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Inhibition of phosphoantigen-mediated γδ T-cell proliferation by $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells

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Summary

Tumour growth promotes the expansion of $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T cells (Tregs) which suppress various arms of immune responses and might therefore contribute to tumour immunosurveillance. In this study, we found an inverse correlation between circulating Treg frequencies and phosphoantigen-induced γδ T-cell proliferation in cancer patients, which prompted us to address the role of Tregs in controlling the γδ T-cell arm of innate immune responses. *In vitro*, human Treg–peripheral blood mononuclear cell (PBMC) co-cultures strongly inhibited phosphoantigen-induced proliferation of γδ T cells and depletion of Tregs restored the impaired phosphoantigen-induced γδ T-cell proliferation of cancer patients. Tregs did not suppress other effector functions of γδ T cells such as cytokine production or cytotoxicity. Our experiments indicate that Tregs do not mediate their suppressive activity via a cell–cell contact-dependent mechanism, but rather secrete a soluble non-proteinaceous factor, which is independent of known soluble factors interacting with amino acid depletion (e.g. arginase-diminished arginine and indolamine 2,3-dioxygenase-diminished tryptophan) or nitric oxide (NO) production. However, the proliferative activity of αβ T cells was not affected by this cell–cell contact-independent suppressive activity induced by Tregs. In conclusion, these findings indicate a potential new mechanism by which Tregs can specifically suppress γδ T cells and highlight the strategy of combining Treg inhibition with subsequent γδ T-cell activation to enhance γδ T cell-mediated immunotherapy.

Keywords: cancer; γδ T cells; innate immunity; regulatory T cells (Tregs)

Introduction

Regulatory T cells (Tregs) play an important role in the maintenance of immune tolerance and have been shown to suppress immune responses to infectious agents, transplants and tumours in both the priming and the effector phases.[1–3] The ability of Tregs to regulate immunity has prompted attempts to stimulate Tregs to treat autoimmunity and induce transplant tolerance on the one hand or to dampen Tregs to increase immunity to pathogens and tumours on the other hand. Current evidence suggests the existence of several phenotypically distinct Treg populations [naturally occurring $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs, induced Tregs such as type 1 T regulatory (Tr1) cells and T helper type 3 (Th3) cells, $CD4^-$ $CD8^-$ DN αβ T cells, $CD8^+$ $CD28^-$ Tregs, mesenchymal stem cell-induced Tregs and others].[4,5] Among the different types of Treg, the classic Tregs are thymus-derived $CD4^+$ $CD25^+$ $FoxP3^+$ T lymphocytes (termed Tregs throughout this study), representing 5–10% of total peripheral blood $CD4^+$ T cells in humans, and are usually phenotypically characterized by the constitutive expression of CD25, transcription factor FoxP3, cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and glucocorticoid-induced tumour necrosis factor receptor (TNFR)-related protein (GITR) and, as recently revealed, by low expression of CD127 [interleukin-7 receptor α (IL-7Rα)].[6] There is now considerable evidence that FoxP3 is a key control molecule for Treg development and function, and is an excellent marker for the study of Tregs.[7] Tregs inhibit proliferation and other effector functions of various immune cells of both the adaptive ($CD4^+$ and $CD8^+$ αβ T cells and B cells) and the innate [natural killer (NK) cells, NKT cells, monocytes/macrophages and dendritic cells (DCs)] immune system.[8–14] Multiple suppressive mechanisms of Tregs, including direct (cell–cell contact dependent) and indirect

(soluble factors) mechanisms, have been proposed; the relative importance of these mechanisms may depend on the experimental *in vitro* or *in vivo* model. Possible inhibitory effects of Tregs include (1) cell–cell contact-dependent effects produced by membrane-bound transforming growth factor (TGF)-β or intracellular transport of cAMP via gap junctions, (2) secretion or induction of immunosuppressive cytokines such as TGF-β and IL-10, (3) competitive consumption of IL-2, (4) down-regulation of immunostimulatory B7 family members (CD80 and CD86) or induction of immunosuppressive B7 family members (e.g. B7-H4) on antigen-presenting cells (APCs), (5) CTLA-4- or prostaglandin E_2 (PGE_2)-mediated induction of indoleamine 2,3-dioxygenase (IDO)-expressing APCs, (6) depletion of arginine by induction of arginase in myeloid cells, and (7) induction of nitric oxide (NO) by activation of inducible NO synthase (iNOS).[15–19] However, none of the proposed mechanisms can explain all aspects of Treg-mediated suppression and it is possible that various combinations of several mechanisms or undiscovered modes of action are operating.

