

# Mycobacteria activate $\gamma\delta$ T-cell anti-tumour responses via cytokines from type 1 myeloid dendritic cells: a mechanism of action for cancer immunotherapy

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**Abstract** Attenuated and heat-killed mycobacteria display demonstrable activity against cancer in the clinic; however, the induced immune response is poorly characterised and potential biomarkers of response ill-defined. We investigated whether three mycobacterial preparations currently used in the clinic (BCG and heat-killed *Mycobacterium vaccae* and *Mycobacterium obuense*) can stimulate anti-tumour effector responses in human  $\gamma\delta$  T-cells.  $\gamma\delta$  T-cell responses were characterised by measuring cytokine production, expression of granzyme B and cytotoxicity against tumour target cells. Results show that  $\gamma\delta$  T-cells are activated by these mycobacterial preparations, as indicated by upregulation of activation marker expression and proliferation. Activated  $\gamma\delta$  T-cells display enhanced effector responses, as shown by upregulated granzyme B expression, production of the  $T_H1$  cytokines IFN- $\gamma$  and TNF- $\alpha$ , and enhanced degranulation in response to susceptible and zoledronic acid-treated resistant tumour cells. Moreover,  $\gamma\delta$  T-cell activation is induced by IL-12, IL-1 $\beta$  and TNF- $\alpha$  from circulating type 1 myeloid dendritic cells (DCs), but not from type 2 myeloid DCs or plasmacytoid DCs. Taken together, we show that BCG, *M. vaccae* and *M. obuense* induce  $\gamma\delta$  T-cell anti-tumour effector responses indirectly via a specific subset of circulating DCs and suggest a mechanism for the potential immunotherapeutic effects of BCG, *M. vaccae* and *M. obuense* in cancer.

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## Introduction

The immune system's intrinsic ability to recognise and destroy tumour cells is an attractive target for therapeutic intervention. Moreover, cancer patients are often immunocompromised due to the immunosuppressive nature of the tumour and collateral effects of certain chemotherapies. Cancer immunotherapy aims to bolster appropriate anti-tumour immune responses; an approach that has great potential as a stand-alone treatment or combinatorial partner of standard chemotherapy. Indeed, cancer immunotherapy has had many successes, with one of the most prominent milestones to date being the use of Bacillus Calmette Guérin (BCG) to treat bladder cancer [1].

The remarkable success of BCG therapy in bladder cancer highlights the potential for mycobacterial preparations as cancer immunotherapeutic agents or as adjuvants to existing chemotherapies. There has also been considerable interest in a heat-killed preparation of *Mycobacterium vaccae* for the treatment of cancers of the prostate, lung, kidney and skin [2–5]. In particular, *M. vaccae* combined with chemotherapy increased survival and improved quality of life in phase III trials for adenocarcinoma of the lung [6, 7]. Recently, attention has turned to heat-killed preparations of *M. obuense*, which are currently undergoing early phase trials in melanoma [8]. Although mycobacterial preparations are currently under clinical investigation, mechanistic studies are limited and biomarkers of response ill-defined, which is hampering their success. In vitro studies are needed to identify candidate immune cells for immunological monitoring during clinical trials.  $\gamma\delta$  T-cells have a number of properties

that highlight them as a potential mechanism by which mycobacterial preparations elicit their anti-cancer effects. In particular,  $\gamma\delta$  T-cells play a demonstrable role in protective immunosurveillance against cancer [9–11]; are highly reactive against mycobacterial antigens [12]; and display potent cross-reactivity against tumour cells and mycobacteria [13]. However, the capacity for clinical preparations of BCG, *M. vaccae* and *M. obuense* to elicit effector responses in  $\gamma\delta$  T-cells has been largely overlooked and remains poorly characterised.

$\gamma\delta$  T-cells can elicit protective immune responses against cancer and are an essential component of the anti-tumour immune response. In vitro, human  $\gamma\delta$  T-cells display potent cytotoxicity against tumour cells from a broad range of epithelial and haematologic malignancies [14–16]. They also produce interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$  in response to mycobacteria and tumour, which potentiate protective cell-mediated immune responses against cancer [17]. Moreover,  $\gamma\delta$  T-cell responses to antigenic challenge are rapid and memory-like, thus providing an early defence mechanism that complements the delayed immune response of  $\alpha\beta$  T-cells [18]. In contrast to  $\alpha\beta$  T-cells,  $\gamma\delta$  T-cells recognise phosphoantigens independently of major histocompatibility complex (MHC) class I, which is often down-modulated in a range of cancers, thus reinforcing the value of  $\gamma\delta$  T-cells in cancer immunotherapy [19].  $\gamma\delta$  T-cells also express the natural killer activatory receptor NKG2D; this receptor interacts with MHC class I-related stress molecules such as MICA and MICB, which are frequently upregulated on tumours [20].

Our aim was to examine whether BCG and heat-killed *M. vaccae* and *M. obuense* can prime  $\gamma\delta$  T-cells for an anti-tumour effect. Data presented herein suggest that these mycobacterial preparations stimulate anti-tumour responses in  $\gamma\delta$  T-cells, as shown by production of  $T_H1$  cytokines, upregulation of granzyme B and increased cytotoxicity against tumour cells. Furthermore, data suggest that  $\gamma\delta$  T-cell responses are indirectly stimulated by IL-12, IL-1 $\beta$  and TNF- $\alpha$  from circulating type 1 myeloid dendritic cells (mDC1s). Taken together, our study is the first to demonstrate that BCG, *M. vaccae* and *M. obuense* may enhance the effector responses of  $\gamma\delta$  T-cells by stimulating mDC1s to produce IL-12, IL-1 $\beta$  and TNF- $\alpha$ , which sheds light on the mechanism of action for the anti-cancer effects of these immunotherapies.

## Materials and methods

### Mycobacteria

Heat-killed *M. vaccae* and *M. obuense* were supplied by Professor John Stanford (University College London).

Mycobacteria were heat-killed by autoclaving at 121°C for 15 min in borate-buffered solution. Lyophilised BCG vaccine (Danish strain 1331; Statens Serum Institut) was resuspended in phosphate-buffered saline (PBS; Sigma) and heat-killed as described above. Mycobacteria were added to cell cultures using optimised doses of  $1 \times 10^5$  culturable particles/ml BCG, 100  $\mu\text{g/ml}$  *M. vaccae* and 100  $\mu\text{g/ml}$  *M. obuense* (supplementary fig. 1).

### Cell isolation/depletion

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by density-adjusted centrifugation using Histopaque –1,077 (Sigma). Contaminating red blood cells were lysed with hypotonic ammonium chloride (5 Prime) and platelets removed by centrifugation at 200 g. Specific cell populations were isolated or depleted from PBMCs using magnetic microbeads against TCR $\gamma\delta$ , CD14, CD4, CD8, CD19, CD56 and/or CD1c (Miltenyi Biotec) according to the manufacturer's instructions. Purities for cell isolations were analysed by flow cytometry and were consistently >95%.

### Cell culture

All cell cultures were performed at 37°C with 5% CO<sub>2</sub>.  $1 \times 10^6$  PBMCs in 200  $\mu\text{l}$  complete medium RPMI-1640 (Sigma) with 5% heat-inactivated human A/B serum (Lonza) and 2 mM L-glutamine (Sigma), were cultured in 96-well, flat-bottomed tissue culture plates. For proliferation assays, PBMCs were stained with 400 nM carboxy-fluorescein diacetate succinimidyl ester (CFSE; Invitrogen) and  $1 \times 10^6$  cells in 1 ml of complete medium cultured in 24-well tissue culture plates for 6 days. The percentage of cells with CFSE fluorescence lower than the untreated controls was used as a measure of proliferation.  $2\text{--}5 \times 10^4$  purified  $\gamma\delta$  T-cells in 200  $\mu\text{l}$  of complete medium were cultured in 96-well, round-bottomed tissue culture plates alone or with CD56<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> or CD1c<sup>+</sup> cells. For some experiments,  $\gamma\delta$  T-cells were cultured overnight with recombinant human IL-12 (Miltenyi Biotec), IL-1 $\beta$  and TNF- $\alpha$  (both Peprotech) at concentrations indicated in figure legends.  $1 \times 10^6$  CD4<sup>+</sup> cells in 200  $\mu\text{l}$  complete medium were cultured in 96-well, flat-bottomed tissue culture plates. For some experiments, supernatants were collected and passed through 0.2- $\mu\text{m}$  filters prior to use. For intracellular cytokine staining, 1  $\mu\text{g/ml}$  of Brefeldin A (Sigma) was added for the last 4 h of culture.

