup>+</sup> T cells stimulated via the T cell receptor in the absence of co-stimulation [8]. Thus, in certain clinical settings, such as in the treatment of cancer patients, Thd may act as an

adjuvant to promote T-cell responses. However, Thd treatment has also been known to increase circulating levels of TNF- $\alpha$  [9–12] and IL-12 [13] and it appears likely that the effects of Thd vary, depending on the relative predominance of monocyte/macrophage and T cell activation. Furthermore, the effects of Thd may also vary depending on the activation of particular signalling pathways [14].

Significant side effects such as somnolence and peripheral neuropathy are associated with clinically effective Thd treatment. However, the design and synthesis of Thd analogues has yielded compounds with enhanced activity/toxicity profiles [15,16]. These analogues are currently being characterized and have been shown to segregate into at least two distinct classes. The SelCIDs<sup>TM</sup> (selective cytokine inhibitory drugs) consist of phosphodiesterase type 4 (PDE4) inhibitors [17]. The IMiDs<sup>TM</sup> (immunomodulatory drugs) are thought to be mechanistically similar to Thd, although with enhanced potency and therapeutic indices, and act via as yet

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unknown mechanism(s) [18–24]. Both groups of compounds are potent inhibitors of monocyte/macrophage-derived TNF- $\alpha$ , although T cell co-stimulatory activity is limited to the IMiD group [17].

Thd and its co-stimulatory IMiD analogues, in particular CC-4047 and CC-5013, are currently being assessed in the treatment of patients with advanced multiple myeloma and patients with advanced solid tumours [25–32]. Furthermore, we have shown recently that CC-4047, when co-administered with autologous or allogeneic tumour cell vaccination, is able to enhance protective and long-lasting immunity significantly in a murine model of colorectal cancer [33].

Thd and its derivatives are known to suppress TNF- $\alpha$  during the activation of inflammatory pathways by LPS, but it is not known whether this is the case during the direct stimulation of T cell activation pathways. Therefore, in this study we planned to determine the effect of these compounds, plus the non-co-stimulatory analogue CC-3052, on the expression of TNF- $\alpha$  and its receptors during co-stimulation *in vitro*. Furthermore, we wanted to assess the co-stimulatory effect on CD4<sup>+</sup> and CD8<sup>+</sup> populations because previous results suggested that CD8<sup>+</sup> T cells were activated preferentially. Because signals generated via the TNF system are important in T cell homeostatic control we hoped to provide insights into the T cell co-stimulatory mechanism of these novel compounds. Finally, we hoped to show that any effects on the TNF system were relevant for clinical non-inflammatory disease and to this end we obtained sera from a small phase I study of CC-5013 in the treatment of advanced cancer.

## MATERIALS AND METHODS

### *PBMC and whole blood culture*

Thd, two IMiDs (CC-4047 and CC-5013) and a SelCID (CC-3052) (provided by Celgene Corporation, NJ, USA) were dissolved freshly in DMSO to make a 20 mg/ml stock solution. The compounds were then diluted directly into the tissue culture wells at the required concentrations.

Venous blood was collected into sodium heparin vacutainers (Becton Dickinson, Oxford, UK) and PBMC were prepared subsequently by density centrifugation over Ficoll Hypaque (Sigma, Poole, UK). After centrifugation at 700 g for 20 min cells were collected from the interface, washed three times in HBSS (Sigma), counted and resuspended at  $1\text{--}2 \times 10^6/\text{ml}$  in complete RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 2 mM L-glutamine (all Sigma). PBMC were cultured  $\pm$  Thd/analogues (1 ng–10  $\mu\text{g}/\text{ml}$ ; 0.05% v/v final DMSO concentration) at 1 ml/well in 24-well plates, precoated with anti-CD3 mAb (2.5  $\mu\text{g}/\text{ml}$ ; R&D systems, Minneapolis, USA) in bicarbonate coating buffer pH 9.5. PBMC were incubated for 12 h–7 days at 37°C in 5% CO<sub>2</sub>. For some experiments PBMC were co-incubated with neutralizing anti-IL-2 mAb (0.005–0.1  $\mu\text{g}/\text{ml}$ ; R&D systems). Cell-free supernatants were collected and stored in aliquots at –70°C until assayed by ELISA. Cells were harvested and resuspended in PBS/BSA prior to processing as described below.