Among innate effector lymphocytes, γδ T cells represent a unique subset of unconventional lymphocytes, because they display features of both conventional αβ T cells and NK cells.[20,21] With increasing knowledge regarding their antigen specificity and function, human γδ T cells have attracted more and more interest for clinical immunotherapy approaches. Vγ9Vδ2 T cells, which represent the vast majority of human circulating γδ T cells (50–70% of γδ T cells and 0·5–7% of $CD3^+$ T cells, respectively), recognize phosphorylated, non-peptide compounds (so-called phosphoantigens), which naturally occur in the microbial non-mevalonate pathway of isoprenoid biosynthesis (the Rohmer pathway).[22,23] The capacity of Vγ9Vδ2 T cells to be activated by these naturally occurring phosphoantigens facilitates a specific immune response to numerous microbial pathogens through targeting of a distinctive and essential metabolic route in these organisms. In addition to these natural phosphoantigens, several synthetic compounds have been identified which selectively stimulate Vγ9Vδ2 T cells either by mimicking the highly potent natural phosphoantigens [e.g. bromohydrinpyrophosphate (BrHPP)] or by inducing accumulation of cross-reactive endogenous mevalonate metabolites [e.g. isopentenylpyrophosphate (IPP)] via inhibition of the mevalonate pathway (aminobisphosphonates).[24–26] γδ T cells not only recognize microbial antigens, but are also capable of exerting significant major histocompatibility complex (MHC)-unrestricted anti-tumour activity against a broad spectrum of tumour cells *in vitro*.[21,27] Previous studies by our group and recent data from Dieli *et al.* have provided additional evidence that selective stimulation of γδ T cells by phosphoantigens can induce anti-tumour activity *in vivo*.[28,29] However, γδ T cell-mediated immunotherapy is limited by the fact that phosphoantigen-induced proliferation of γδ T cells has been shown to be frequently impaired in patients with haematological malignancies.[28] This kind of γδ T-cell anergy has also been described in certain chronic infectious diseases such as HIV-infection or tuberculosis, although the cause of this γδ T-cell anergy has not yet been discovered.[30,31]

To date, the role of Tregs in hampering γδ T-cell function has not been investigated. In this study we provide the first direct evidence that Tregs potently suppress phosphoantigen-induced γδ T-cell proliferation via a novel cell–cell contact-independent mechanism. In the light of this finding, we speculate that the increased Treg:γδ T-cell ratio in cancer patients may contribute to the state of apparent immunological unresponsiveness to phosphoantigens found in many types of cancer. Thus, our data are compatible with the hypothesis that eliminating Tregs in cancer patients might constitute a rational approach to stimulating not only the adaptive immune response to tumour-associated antigens, but also the γδ T-cell arm of the innate immune system.

Patients, materials and methods

Patients

Blood samples were drawn from 42 healthy donors and 336 patients with different types of cancer [multiple myeloma (MM), $n = 198$; B-cell non-Hodgkin lymphoma (B-NHL), $n = 65$; metastatic renal cell cancer (mRCC), $n = 22$; metastatic malignant melanoma (mMEL), $n = 40$; metastatic colorectal cancer (mCRC), $n = 7$; metastatic breast cancer (mBC), $n = 4$] and 19 patients with monoclonal gammopathy of unknown significance (MGUS) having obtained their written informed consent. Ethical approval for this study was obtained from the Ethics Committee of the University of Würzburg. All patients included in this study had not received any cancer-specific or immunosuppressive (i.e. steroid) treatment for at least 3 months and had a performance status Eastern Cooperative Oncology Group (ECOG) 0-1. Peripheral blood mononuclear cells (PBMC) were analysed by flow cytometry and the number of $Vδ2^+$ T cells and Tregs ($CD4^+$ $CD25^+$ $FoxP3^+$ Tregs) was assessed as described below.