The following positive controls were used: isopentenyl pyrophosphate (IPP; 10  $\mu\text{g/ml}$ ); phorbol myristate acetate (PMA; 25 ng/ml); ionomycin (I; 1  $\mu\text{g/ml}$ ); phytohaemagglutinin-leucoagglutinin (PHA-L; 1  $\mu\text{g/ml}$ ); lipopolysaccharide (LPS; *E. coli*-derived; 1  $\mu\text{g/ml}$ ); R848 (2.5  $\mu\text{g/ml}$ ) (all from Sigma); and IL-2 (5U/ml; Peprotech).

The Burkitt's Lymphoma cell line Daudi and lung carcinoma cell line A549 (both ECACC) were cultured in RPMI-1640 or Dulbecco's modified eagle media (DMEM; Sigma), respectively, supplemented with 10% foetal bovine serum (FBS; Invitrogen) and 2 mM L-glutamine.  $2 \times 10^6$  A549 cells were added per 75 cm<sup>2</sup> tissue culture flask and allowed to adhere for 24 h prior to overnight culture with 0.1, 1 or 10  $\mu$ M of zoledronic acid (Novartis).

#### Flow cytometry

Cells were stained in buffer (PBS with 1% albumin bovine serum (Sigma) and 0.1% sodium azide (Sigma) for 30 min at 4°C with the following monoclonal antibodies: CD3-FITC, CD3-PERCP, TCR $\gamma\delta$ -PE, V $\delta$ 2-PE, CD69-FITC, CD69-PE, CD69-APC, HLA-DR-PERCP, CD25-APC, CD4-PE, CD123-PE, CD123-PECy5 and CD14-APC (all from Becton–Dickinson); CD11c-FITC, CD11c-PE and CD1c-PE (all from Miltenyi Biotec); and V $\delta$ 1-FITC (Thermo Scientific). Matched isotype control antibodies were used to determine background staining. Cells were then fixed in 4% paraformaldehyde (BD Cellfix; Becton–Dickinson) for 20 min at 4°C. For intracellular cytokine staining, cells were simultaneously fixed and permeabilised in 4% paraformaldehyde and 0.1% saponin (BD Cytotfix/Cytoperm; Becton–Dickinson) for 20 min at 4°C and then resuspended in 0.1% saponin with IFN- $\gamma$ -APC, TNF- $\alpha$ -APC, IL-10-APC, IL-12-APC (all from Miltenyi Biotec), IFN- $\gamma$ -PECy7 (Becton–Dickinson), IL-1 $\beta$ -APC (R and D Systems) or Granzyme B-APC (Caltag-MedSystems) for 10 min at room temperature. Expression levels were analysed using FACScalibur or LSRII with CellQuest Pro or FACs DIVA software, respectively (all Becton–Dickinson).

#### Tritiated <sup>3</sup>H incorporation

Tritiated [<sup>3</sup>H]-thymidine incorporation was used to assess proliferation of purified  $\gamma\delta$  T-cells. Cells were cultured with mycobacteria for 6 days prior to the addition of 2.5  $\mu$ Ci/ml [<sup>3</sup>H]-thymidine (GE Healthcare) for the last 18 h of culture. Cells were osmotically lysed and DNA collected onto filters (Perkin Elmer). Scintillation fluid (Perkin Elmer) was added prior to measuring beta emission (photon counts per minute) using a liquid scintillation counter (Perkin Elmer).

#### Cytokine analysis of culture supernatants

T<sub>H</sub>1/T<sub>H</sub>2 cytometric bead arrays (CBAs; Becton–Dickinson) were used to measure the concentration of IFN- $\gamma$ , TNF- $\alpha$ , IL-10, IL-5, IL-4 and IL-2 in cell culture supernatants according to the manufacturer's instructions. Supernatants were also analysed by multi-analyte profiling of inflammation-related analytes by Rules Based Medicine (USA).

#### Cytotoxicity assays

Mycobacteria-stimulated PBMCs were co-cultured with Daudi or zoledronic acid-treated A549 cells at a pre-optimised effector:target cell ratio of 2:1. Antibodies to CD107b-FITC and CD107a-APC or IgG1 $\kappa$ -FITC and IgG1 $\kappa$ -APC (Becton–Dickinson) were added directly to the wells. Co-cultures were incubated for 1 h prior to addition of 1  $\mu$ g/ml monensin (Sigma) to neutralise intracellular acidity. After a further 5 h of culture, V $\delta$ 2 T-cell expression of CD107a and CD107b was assessed by flow cytometry. Alternatively, cytokine-treated  $\gamma\delta$  T-cells were co-cultured overnight with Daudi cells at target:effector cell ratios of 1:10, 1:20 and 1:50 in 96-well, V-bottomed tissue culture plates. Cytotoxicity was assessed using the lactate dehydrogenase assay (Promega) according to the manufacturer's instructions. Percentage cytotoxicity was calculated against maximum target cell release using 9% Triton X-100 (Promega).

#### Blocking experiments

PBMCs were cultured overnight with mycobacteria in the presence of blocking antibodies to IL-12, IL-1 $\beta$  and TNF- $\alpha$  (R and D Systems) each at 100  $\mu$ g/ml. Also, culture supernatants from mycobacteria-treated CD4<sup>+</sup> cells were pre-treated for 1 h with blocking antibodies to IL-12, IL-1 $\beta$  and/or TNF- $\alpha$  each at 100  $\mu$ g/ml prior to culturing with  $\gamma\delta$  T-cells. Goat IgG1 isotype antibodies (R and D Systems) were used as controls throughout.

#### Statistical analyses

Statistical testing was carried out using SigmaStat (SPSS Inc.) analytical software. Statistical differences between conditions were determined using one-way ANOVA (for parametric data with equal variances and normal distributions) or Kruskal–Wallis ANOVA on ranks (for non-parametric data) followed by either Holm–Sidak or Dunn's Tests, respectively. For some experiments, statistical differences between conditions were determined using the student's paired t test. Differences with *P* values less than 0.05 were deemed significant.

## Results

$\gamma\delta$  T-cells within mycobacteria-treated PBMCs produce granzyme B and T<sub>H</sub>1 cytokines

To determine the effects of mycobacterial adjuvants on  $\gamma\delta$  T-cells, we measured activation, proliferation, granzyme B expression and cytokine production by  $\gamma\delta$  T-cells within BCG-, *M. vaccae*- and *M. obuense*-treated PBMCs.

Preliminary experiments show that live and heat-killed BCG were equipotent at upregulating CD69 on  $\gamma\delta$  T-cells (supplementary fig. 1a). Using the pre-optimised concentrations determined in supplementary figs. 1a and 1b, heat-killed BCG, *M. vaccae* and *M. obuense* upregulated CD69, CD25 and HLA-DR expression on  $\gamma\delta$  T-cells (Fig. 1a). Activation was restricted to the  $V\delta 2^+$  subset, as shown by upregulated CD69 expression in  $V\delta 2^+$  but not  $V\delta 1^+$  cells (Fig. 1b). The mycobacteria induced proliferation in  $\gamma\delta$  T-cells; however, levels of proliferation against BCG and *M. obuense* were markedly higher than against *M. vaccae* (Fig. 1c). Changes in the percentage of  $\gamma\delta$  T-cells within PBMCs after 6 days of mycobacterial stimulation were only minor and not significant (data not shown), suggesting that other cell types are also proliferating. Moreover, the mycobacteria upregulated  $\gamma\delta$  T-cell expression of granzyme B (Fig. 1d). Analysis of supernatants from mycobacteria-treated PBMCs indicated detectable levels of IFN- $\gamma$ , TNF- $\alpha$  and IL-10, but relatively low levels of IL-5, IL-4 and IL-2 (data not shown). Intracellular cytokine staining revealed that  $\gamma\delta$  T-cells produced IFN- $\gamma$  and TNF- $\alpha$ , but not IL-10 (Fig. 1e).