For lipopolysaccharide (LPS; Sigma, *Escherichia coli* serotype 0127:B8)-stimulated cultures heparinized venous blood was diluted 1 : 4 in complete RPMI-1640 medium (without FCS) and stimulated in 24-well plates at 1 ml/well with LPS (1  $\mu\text{g}/\text{ml}$ ). All cultures were incubated at 37°C in 5% CO<sub>2</sub>  $\pm$  Thd/analogues (10  $\mu\text{g}/\text{ml}$ ). After 24–48 h cell-free supernatants were collected by

microcentrifugation and stored in aliquots at –70°C until assayed by ELISA.

### *Clinical CC-5013 study: patient selection, treatment and sample collection*

We collected serum from a small phase I study designed to assess the safety, tolerability and efficacy of REVIMID™ (CC-5013) in the treatment of patients with advanced and heavily pretreated cancer. Of the 20 patients enrolled we obtained samples from 11 subjects, aged between 18 and 75 years, with histologically proven stage IV metastatic melanoma ( $n = 6$ ), histological or cytological proof of adenocarcinoma of the exocrine pancreas ( $n = 2$ ) or other malignancy (one each of breast, renal and lung cancer;  $n = 3$ ). Before initiating therapy, patients were subject to a complete medical history, physical examination and baseline evaluation of signs and symptoms, including full blood counts and neurological examination. These were repeated at weekly intervals during the study period and also at the end of the study.

All patients gave written informed consent prior to participation in the study. The protocol was approved by the Wandsworth Local Research Ethics Committee (LREC). All patients were treated with 5 mg oral REVIMID™ daily (tablets were taken every evening) for 1 week with the dose escalating to 10 mg daily at week 2, 25 mg daily at week 3 and 50 mg daily at week 4 at which the treatment was maintained indefinitely depending on tolerance.

Blood was taken at the same time at each visit (and therefore at the same time relative to drug administration), collected into serum separator (SST; Vacutainer, BD) tubes and left to clot for ~30 min. Tubes were spun at 950 g for 10 min and serum collected. Sera were frozen in aliquots at –70°C until being assayed for TNF- $\alpha$  and sIL-2 receptor  $\alpha$  (sIL-2R) by ELISA. Serum was collected at baseline, during the first 1–3 weeks (10–25 mg daily) of treatment and again at weeks 4–5 (50 mg daily).

REVIMID™ tablets were manufactured by Penn pharmaceuticals (Tredegar, Wales, UK) and supplied by Celgene Corporation (Warren, NJ, USA).

### *Enzyme-linked immunosorbent assay (ELISA)*

Culture supernatants were assayed for sTNFR1, sTNFR2, sIL-2R using kits provided by R&D Systems (Minneapolis, MN, USA) and for IL-2 and TNF- $\alpha$  using an assay procedure and reagents provided by Pharmingen (BD). Briefly, 96-well flat-bottomed ELISA plates (Nunc) were precoated overnight at 4°C with anti-cytokine capture mAb in bicarbonate coating buffer, pH 9.5 and washed (with PBS/Tween<sub>20</sub>) prior to incubation of standards and test (supernatant) samples overnight at 4°C. After washing, plates were incubated with biotinylated anticytokine mAb for 1 h. After further washing, plates were developed using chromogen (tetramethylbenzidine)/hydrogen peroxide for 20–30 min at RT. Standard absorbances (405 nm) of duplicate wells (minus control zero standard) were used to calculate concentration of cytokine/receptor levels which were corrected for any dilution factor. Data are expressed as percentage compared to control (DMSO alone), which is represented as 100%.