Reagents

The following compounds, monoclonal antibodies (mAbs), assays and kits were used in this study: BrHPP (kindly provided by Innate Pharma, Marseilles, France), recombinant human IL-2 (Proleukin; Novartis, Basel, Switzerland), the anti-human FoxP3 Staining Kit (eBiosciences, San Diego, CA), interferon (IFN)-γ secretion assay, $CD4^+$ $CD25^+$ Treg isolation kit and anti-PE microbeads (all from Miltenyi Biotec, Bergisch Gladbach,

Germany), recombinant human (rh) cytomegalovirus (CMV) pp65 protein (Miltenyi Biotec) 1-methyl-L-tryptophan, phytohaemagglutinin (PHA), the PKH26 red fluorescent cell linker mini kit (Sigma-Aldrich, Taufkirchen, Germany), anti-TGF-β mAb, recombinant human TGF-β soluble receptor II (sRII), anti-IL-10 mAb, human latency-associated petide (LAP; R&D Systems, Wiesbaden, Germany), proteinase K (Merck, Darmstadt, Germany), ToPro-3 iodide, the Vybrant®CFDA SE cell tracer kit (Molecular Probes, Eugene, OR) granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-4, IL-15, IL-1β, IL-6, TNF-α (Tebu-Bio, Offenbach, Germany), prostaglandin E2 (Biomol, Hamburg, Germany), iTAg Tetramer/PE-HLA-A*0201 CMV pp65 (NLVPMVATV; Beckmann Coulter Immunomics Operations, Villepinte Roissy, France), N^G-monomethyl-L-arginine (L-NMMA) and N^{ω}-hydroxy-nor-L-arginine (nor-NOHA; Axxora, Grünberg, Germany).

Flow cytometric analysis

Cells were harvested after the indicated* culture periods and analysed by two- or three-colour flow cytometry (FACScan flow cytometer; Becton Dickinson, Heidelberg, Germany) using the CELLQUEST program (Becton Dickinson). Cells were stained with the appropriate concentrations of following mABs: fluorescein isothiocyanate (FITC)-conjugated anti-pan γδ T-cell receptor (TCR), anti-Vδ2 TCR, anti-Vγ9 TCR, anti-CD8, anti-CD4, phycoerythrin (PE)-conjugated anti-CD14, anti-CD25, anti-CD3, anti-pan γδ TCR, anti-CD69 (Beckman Coulter, Krefeld, Germany), anti-CD25, anti-αβ TCR (Miltenyi Biotec), anti-FoxP3 (eBiosciences) and peridinin chlorophyll protein (PerCP)-conjugated anti-CD3 (Becton Dickinson). Lymphocytes were gated using forward/sideward scatter analysis.

Isolation of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs

$CD4^+$ $CD25^+$ Tregs were purified from fresh PBMC either by magnetic cell separation (Miltenyi Biotec) according to the manufacturer's instructions or by using a cell sorter cytometer (FACSVantage; Becton Dickinson) and cultivated in RPMI-1640 medium supplemented with 10% pooled human AB serum (PAN Biotech, Aidenbach, Germany), 2 mmol/l L-glutamine and 100 U/ml IL-2 before further use. The purity of Tregs was assessed by flow cytometry using mAb against CD4 and CD25 (Becton Dickinson). In addition, FoxP3 expression in isolated $CD4^+$ $CD25^+$ cells was determined using the anti-human FoxP3 Staining Kit (eBiosciences).

*Wherever indicated is used in the 'patients, material, and methods' section, the exact ratios or culture conditions are described in detail in figure legends or results section.