#### Mycobacteria-activated $V\delta 2^+$ cells have enhanced cytotoxicity against tumour cells

Upregulation of granzyme B suggests that mycobacteria-activated  $\gamma\delta$  T-cells may have enhanced cytotoxic properties. We investigated the ability of mycobacteria-activated  $\gamma\delta$  T-cells to degranulate in the presence of tumour cells. The Burkitt's lymphoma cell line Daudi and the lung cancer cell line A549 were selected as  $\gamma\delta$  T-cell susceptible and resistant tumour target cells, respectively. Resting  $V\delta 2^+$  cells increased CD107a/b expression when exposed to Daudi but not A549 target cells (Fig. 2a). Mycobacteria-activated  $V\delta 2^+$  cells had higher levels of Daudi-induced CD107a/b expression than untreated  $V\delta 2^+$  cells (Fig. 2b–d). Pre-treating A549 cells with zoledronic acid increased their capacity to induce degranulation in resting  $V\delta 2^+$  cells (Fig. 2e). Moreover, mycobacteria-activated  $V\delta 2^+$  cells had increased percentage expression of CD107a/b and mean fluorescence intensity (MFI) expression of CD107a in response to A549 cells pre-treated with zoledronic acid (Fig. 2e). Taken together, mycobacterial adjuvants enhance  $\gamma\delta$  T-cell degranulation in the presence of susceptible tumours but further treatment was required to expose this effect in tumours that are refractory to  $\gamma\delta$  T-cell killing.

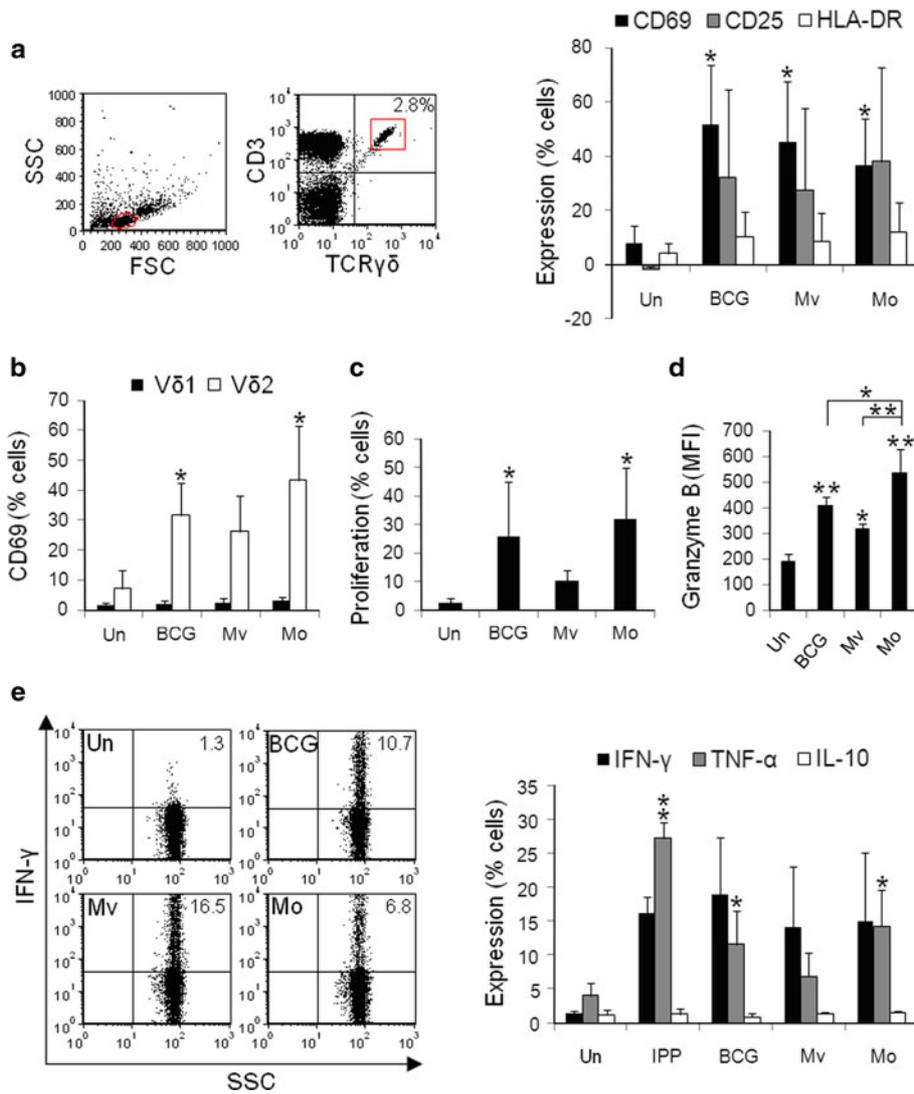
#### $V\delta 2^+$ cell responses to mycobacteria are dependent on CD4<sup>+</sup> cells

A range of cell types have been implicated in mediating  $\gamma\delta$  T-cell activation [21–23]. To determine whether the effect

of BCG, *M. vaccae* and *M. obuense* on  $\gamma\delta$  T-cells is direct or mediated by another cell type, the capacity for these mycobacterial preparations to activate highly purified  $\gamma\delta$  T-cells was assessed. Purified  $\gamma\delta$  T-cells (mean  $\pm$  SD:  $98.2 \pm 1.1\%$ ) were not activated by the mycobacteria, as shown by baseline levels of CD69 expression, cytokine production and proliferation (Fig. 3a–c). To determine the cell type required for activation, PBMCs were sequentially depleted of CD14<sup>+</sup> cells (monocytes), CD19<sup>+</sup> cells (B-cells) and CD56<sup>+</sup> cells (NK and NKT cells) prior to overnight stimulation and analysis of CD69 expression on  $V\delta 2^+$  cells. Depletion of CD14<sup>+</sup> monocytes augmented mycobacteria-induced  $V\delta 2^+$  cell expression of CD69 (Fig. 3d) and IFN- $\gamma$  (data not shown), suggesting that in our system monocytes are inhibitory. Furthermore, co-culturing  $\gamma\delta$  T-cells with monocytes failed to restore their response to the mycobacteria (data not shown). Subsequent depletion of CD19<sup>+</sup> B-cells had no effect, whereas depletion of CD56<sup>+</sup> NK and NKT cells reduced mycobacteria-induced CD69 expression on  $V\delta 2^+$  cells (Fig. 3d). Purified  $\gamma\delta$  T-cells were then stimulated with mycobacteria in the presence of either CD56<sup>+</sup> cells or a combination of CD4<sup>+</sup> and CD8<sup>+</sup> cells prior to flow cytometric analysis of IFN- $\gamma$  production. In the presence of CD4<sup>+</sup>/CD8<sup>+</sup> cells but not CD56<sup>+</sup> cells, the mycobacteria induced IFN- $\gamma$  production by  $V\delta 2^+$  cells (Fig. 3e). Furthermore, CD4<sup>+</sup> but not CD8<sup>+</sup> cells were responsible for restoring  $\gamma\delta$  T-cell responses to the mycobacterial preparations (Fig. 3f).

#### $V\delta 2^+$ cells are activated by IL-12, IL-1 $\beta$ and TNF- $\alpha$ released from mycobacteria-stimulated CD4<sup>+</sup> cells

We assessed whether soluble mediators are responsible for  $\gamma\delta$  T-cell activation. Culture supernatants from mycobacteria-stimulated CD4<sup>+</sup> cells were added to purified  $\gamma\delta$  T-cells, and IFN- $\gamma$  production by the  $V\delta 2^+$  subset was measured. Results show that culture supernatants from treated CD4<sup>+</sup> cells upregulated IFN- $\gamma$  production by  $V\delta 2^+$  cells (Fig. 4a). Multi-analyte profiling of culture supernatants from mycobacteria-treated CD4<sup>+</sup> cells revealed the presence of a number of cytokines (data not shown). Of these, IL-12, IL-1 and TNF have been previously shown to mediate  $\gamma\delta$  T-cell activation [24–26]. To determine the role played by these three cytokines, PBMCs were stimulated with mycobacteria in the presence of blocking antibodies to IL-12, IL-1 $\beta$  and TNF- $\alpha$ . This resulted in a marked reduction in  $V\delta 2^+$  cell IFN- $\gamma$  production (Fig. 4b), indicating that these cytokines are key mediators in  $\gamma\delta$  T-cell activation. To confirm this observation, isolated  $\gamma\delta$  T-cells were stimulated with conditioned media from mycobacteria-treated CD4<sup>+</sup> cells in the presence of blocking antibodies to IL-12, IL-1 $\beta$  and TNF- $\alpha$ . Results show that blocking these cytokines reduced  $V\delta 2^+$  cell IFN- $\gamma$  production (Fig. 4c, d). Analysis of these block-