Sera from patients were assayed for TNF- $\alpha$ , sTNFR2, IL-2 and sIL-2R using kits provided by R&D Systems. In each case, the manufacturer's instructions were followed exactly. Data are expressed as pg/ml at baseline (pretreatment) compared to follow-up at 1–3 and 4–5 weeks.

#### Flow cytometric analysis of surface CD25, CD69, TNFR1 and TNFR2

PBMC were harvested from 24-well plates by gentle aspiration, washed and suspended in wash buffer (PBS/0.5% BSA/0.1% sodium azide). Three-colour phenotypical analysis of  $\alpha$ CD3-stimulated PBMC was performed by surface staining cells (by incubating cells for 30 min at 4°C) with the following fluorochrome conjugated monoclonal antibodies: anti-CD25 FITC (Becton Dickinson Immunocytometry Systems, BDIS, Oxford, UK), anti-CD69 FITC (L78; BDIS) or anti-TNFR1 FITC (16803-161; R&D Systems) and anti-TNFR2 PE (22235-311; R&D Systems) with either anti-CD4 PerCP (SK3; BDIS) or anti-CD8 PerCP (SK1; BDIS) plus appropriate isotype-matched and compensation controls. Cells were then washed once in 2 ml wash buffer and fixed for analysis in 200  $\mu$ l Cellfix™ (BDIS) prior to flow cytometric analysis. Upon analysis lymphocytes were gated on forward scatter (FSC) versus side scatter (SSC) properties. PerCP-positive T cell subsets were then selected by gating on SSC versus FL-3 dot-plots and displayed as two-colour dot-plots, with quadrants set according to isotype-matched controls. For each sample 10 000 lymphocytes were acquired on a Becton Dickinson FACScan using CellQuest™ software.

#### Analysis of intracellular TNFR2, IL-2 and TNF- $\alpha$

PBMC were stimulated with  $\alpha$ CD3  $\pm$  CC-4047 for 24–96 h with the protein transport inhibitor Brefeldin A (10  $\mu$ g/ml; Sigma) present for the last 6 h of culture to allow intracellular accumulation of protein. Cells were harvested, washed and suspended in wash buffer, then fixed and permeabilized using the specially formulated Becton Dickinson FACS lysis and permeabilization solutions as per the manufacturer's instructions. Cells were then stained with anti-TNFR2 FITC, anti-IL-2 FITC (5344-111, BDIS) or anti-TNF- $\alpha$  FITC (6401-1111, BDIS) and anti-CD4 PE or anti-CD8 PE plus appropriate isotype-matched and compensation controls for 30 min before washing once in 2 ml wash buffer followed by a final resuspension in 200  $\mu$ l Cellfix™. Stained samples were kept in the dark at 4°C prior to two-colour analysis on the permeabilized cells. Upon flow cytometric analysis lymphocytes were gated on forward scatter (FSC) versus side scatter (SSC) properties and displayed as two-colour dot-plots, with quadrants set according to isotype-matched controls. For each sample 10 000 lymphocytes were acquired on a Becton Dickinson FACScan using CellQuest™ software.

#### Proliferation assay

PBMC ( $1-2 \times 10^5$ ) were resuspended in complete RPMI-1640 ( $\pm$  Thd/analogues) onto  $\alpha$ CD3 precoated 96-well round-bottomed plates (Falcon, BD) to a final volume of 200  $\mu$ l per well. Cells were cultured at 37°C in 5% CO<sub>2</sub> for 5 days with [<sup>3</sup>H]-thymidine (NEN Life Science Products, Hounslow, UK) added for the last 24 h (2 mCi/well). Cells were harvested onto glass fibre filter paper and counted in a matrix 96 direct beta counter (Canberra Packard). Uptake of [<sup>3</sup>H]-thymidine was determined as mean cpm of sextuplet culture wells and expressed as percentage change compared to control (DMSO alone).