γδ T-cell proliferation assay

The γδ T-cell proliferation assay was described previously.[25] Briefly, 1×10^5 PBMC from healthy donors were cultivated in triplicate in 100 μl of RPMI-1640 medium per well (Biochrom, Berlin, Germany), 10% pooled human AB serum, and 100 U/ml IL-2 in 96-well round-bottom microtitre plates (Nunc, Wiesbaden, Germany). For determination of phosphoantigen-induced γδ T-cell proliferation, BrHPP (Innate Pharma) was added in escalating concentrations on day 0. Cells were harvested on day 7 and double or triple stained with FITC, PE or PerCP Cy5·5-conjugated monoclonal CD3, TCR pan γδ, TCR Vγ9 or TCR Vδ2 (Beckman Coulter) antibodies. Using a FACScan supported with CELLQUEST acquisition and data analysis software (Becton Dickinson), 1×10^4 cells from each sample were analysed. The lymphocytes were gated using forward/sideward scatter analysis. The amplification rate (A) was calculated as $A = (\%\ \gamma\delta\ \text{day}\ 7 \times \text{total cell number day}\ 7)/(\%\ \gamma\delta\ \text{day}\ 0 \times \text{total cell number day}\ 0)$. A more than 50-fold amplification rate was considered as proliferation positive. To deplete $CD25^+$ cells, PBMC were labelled with anti-CD25 PE mAb (Beckman Coulter) and further incubated with anti-PE microbeads (Miltenyi Biotec). The increase in γδ T cell numbers was calculated by analysing the relative and absolute numbers of viable cells per well by cytofluorometric identification of γδ T cells using fluorescence-activated cell sorter (FACS) analysis on day 7 of culture. In some experiments, γδ T cell-enriched fractions of PBMC were used after depletion of αβ T cells, B cells, NK cells or monocytes using PE-conjugated anti-αβ TCR, anti-CD19, anti-CD56 or anti-CD14 mAb and anti-PE microbeads (Miltenyi Biotec).

To determine the suppressive effects of Tregs on phosphoantigen-induced γδ T-cell proliferation, 2×10^4 PBMC treated with a single dose of BrHPP (1 μM) were co-cultured with purified $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs or supernatants from overnight IL-2 activated (100 U/ml) Tregs at indicated ratios. Where indicated, Tregs were additionally activated with PHA (2·5 μg/ml). To further investigate the suppressive mechanisms, Tregs or supernatants from Tregs were treated with anti-IL-10 (25 ng/ml), TGF-β sRII (150 ng/ml), LAP (800 ng/ml), L-NMMA (0·5 mM, 2 mM), nor-NOHA (0·1 mM, 0·5 mM) or 1-methyl-L-tryptophan (1 mM, 2 mM, 5 mM) or proteins in the supernatants were inactivated by heat (15 min at 56° or 99°) or by proteinase K digestion (2 hr at 37°; inactivation of enzyme for 15 min at 99°). Transwell experiments were performed in Nunc Tissue Culture Inserts (0·2 μm pore size; Nunc). PBMC (1×10^5) were cultured in the upper chamber, and purified $CD4^+$ $CD25^+$ Tregs at indicated ratios were placed in the bottom chamber. Each γδ T-cell proliferation assay was carried out in RPMI-1640 medium + 10% AB serum + IL-2 (100 U/ml).

CMV pp65-specific αβ T-cell proliferation assay

Immature dendritic cells (iDCs) were derived with GM-CSF (800 U/ml) and rhIL-4 (800 U/ml) after CD14-positive magnetic antibody cell sorting (MACS; purity > 98%) from fresh PBMC using anti-CD14 PE (Beckmann Coulter) and anti-PE microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Further iDCs were loaded with CMV pp65 recombinant protein (20 μl per 1×10^7 cells) (Miltenyi Biotec) and incubated in RPMI-1640 medium + 10% AB serum and fresh cytokines (800 U/ml IL-4 and 800 U/ml GM-CSF) overnight, and then a maturing cocktail was added to the DCs (2 ng/ml IL-1β, 1 μg/ml PE2, 10 ng/ml TNF-α and 5 ng/ml IL-6). On the next day, mature DCs were washed twice with 1 × Hanks' balanced salt solution (HBSS), re-suspended in RPMI-1640 medium supplemented with 10% AB serum and 100 U/ml IL-2, and co-cultivated either with autologous PBMC (ratio mDCs:PBMC = 1 : 10) alone or together with autologous purified Tregs or supernatants from overnight-cultivated Tregs at indicated ratios. Proliferation of CMV pp65-specific $CD8^+$ αβ T cells was determined on day 7 by flow cytometry using FITC-conjugated anti-CD8 (Beckman Coulter) and iTAg Tetramer/PE-HLA-A*0201 CMV pp65 (NLVPMVATV; Beckmann Coulter Immunomics Operations).