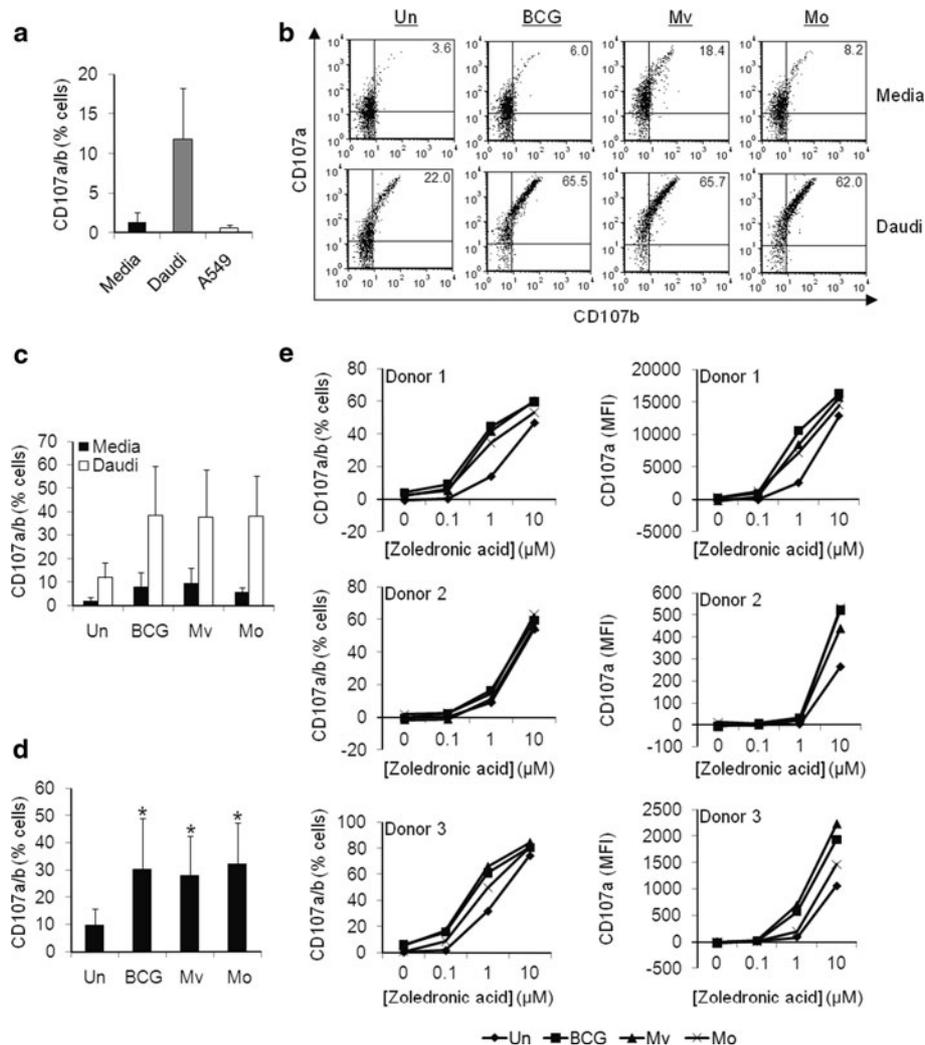
**Fig. 1**  $\gamma\delta$  T-cells within BCG-, *M. vaccae*- and *M. obuense*-treated PBMCs produce granzyme B and T<sub>H</sub>1 cytokines. PBMCs were cultured with heat-killed BCG, *M. vaccae* (Mv) and *M. obuense* (Mo) and responses measured within gated  $\gamma\delta$  T-cells. Untreated (un) cells were used as a negative control. **a** Gating strategy used to identify  $\gamma\delta$  T-cells within PBMCs. Lymphocytes were gated according to size (forward scatter; FSC) and granularity (side scatter; SSC). Within lymphocytes,  $\gamma\delta$  T-cells were gated as CD3<sup>+</sup>TCR $\gamma\delta$ <sup>+</sup>. Mean percentages of  $\gamma\delta$  T-cells expressing CD69 (at 24 h; n = 5), CD25 and HLA-DR (both at 48 h; n = 3) are shown. **b** Mean percentages of V $\delta$ 1<sup>+</sup> and V $\delta$ 2<sup>+</sup> cells express-

ing CD69 (at 24 h; n = 3). **c** CFSE<sup>+</sup> PBMCs were cultured for 6 days with mycobacteria and proliferation measured within the  $\gamma\delta$  T-cell compartment. Mean percentages of  $\gamma\delta$  T-cells proliferating are shown (n = 6). **d** Mean fluorescent intensities (MFI) of granzyme B expression within  $\gamma\delta$  T-cells (at 24 h; n = 3). **e** Percentages of  $\gamma\delta$  T-cells expressing IFN- $\gamma$ , TNF- $\alpha$  and IL-10 were measured after 24 h of stimulation. Representative flow cytometric dot plots from one donor and mean values for n = 3 are shown. Error bars represent SD. \* and \*\* indicates P values of <0.05 and <0.001, respectively, for statistical comparisons between treated and untreated cells

ing antibodies either individually or in various combinations revealed a combined effect of all three cytokines (Fig. 4d).

To confirm the key role of these cytokines, we measured  $\gamma\delta$  T-cell IFN- $\gamma$  production, granzyme B expression and cytotoxicity in response to recombinant IL-12, IL-1 $\beta$  and TNF- $\alpha$ .  $\gamma\delta$  T-cells cultured with all three cytokines resulted in the greatest IFN- $\gamma$  response in the V $\delta$ 2<sup>+</sup> subset (Fig. 5a). In accordance with data shown in Fig. 1b, these cytokines

did not induce IFN- $\gamma$  production in V $\delta$ 2<sup>-</sup>  $\gamma\delta$  T-cells (Fig. 5a). IL-12, IL-1 $\beta$  and TNF- $\alpha$  upregulated intracellular expression of granzyme B (Fig. 5b), yet did not induce degranulation, as shown by baseline levels of CD107a expression (data not shown).  $\gamma\delta$  T-cells activated with these cytokines showed a marked increase in cytotoxicity against Daudi target cells (Fig. 5c). Together, these data demonstrate that IL-12, IL-1 $\beta$  and TNF- $\alpha$  can induce effector responses in  $\gamma\delta$  T-cells.



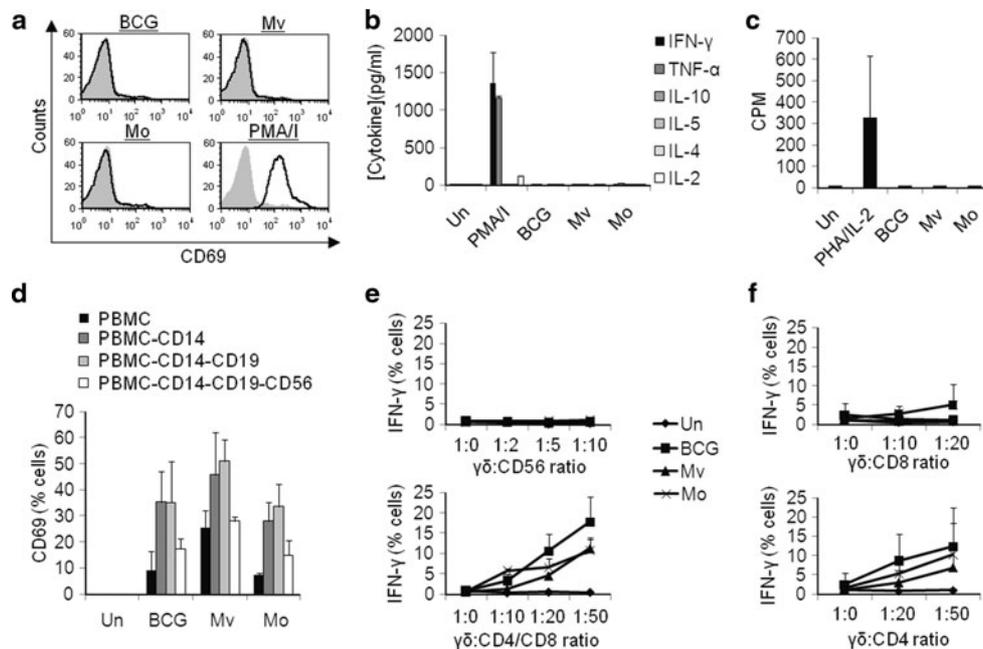
**Fig. 2** BCG, *M. vaccae* and *M. obuense* enhance  $\gamma\delta$  T-cell cytotoxicity. PBMCs were cultured overnight with heat-killed BCG, *M. vaccae* (Mv) and *M. obuense* (Mo). Untreated (un) cells were used as a negative control. PBMCs were then co-cultured for 6 h with Daudi or A549 cells at an effector:target cell ratio of 2:1 and CD107a/b expression measured within gated  $V\delta 2^+$  cells. **a** Mean percentage of  $V\delta 2^+$  cells expressing CD107a/b in the absence (media) or presence of Daudi or A549 target cells ( $n \geq 5$ ). **b** Representative flow cytometric dot plots from one donor showing CD107a/b expression on gated  $V\delta 2^+$  cells. **c** Mean percentage of  $V\delta 2^+$  cells expressing CD107a/b in the absence (Media) or presence of Daudi target cells ( $n = 5$ ). **d** Media test scores