#### Statistical analysis

For analysis of serum data comparisons between groups were examined by the Kruskal–Wallis test with inclusion of the Dunn's

multiple comparison test. The statistics were performed using Primer of Biostatistics (3.02) statistical software.

## RESULTS

#### *Co-stimulatory IMiD CC-4047 strongly inhibits the secretion of sTNFR2 by $\alpha$ CD3-stimulated PBMC cultures whereas non-co-stimulatory SelCID CC-3052 augments sTNFR2*

A single treatment of CC-4047 leads to potent inhibition of sTNFR2 levels in  $\alpha$ CD3-stimulated PBMC supernatants at 24 h (Fig. 1a). This effect was consistent in four of four experiments (24 h: mean  $\alpha$ CD3 alone, 2676 pg/ml; + CC-4047 = 32.3%  $\pm$  23.5% of control). Inhibition was decreased at 48 h (mean  $\alpha$ CD3 alone, 4436 pg/ml; + CC-4047 = 52.7%  $\pm$  13% of control), although a small inhibitory effect was still apparent after 72 h. Soluble TNFR2 was not consistently detectable in 12 h cultures (data not shown).

We found that the non-co-stimulatory analogue, CC-3052, had the opposite effect on sTNFR2 when compared to CC-4047 in all experiments (24 h: + CC-3052 = 222%  $\pm$  141% of control). The effect of CC-3052 also decreased at 48 h (+ CC-3052 = 159%  $\pm$  50.4%) and a smaller augmentary effect was apparent at 72 h. Thd had very little overall effect on sTNFR2 (for example, at 48 h: + Thd = 102.7%  $\pm$  9.3% of control).

In contrast to the effects on sTNFR2 none of the compounds had a consistent or significant effect on the levels of sTNFR1 at 24 h (+ CC-4047, mean 110%  $\pm$  10.9%; CC-3052, mean 86.8%  $\pm$  13.6%; Thd = 104%  $\pm$  11.2%; Fig. 1b). However, CC-3052 did inhibit sTNFR1 at 48 and 72 h.

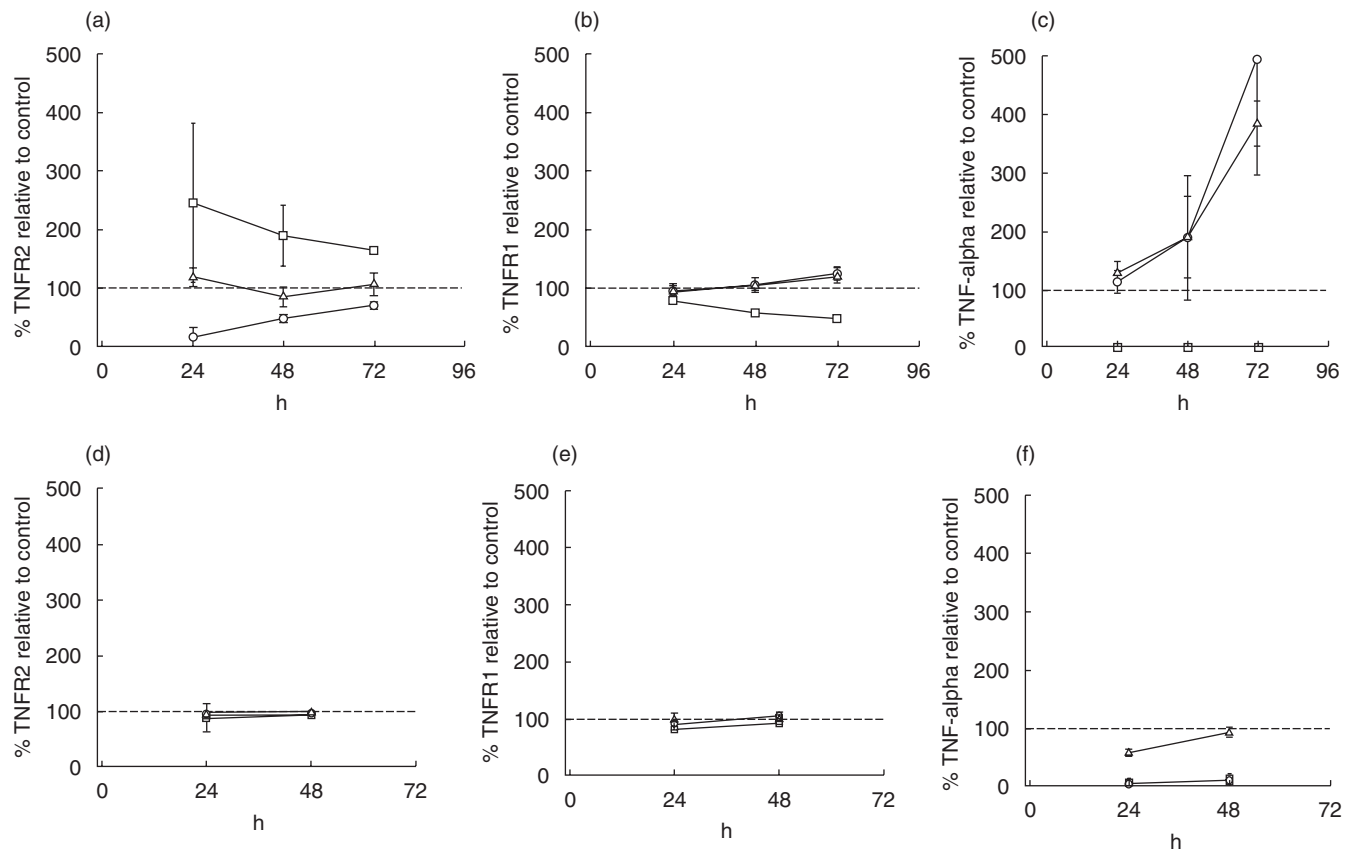
We also found that CC-4047 and Thd strongly induced the production of TNF- $\alpha$  at 72 h (mean  $\alpha$ CD3 alone control, 1443 pg/ml; + CC-4047 = 365%  $\pm$  233%; + Thd = 247%  $\pm$  122%; Fig. 1c), although there was very little effect at 24 h ( $\alpha$ CD3, 2753 pg/ml; CC-4047 = 114%  $\pm$  37.8%; Thd = 129%  $\pm$  39.8%).

#### *sTNFR2 production by LPS-stimulated whole blood cultures was not affected by CC-4047, Thd or CC-3052*

We next determined whether the divergent effects of CC-4047 and CC-3052 on sTNFR2 production seen during T cell co-stimulation with  $\alpha$ CD3 were also apparent during LPS activation of monocyte/macrophage cell populations. In contrast to the effect during T cell activation we found that there was no effect of any compound on sTNFR1 or sTNFR2 production by LPS-stimulated whole blood cultures at any time-point (Fig. 1d,e). As expected, CC-3052 and CC-4047 both strongly inhibited LPS-induced TNF- $\alpha$  production (Fig. 1f) and were more effective than Thd in this respect.

#### *CC-4047 and CC-5013 strongly reduced the surface expression of TNFR2 on $\alpha$ CD3-stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells during PBMC co-stimulation*

We then determined whether the inhibitory effect of CC-4047 on supernatant levels of sTNFR2 during co-stimulation was due to reduced surface TNFR2 expression or to reduced receptor shedding. We found that CC-4047 and CC-5013 (and to a lesser extent Thd) reduced the surface expression of TNFR2 and that this correlated with the induction of surface CD25 (IL-2 receptor) (Fig. 2). Furthermore, this was seen equally in both CD4<sup>+</sup> (Fig. 2a) and CD8<sup>+</sup> populations (Fig. 2b). Results from two dose–response experiments indicated that co-stimulation (gated by CD69