were subtracted from Daudi test scores. Mean values are shown for  $n = 5$ . Error bars represent SD and \* indicates a  $P$  value of  $<0.05$  for statistical comparisons between untreated and treated conditions. For statistical testing, data were standardised by subtracting the untreated scores from test scores. **e** Percentage of  $V\delta 2^+$  cells expressing CD107a/b and mean fluorescent intensity (MFI) expression of CD107a on  $V\delta 2^+$  cells in the presence of zoledronic acid-treated A549 cells. Background levels of degranulation were subtracted from A549-induced degranulation. Individual experiments for three donors are shown

IL-12, IL-1 $\beta$  and TNF- $\alpha$  are produced by CD4 $^+$  type 1 myeloid DCs

We have determined the importance of CD4 $^+$  cells and their cytokines in mycobacteria-induced stimulation of  $\gamma\delta$  T-cells. However, CD4 is predominantly expressed on  $\alpha\beta$  T-cells, which typically do not produce IL-12 or IL-1 $\beta$ . These cytokines are mostly associated with antigen presenting cells (APCs) such as monocytes and DCs, which express low levels of CD4. Therefore, we assessed whether APCs are

present in the CD4 $^+$  cell population and measured intracellular expressions of IL-12, IL-1 $\beta$  and TNF- $\alpha$ . Monocytes were depleted from the PBMC preparations prior to CD4 $^+$  cell isolation and therefore were not present in the CD4 $^+$  cell population. Flow cytometric analysis of CD4, CD3, CD11c, CD123 and CD14 revealed three populations of cells: T-cells (CD4 $^{\text{high}}$ CD3 $^+$ ), myeloid DCs (mDCs; CD4 $^{\text{low}}$ CD3 $^-$ CD11c $^+$ CD123 $^{-/\text{low}}$ CD14 $^-$ ) and plasmacytoid DCs (pDCs; CD4 $^{\text{low}}$ CD3 $^-$ CD11c $^-$ CD123 $^{\text{high}}$ CD14 $^-$ ) (Fig. 6a). Intracellular cytokine staining of mycobacteria-treated CD4 $^+$  cells



**Fig. 3** BCG-, *M. vaccae*- and *M. obuense*-induced activation of  $\gamma\delta$  T-cells is dependent on CD4<sup>+</sup> cells. **a**  $\gamma\delta$  T-cells were cultured overnight with PMA/I, heat-killed BCG, *M. vaccae* (Mv) and *M. obuense* (Mo) and CD69 expression measured. Representative flow cytometric histogram plots from one of two donors are shown. Expression for untreated cells is shown in grey fill. **b** Culture supernatants from  $\gamma\delta$  T-cells were screened for their cytokine content using cytometric bead arrays. Mean values for  $n = 3$  are shown. **c**  $\gamma\delta$  T-cells were cultured with PHA/IL-2, BCG, Mv and Mo for 6 days and <sup>3</sup>H incorporation measured for the last 16 h of culture. Mean counts per minute (CPM) are shown for  $n = 2$ . **d** PBMCs were sequentially depleted of CD14<sup>+</sup>, CD19<sup>+</sup> and

CD56<sup>+</sup> cells and stimulated overnight with BCG, Mv and Mo prior to measuring CD69 expression on gated V $\delta$ 2<sup>+</sup> cells. Data were standardised by subtracting untreated scores from test scores. Mean values for  $n = 3$  are shown. **e**  $\gamma\delta$  T-cells were co-cultured overnight with CD56<sup>+</sup> cells (*top graph*) or a combination of CD4<sup>+</sup> and CD8<sup>+</sup> cells (*bottom graph*) in the presence of mycobacteria prior to measuring IFN- $\gamma$  expression on gated V $\delta$ 2<sup>+</sup> cells. Mean values for  $n \geq 2$  are shown. **f**  $\gamma\delta$  T-cells were co-cultured overnight with either CD8<sup>+</sup> (*top graph*) or CD4<sup>+</sup> (*bottom graph*) in the presence of mycobacteria prior to measuring IFN- $\gamma$  expression on gated V $\delta$ 2<sup>+</sup> cells. Mean values for  $n = 3$  are shown. *Error bars* represent SD

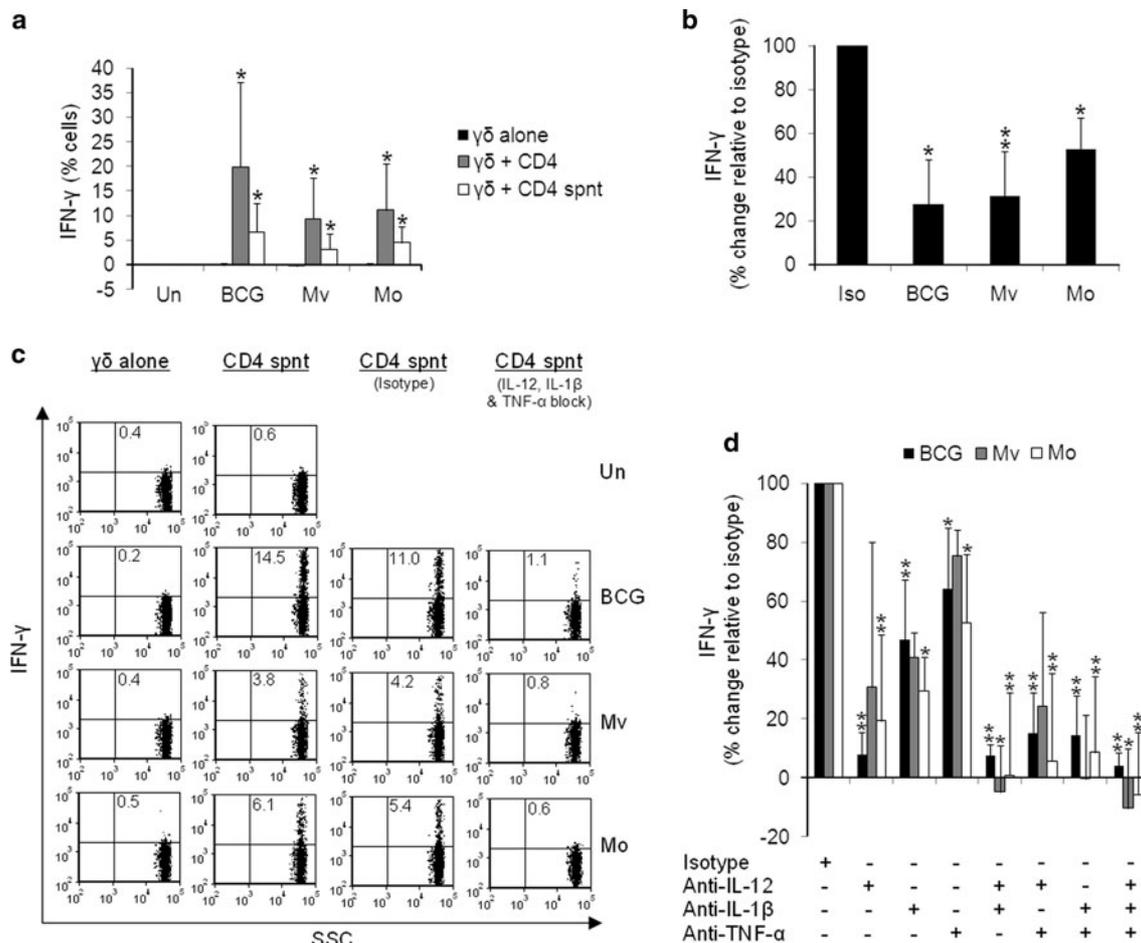
revealed that IL-12, IL-1 $\beta$  and TNF- $\alpha$  were expressed predominantly in mDCs (Fig. 6b).

Two types of mDC have been defined based on expression of CD123: type 1 (CD123<sup>low</sup>; mDC1) and type 2 (CD123<sup>-</sup> mDC2) [27]. Analysis of CD123 expression revealed that CD123<sup>+</sup> but not CD123<sup>-</sup> mDCs produced IL-12, IL-1 $\beta$  and TNF- $\alpha$  in response to the mycobacteria, suggesting that mDC1s and not mDC2s are involved in the  $\gamma\delta$  T-cell response to these mycobacterial preparations (data not shown). Accordingly, the majority of the mDC population expressed CD1c, a specific marker for mDC1s (Fig. 6c). To confirm the role of mDC1s in the  $\gamma\delta$  T-cell response to mycobacteria, we depleted CD1c<sup>+</sup> cells from the CD4<sup>+</sup> cell population and assessed the effects on  $\gamma\delta$  T-cell activation. Results show that when CD1c<sup>+</sup> cells were depleted from  $\gamma\delta$  T-cell and CD4<sup>+</sup> cell co-cultures, IFN- $\gamma$  production by V $\delta$ 2<sup>+</sup> cells in response to mycobacteria was lost (Fig. 6d). Taken together, data suggest that mDC1s are activated by mycobacteria to produce cytokines that activate  $\gamma\delta$  T-cell responses.

## Discussion

As part of our ongoing studies into the mechanisms of action for BCG, *M. vaccae* and *M. obuense* cancer immunotherapy, we investigated the potential role of  $\gamma\delta$  T-cells. Our data suggest that BCG, *M. vaccae* and *M. obuense* activate an anti-tumour programme in peripheral blood V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T-cells that is characterised by T<sub>H</sub>1 cytokine production and enhanced cytotoxic responses against tumour. Moreover, our data suggest that these responses are indirectly mediated by IL-12, IL-1 $\beta$  and TNF- $\alpha$  from circulating mDC1s.

$\gamma\delta$  T-cells are highly responsive to mycobacteria; however, studies have primarily focussed on the stimulatory capacity of live mycobacterial infections and lysate preparations. We focussed on live attenuated BCG and heat-killed *M. vaccae* and *M. obuense*, which are preparations of mycobacteria that are currently used as cancer immunotherapies in the clinic. Little is known about whether these preparations can stimulate anti-tumour effector responses in  $\gamma\delta$  T-cells nor the mechanisms involved. We show here that



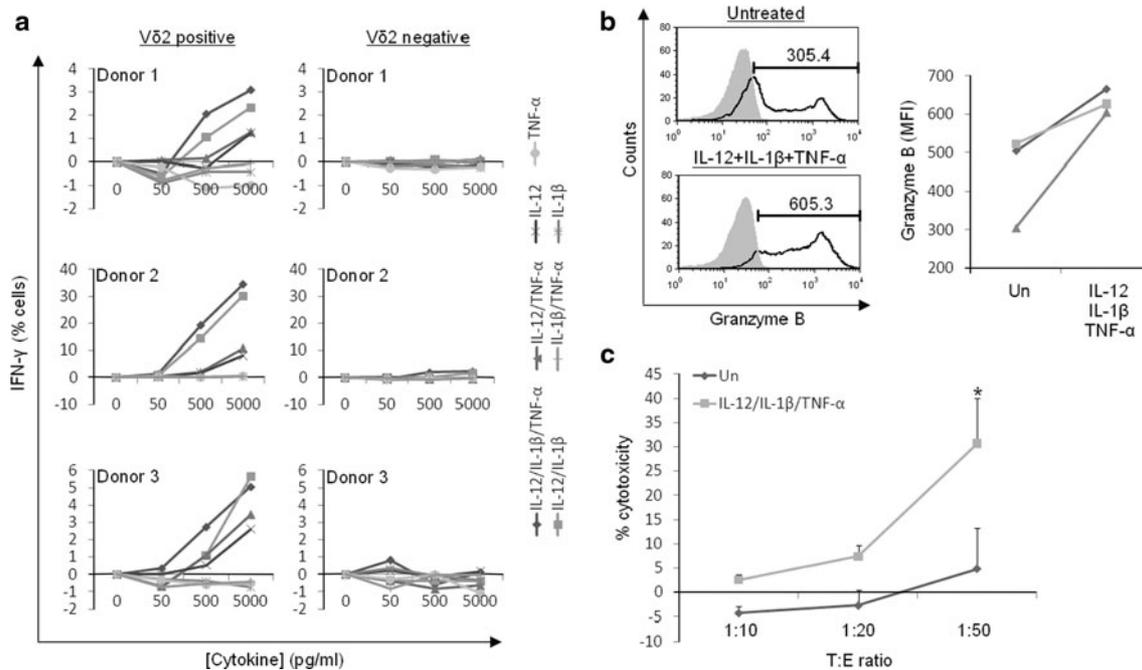
**Fig. 4** IL-12, IL-1 $\beta$  and TNF- $\alpha$  from BCG-, *M. vaccae*- and *M. obuense*-treated CD4<sup>+</sup> cells activate V $\delta$ 2<sup>+</sup> cells. **a** CD4<sup>+</sup> cells were cultured overnight with heat-killed BCG, *M. vaccae* (Mv) and *M. obuense* (Mo).  $\gamma\delta$  T-cells were then cultured overnight in culture supernatant (spnt) from treated CD4<sup>+</sup> cells prior to measuring IFN- $\gamma$  expression on gated V $\delta$ 2<sup>+</sup> cells. IFN- $\gamma$  expression was also measured on  $\gamma\delta$  T-cells that had been cultured directly with mycobacteria in the presence or absence of CD4<sup>+</sup> cells. Data were standardised by subtracting untreated scores from test scores. **b** PBMCs were cultured overnight with mycobacteria in the presence of blocking antibodies to IL-12, IL-1 $\beta$  and TNF- $\alpha$  each at 100  $\mu$ g/ml. 300  $\mu$ g/ml of isotype

control antibodies were used as a control. IFN- $\gamma$  expression on gated V $\delta$ 2<sup>+</sup> cells was then measured. **c**  $\gamma\delta$  T-cells were cultured overnight with spnts from mycobacteria-treated CD4<sup>+</sup> cells in the presence of blocking antibodies to IL-12, IL-1 $\beta$  and TNF- $\alpha$  each at 100  $\mu$ g/ml prior to measuring IFN- $\gamma$  expression on gated V $\delta$ 2<sup>+</sup> cells. Representative flow cytometric dot plots are shown. **d** Mean percentage of V $\delta$ 2<sup>+</sup> cells expressing IFN- $\gamma$  relative to isotype control for experiments conducted in **c**. Data points are mean values ( $n = 7$  for **a**;  $n = 3$  for Mv and Mo in **b**;  $n = 2$  for BCG in **b**; and  $n = 3$  for **d**) where error bars represent SD and \* and \*\* indicate  $P$  values of  $<0.05$  and  $<0.001$ , respectively, for statistical comparisons between treated and untreated cells

BCG-, *M. vaccae*- and *M. obuense*-activated PBMCs contain  $\gamma\delta$  T-cells that produce the T<sub>H</sub>1 cytokines IFN- $\gamma$  and TNF- $\alpha$ , both of which play a demonstrable role in anti-tumour immunity. Documented effects of these cytokines include the following: MHC class I upregulation on tumour cells, which enhances recognition by cytotoxic  $\alpha\beta$  T-cells [28, 29]; cell cycle blockade and pro-apoptotic signalling, which hampers tumour cell growth [30, 31]; and T<sub>H</sub>1 differentiation, which is critical for generating protective immune responses against cancer [32, 33]. Evidence suggests that tumours can evade immune responses by upregulating T<sub>H</sub>2 and downmodulating T<sub>H</sub>1 immune responses; indeed, a T<sub>H</sub>2 bias correlates with disease progression in

certain cancers [34]. Therefore,  $\gamma\delta$  T-cell production of IFN- $\gamma$  and TNF- $\alpha$  in response to BCG, *M. vaccae* and *M. obuense* immunotherapy could counteract this tumour escape mechanism and restore the T<sub>H</sub>1 immune responses required to promote anti-cancer immunity.