**Fig. 1.** Co-stimulatory CC-4047 strongly inhibits the secretion of sTNFR2 by  $\alpha$ CD3-stimulated PBMC cultures (but not LPS-stimulated cultures) whereas non-co-stimulatory SelCID CC-3052 augments sTNFR2. Divergent effects of CC-4047 (O), CC-3052 ( $\square$ ), and Thd ( $\Delta$ ) on the production of sTNFR2, sTNFR1 & TNF- $\alpha$  by PBMC-stimulated with  $\alpha$ CD3 (a-c) and whole blood cultures stimulated with LPS (d-f). Cultures were incubated  $\pm$  Thd/analogues (10  $\mu$ g/ml) and supernatants collected at the indicated times as per Materials and methods. Results are expressed as percentage change compared to control cultures with DMSO alone. Data presented are from four normal donors used in two separate experiments.

expression) and inhibition of surface TNFR2 could both be detected at CC-4047 concentrations as low as 10 ng (data not shown).

We were unable to detect the presence of surface TNFR1 either with  $\alpha$ CD3 alone or in the presence of the co-stimulatory compounds using cells from a total of four donors in two separate experiments (data not shown).

#### CC-4047 did not alter the level of total cellular TNFR2 (intracellular plus surface) in T cells during PBMC co-stimulation

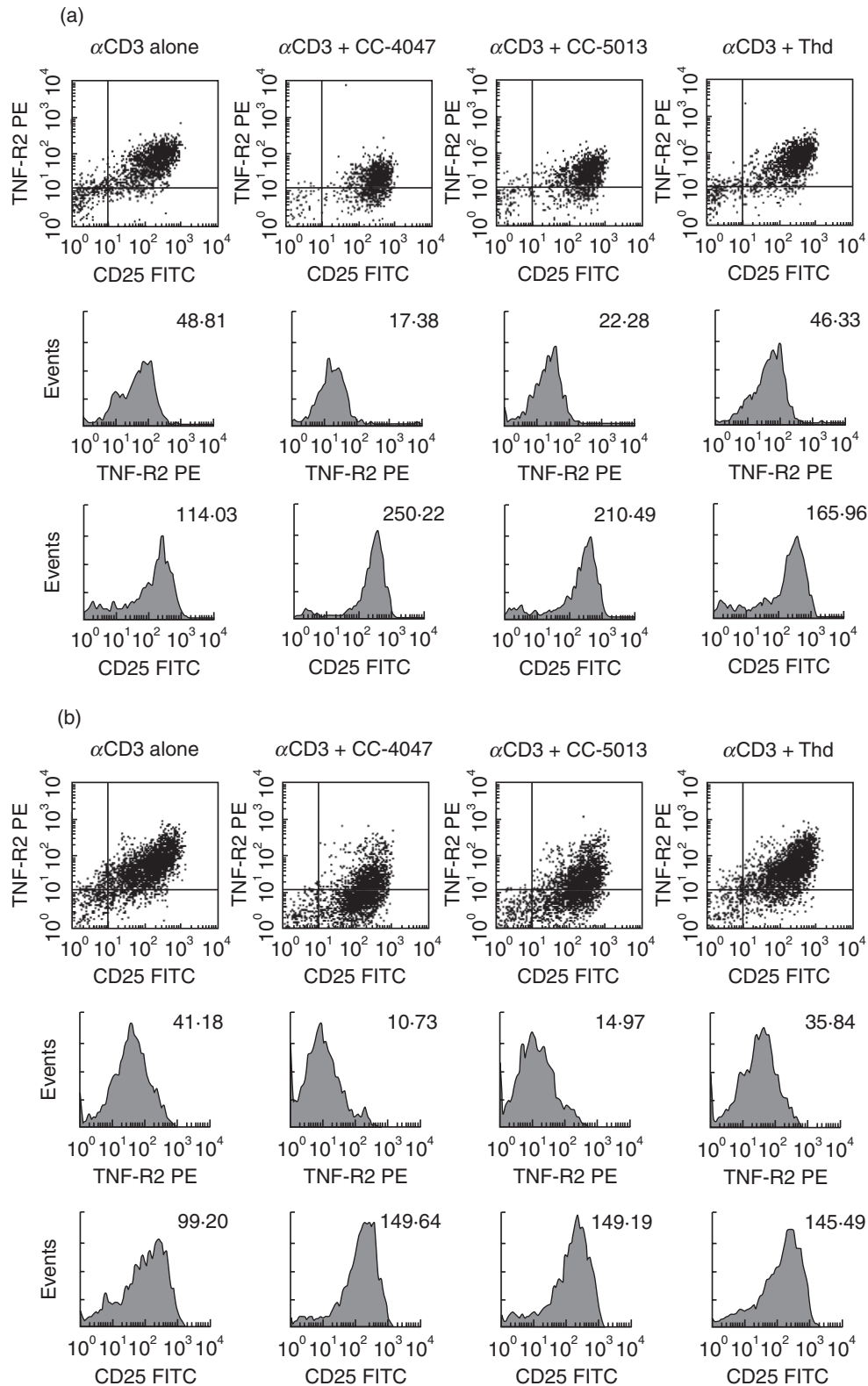
We investigated whether the inhibitory effect of CC-4047 on surface TNFR2 was due to the inhibition of TNFR2 protein production. We utilized the protein transport inhibitor Brefeldin A and two-colour flow cytometry in order to measure the total amount of cellular TNFR2 within CD4<sup>+</sup> and CD8<sup>+</sup> cells during PBMC co-stimulation. The data presented in Fig. 3 show that when total protein was analysed CC-4047 did not appear to exert an inhibitory effect on TNFR2. The data presented suggest strongly that TNFR2 protein production itself is not being affected. Therefore, it is likely that trafficking to the cell surface that is being inhibited. Furthermore, our data also rule out an inhibitory effect on TNFR2 transcription.