We also investigated the effects of these mycobacterial preparations on  $\gamma\delta$  T-cell cytotoxicity against tumour cells. Studies have shown that V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T-cells are cytotoxic towards tumour cells from a broad range of haematologic and epithelial cancers [14]. Cytotoxicity is primarily dependent on TCR recognition of phosphoantigens and granzyme/perforin-dependent induction of apoptosis [35]. Also, BCG-specific  $\gamma\delta$  T-cell lines are cytotoxic towards



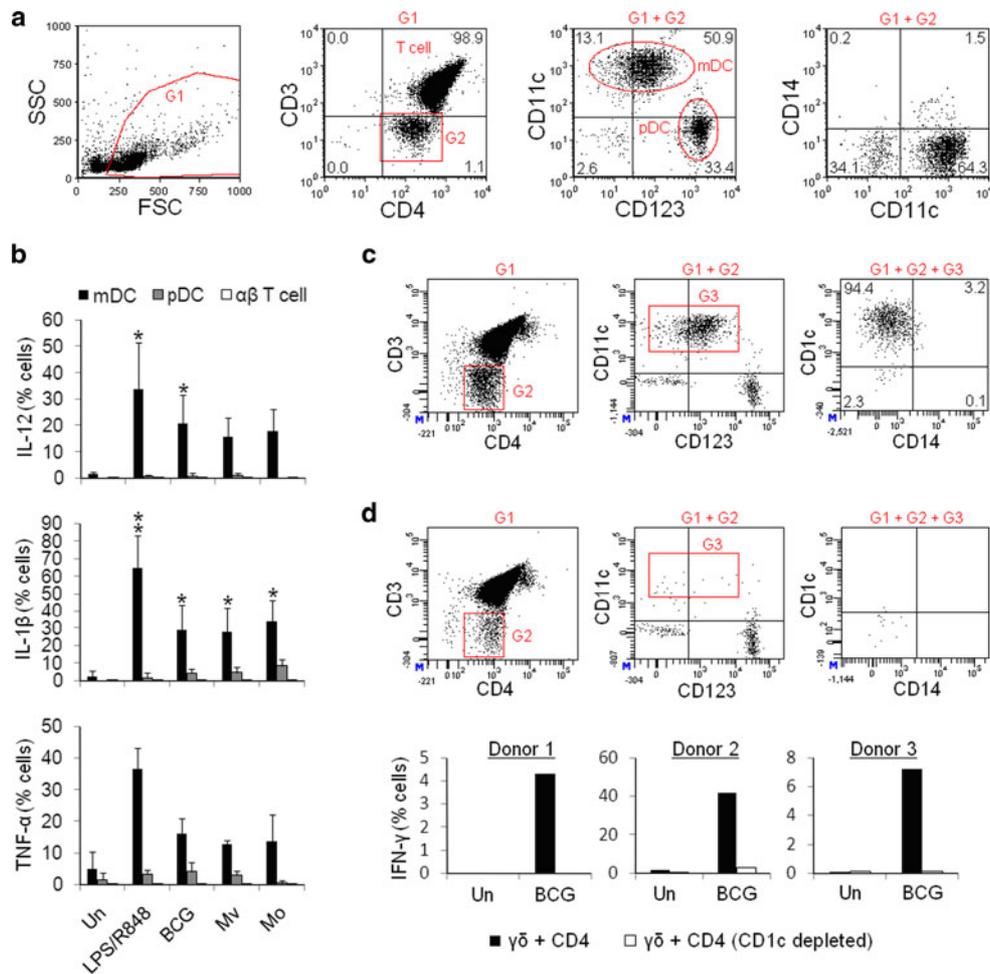
**Fig. 5** IL-12, IL-1 $\beta$  and TNF- $\alpha$  induce  $\gamma\delta$  T-cell IFN- $\gamma$  production and cytotoxicity. **a**  $\gamma\delta$  T-cells were cultured overnight with different concentrations and combinations of IL-12, IL-1 $\beta$  and TNF- $\alpha$  prior to measuring IFN- $\gamma$  expression on gated V $\delta$ 2<sup>+</sup> and V $\delta$ 2<sup>-</sup> cells. Individual experiments from three donors are shown. **b** MFIs for granzyme B expression on gated  $\gamma\delta$  T-cells after overnight culture with 10 ng/ml each of IL-12, IL-1 $\beta$  and TNF- $\alpha$ . *Left*: Representative flow cytometric histogram plots from one donor. *Black fill* is the isotype control antibody. Numbers are MFIs within the marker shown. *Right*: MFI values

for  $n = 3$ . **c** Cytokine-activated  $\gamma\delta$  T-cells were co-cultured overnight with Daudi cells at the target (T) to effector (E) cell ratios shown. Cytotoxicity was assessed by measuring lactate dehydrogenase release into the culture supernatants. Mean values for  $n = 3$  (T:E 1:50) and  $n = 2$  (T:E 1:10 and 1:20) are shown. Untreated (un) cells were used as a negative control throughout. *Error bars* represent SD deviations and \* indicates a  $P$  value of  $<0.05$  for paired  $t$  tests between treatments at the T:E ratio of 1:50

tumour cells, suggesting that  $\gamma\delta$  T-cells are cross-reactive [13]. However, the ability of heat-killed mycobacteria to enhance  $\gamma\delta$  T-cell cytotoxicity has not yet been tested. For the purposes of this study, we used the Burkitt's lymphoma cell line Daudi as a model tumour target as this cell line is routinely used for investigating  $\gamma\delta$  T-cell responses against tumour. We found that mycobacteria-stimulated PBMCs contained  $\gamma\delta$  T-cells with enhanced cytotoxicity against Daudi cells, as shown by increased degranulation. This effect could be explained by upregulation of cytolytic effector molecules; indeed, we found that mycobacteria upregulated  $\gamma\delta$  T-cell expression of granzyme B, which plays a critical role in  $\gamma\delta$  T-cell cytotoxicity [36]. Taken together, our data suggest that heat-killed BCG, *M. vaccae* and *M. obuense* enhance the cytotoxic activity of  $\gamma\delta$  T-cells in PBMCs, which may contribute to the anti-tumour properties of these cancer immunotherapies.

Tumour cell lines that are refractory to  $\gamma\delta$  T-cell killing have been reported; indeed, we found that the A549 tumour cell line failed to induce  $\gamma\delta$  T-cell degranulation. However, pre-treating A549s with the aminobisphosphonate (ABP) zoledronic acid increased their capacity to induce  $\gamma\delta$  T-cell degranulation, which confirms previous studies showing that ABPs upregulate phosphoantigen expression in

tumours, thus enhancing recognition by V $\delta$ 2<sup>+</sup>  $\gamma\delta$  T-cells [37]. We show that PBMCs exposed to heat-killed mycobacteria contain  $\gamma\delta$  T-cells with enhanced cytotoxic activity against zoledronic acid-treated A549s. This suggests that systemic priming of  $\gamma\delta$  T-cells with mycobacteria could be combined with local treatment of tumours with ABPs, which would serve to simultaneously increase the visibility of the tumour whilst augmenting the cytotoxic responses of circulating effector cells. The anti-cancer effects of ABPs are currently under investigation in lymphoma, myeloma, prostate cancer and breast cancer [38–40]; however, ABPs are currently administered via intravenous infusions, which poses a number of problems. Firstly, ABPs are rapidly absorbed by the mineral surfaces of bone, thus necessitating high-dose regimens that are often associated with a range of complications including pyrexia, nephrotoxicity and electrolyte abnormalities [41]. Intratumoural administration of ABPs combined with systemic mycobacterial priming may be a more effective treatment regimen that reduces these complications. Secondly, ABPs upregulate phosphoantigens in peripheral blood monocytes, which may render them susceptible to  $\gamma\delta$  T-cell attack and exhaust  $\gamma\delta$  T-cell cytotoxic function before they reach the tumour [42]. Similar to ABPs, the chemotherapies etoposide, cisplatin and