#### The relative extent of TNFR2 inhibition by CC-4047, CC-5013 and Thd corresponds to co-stimulatory activity as gauged by the production of IL-2, sIL-2R and proliferation in $\alpha$ CD3 stimulated PBMC cultures

We then investigated whether there was a possible connection between the inhibitory effect of these compounds on TNFR2 surface expression and co-stimulatory activity. The co-stimulatory activity of CC-4047, CC-5013 and Thd was assessed by IL-2 and sIL-2R production and also by the incorporation of tritiated thymidine (to measure proliferation).

We found that IL-2 production was not detected in control ( $\alpha$ CD3 alone) cultures. However, after 24 h CC-4047, CC-5013 and, to a lesser extent Thd, strongly induced IL-2 production (Fig. 4a). For CC-4047 this effect was still apparent after 7 days of culture. The increased expression of sIL-2R became apparent after 72 h and followed a very similar pattern (Fig. 4b). Proliferation was assessed at days 5–6 with a consistent pattern of results (Fig. 4c). Therefore, using these three markers of co-stimulation the same relative pattern of effectiveness emerged: CC-4047 > CC-5013 >> Thd.

In contrast to these effects the non-co-stimulatory SelCID, CC-3052, strongly inhibited IL-2 and sIL-2R although it had no effect on proliferation, as we have shown previously [19].



**Fig. 2.** CC-4047 and CC-5013 strongly reduced the surface expression of TNFR2 on  $\alpha$ CD3 stimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells during PBMC co-stimulation. PBMC were incubated  $\pm$  Thd/analogues (10  $\mu$ g/ml) for 48 h. Three-colour flow cytometric analysis was performed by surface staining stimulated PBMC with anti-CD25 FITC, anti-TNFR2 PE and anti-CD4/CD8 PerCP plus appropriate isotype matched and compensation controls as described in Materials and methods. (a) Dot plots represent TNFR2 versus CD25 (IL-2 receptor) gated on (a) CD4 and (b) CD8 T cell subsets. Histograms show mean fluorescent intensity of TNFR2 expression on CD25<sup>+</sup> T cells. This effect was highly consistent when performed in 10 separate experiments each using PBMC from two normal donors although data from a single representative experiment are shown.

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