**Fig. 6** IL-12, IL-1 $\beta$  and TNF- $\alpha$  are produced by type 1 myeloid DCs. **a** Flow cytometry was used to measure the expression of CD4, CD3, CD11c, CD123 and CD14 on purified CD4<sup>+</sup> cells. From left to right: Debris was excluded according to size and granularity using gate (G) 1. Within the G1 population, CD3 and CD4 expression identified CD4<sup>+</sup>  $\alpha\beta$  T-cells (CD3<sup>+</sup>CD4<sup>high</sup>) and non-T-cells (CD3<sup>-</sup>CD4<sup>low</sup>; G2). Within the G1+G2 non-T-cell population, CD11c and CD123 expression identified myeloid DC (mDC) and plasmacytoid DC (pDC) populations, both of which did not express CD14. Representative flow cytometric dot plots from one of two donors are shown. **b** CD4<sup>+</sup> cells were cultured overnight with LPS/R848, heat-killed BCG, *M. vaccae* (Mv) and *M. obuense* (Mo). Untreated (un) cells were used as a negative

control. IL-12, IL-1 $\beta$  and TNF- $\alpha$  expression was measured on gated T-cells (CD3<sup>+</sup>), mDCs (CD3<sup>-</sup>CD11c<sup>+</sup>) and pDCs (CD3<sup>-</sup>CD11c<sup>-</sup>CD123<sup>high</sup>). Mean values for  $n = 3$  are shown (except TNF- $\alpha$ , where  $n = 2$ ). Error bars represent SD. \* and \*\* indicate  $P$  values of <0.05 and <0.001, respectively, for statistical comparisons between treated and untreated conditions. **c** CD1c expression was analysed on gated mDCs as shown. **d** Top panels: CD1c<sup>+</sup> cells were depleted from the CD4<sup>+</sup> cell population. Representative data from one of three donors are shown. Bottom panels:  $\gamma\delta$  T-cells were co-cultured overnight with CD4<sup>+</sup> cells or CD1c-depleted CD4<sup>+</sup> cells in the absence or presence of BCG. Percentage of  $\gamma\delta$  T-cells expressing IFN- $\gamma$  was measured

doxorubicin have also been shown to increase the susceptibility of tumour cell lines to  $\gamma\delta$  T-cell killing [37]. This suggests there may also be potential in combining certain chemotherapies with BCG, *M. vaccae* and *M. obuense* immunotherapy; indeed, a survival benefit was reported in lung cancer patients receiving *M. vaccae* in combination with platinum-based chemotherapy [6, 7].

In comparing the three different bacterial preparations, only minor differences were observed between them in terms of their activity towards  $\gamma\delta$  T-cells. Although this suggests that these different preparations each have the potential to elicit comparable anti-tumour responses in  $\gamma\delta$

T-cells, the potential for differential effects on other immune cells remains to be seen. Moreover, the receptors through which they stimulate mDC1s may differ, which may have bearing on potential combinatorial partners. Further investigations comparing the induced immune responses of these three bacterial preparations are therefore required and are currently underway.

Although  $\gamma\delta$  T-cell responses to BCG have been previously documented, they have been dependent on viable infection of APCs. For example, Martino et al. [23] reported that DCs pre-treated with live, but not heat-killed, BCG activate  $\gamma\delta$  T-cells. We found that heat-killed

mycobacteria can elicit marked  $\gamma\delta$  T-cell responses, suggesting there are alternate mechanisms of activation that have yet to be reported. Such mechanisms may be more relevant to cancer immunotherapy since the bulk of reconstituted lyophilised BCG vaccines consist of non-viable bacilli. Furthermore, BCG is slow growing, thus the effects described during live BCG infections are likely to be outweighed by those elicited by non-viable BCG. Current hypotheses suggest that bacterially infected APCs upregulate expression of  $\gamma\delta$  T-cell-specific phosphoantigens. In support of this, soluble phosphoantigen-specific  $\gamma\delta$  TCRs have been shown to selectively bind to BCG-treated but not untreated DCs [43]. Whether these are mycobacteria-derived or endogenous phosphoantigens remains unclear. As shown by Kistowska et al. [44], mycobacterial infections disrupt isoprenoid biosynthesis in APCs, thus causing accumulation of endogenous phosphoantigens. Although mycobacteria-derived and/or endogenous phosphoantigens presented on infected DCs may activate  $\gamma\delta$  T-cells, their role in  $\gamma\delta$  T-cell activation by heat-killed preparations of mycobacteria is unclear.

We sought to determine the mechanisms by which heat-killed preparations of mycobacteria induce anti-tumour immune responses in  $\gamma\delta$  T-cells. Our data suggest that  $V\delta 2^+$   $\gamma\delta$  T-cells are indirectly activated by these mycobacterial preparations via IL-12, IL-1 $\beta$  and TNF- $\alpha$  produced by mDC1s. Parenthetically, it is interesting to note that depleting monocytes from our system resulted in an increase in  $\gamma\delta$  T-cell activation, which suggests that under certain conditions the bacterial preparations could trigger monocytes to release cytokines that counteract the effects of IL-12, IL-1 $\beta$  and TNF- $\alpha$ . The newly proposed mechanism of activation is contrary to previous reports showing that DCs infected with heat-killed BCG fail to stimulate  $\gamma\delta$  T-cells [23]. However, in this study cytokine-dependent activation of  $V\delta 2^+$   $\gamma\delta$  T-cells by mDC1s was observed in co-cultures consisting of  $\gamma\delta$  T-cells and CD4 $^+$  cells. Therefore, cytokine production by mDC1s in response to mycobacteria may be dependent on other cells within the CD4 $^+$  population. Accordingly, we found that  $\gamma\delta$  T-cells failed to respond to the heat-killed mycobacteria when co-cultured with purified monocytes or mDC1s (data not shown). This is in keeping with previous reports that pathogen-induced IL-12 production by DCs is dependent on costimulatory signals such as CD40 ligation, which may be supplied by the T-cell component of the CD4 $^+$  cell population [45]. The mechanisms underlying mDC1 cytokine production did not fall within the scope of this study and investigations are currently underway.

The observations that heat-killed mycobacteria fail to activate  $\gamma\delta$  T-cells co-cultured with purified DCs (i.e. in the absence of other CD4 $^+$  cells) suggests that phosphoan-

tigen recognition is not involved in the  $\gamma\delta$  T-cell responses observed here (Figs. 1, 2). It is possible that heat-killing stunts the efficacy of phosphoantigens or perturbs their uptake and processing by mDC1s. Seeing as cancer immunotherapies use either heat-killed or low-viability preparations of mycobacteria, the newly identified mechanism of indirect cytokine priming compared with direct phosphoantigen recognition is more clinically relevant. Furthermore, in terms of generating an anti-tumour  $\gamma\delta$  T-cell response, the indirect cytokine-mediated priming described here may elicit more favourable responses compared with direct phosphoantigen-mediated activation. Phosphoantigens are target molecules for  $\gamma\delta$  T-cell cytotoxicity. Therefore, using these target molecules to prime  $\gamma\delta$  T-cells for immunotherapeutic purposes will cause degranulation, which may exhaust  $\gamma\delta$  T-cell cytotoxic responses before they encounter tumour. This has bearing on the use of heat-killed instead of live mycobacteria for cancer immunotherapy; indeed, reports have shown that  $\gamma\delta$  T-cells kill APCs harbouring viable bacterial infections [46]. This also has bearing on immunotherapies that activate  $\gamma\delta$  T-cells via phosphoantigens, either in situ (i.e. via systemic application of ABPs) or ex vivo (i.e. via adoptive transfer of phosphoantigen-expanded  $\gamma\delta$  T-cells). In contrast, heat-killed mycobacteria, which indirectly prime  $\gamma\delta$  T-cells via cytokines from mDC1s, may not exhaust  $\gamma\delta$  T-cell responses before they encounter tumour. This effect could be exploited to improve immunotherapies so as to avoid exhaustion of  $\gamma\delta$  T-cells prior to tumour infiltration; for example, by combining systemic administration of mycobacteria with intratumoural administration of ABPs.

In summary, we demonstrate the potential for heat-killed preparations of BCG, *M. vaccae* and *M. obuense* to prime an anti-tumour effect in  $\gamma\delta$  T-cells. Priming is mediated by cytokines from mDC1s and results in production of T<sub>H</sub>1 cytokines and increased cytotoxicity towards tumour cells. Our data provide a potential explanation for the anti-tumour effects of these mycobacterial preparations in vivo. Furthermore, our data suggest that these immunotherapies can be further developed using combination therapy, for example, combining systemic BCG, *M. vaccae* and *M. obuense* with localised ABPs or chemotherapies that augment  $\gamma\delta$  T-cell susceptibility of target cells. More studies are required to elucidate the full range of effects elicited by these mycobacterial preparations, which are currently underway.

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**Conflict of interest** The authors declare no financial or commercial conflict of interest.

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