Cytokine and cytotoxicity assay

Freshly purified Tregs were activated for 16 hr with 100 U/ml IL-2. Pre-activated Tregs or supernatant from pre-activated Tregs were co-cultured with 2×10^4 autologous PBMC at indicated ratios for 24 hr and stimulated with BrHPP (1 μM) or medium alone as control for a further 16 hr. They were then analysed either for expression of activation marker CD69 by flow cytometry (FACScan) or for IFN-γ production using the IFN-γ Secretion Assay Detection Kit (Miltenyi Biotec) according to the manufacturer's instructions.

A standard cytotoxicity assay was performed.[32] In brief, the target cell line (Daudi) was labelled with 2 μM PKH26 according to the manufacturer's manual (Sigma, St Louis, MO) and 5×10^3 cells/well were incubated in 96-well V-shaped microtitre plates in triplicates with a phosphoantigen-reactive Vγ9Vδ2 T-cell line at the indicated effector:target (E:T) ratios. After 4 hr, cells were harvested, brought up to 400 μl with 1 × phosphate-buffered saline (PBS), stained with the nucleic acid-specific ToPro-3 iodide stain (0·5 μM; 15 min on ice) and further analysed by flow cytometry using FL-2 and FL-4, respectively (FACS Calibur; Becton Dickinson). Percentages of cells killed by cytotoxic activity were calculated as (upper right)/(upper and lower right) × 100 followed by subtraction of the percentage of spontaneous death in the target cells-only control tube. In some experiments, purified $CD4^+$ $CD25^+$ Tregs or the $CD4^+$ $CD25^-$ fraction of PBMC was added at the indicated ratios.

Cell lines

γδ T-cell lines were established by culturing 1×10^5 freshly isolated PBMC per well in 96-well microtitre plates in standard medium (RPMI-1640 medium supplemented with 10% pooled AB human serum, 2 mmol/l L-glutamine, and 100 U/ml rhIL2) with a single dose of BrHPP (1 μM). Cells were periodically re-stimulated with IL-2 (100 U/ml) every 3–4 days and after 2 weeks more than 90% of the cells expressed the Vγ9Vδ2 TCR, as determined by flow cytometry. For proliferation assays, γδ T-cell lines were αβ T cells depleted using anti-αβ TCR PE and anti-PE microbeads (Miltenyi Biotec) on days 7–8 and kept without IL-2 overnight. Further γδ T cells were labelled with 1 μM carboxyfluorescein succinimidyl ester (CFSE; 1×10^6 cells/ml pre-warmed PBS; 15 min at 37°), washed once with PBS and incubated with medium alone or BrHPP and co-cultured with autologous purified Tregs. On day 6, proliferation was assessed by three-colour staining additionally using anti-pan γδ TCR-PE and anti-CD3-PerCP.

The Daudi cell line was obtained from DSMZ (Braunschweig, Germany) and was grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 2 mmol/l L-glutamine.

Mixed lymphocyte reaction (MLR)

To determine the suppressive activity of Tregs on αβ T cells, a mixed lymphocyte reaction (MLR) was performed. PBMC (1×10^5 cells/well) from healthy donors were labelled for 15 min with 1 μM CFSE as described above. Further PBMC were co-cultured with allogenic iDCs (1×10^4 cells/well) alone or together with supernatant from overnight-cultivated autologous Tregs (ratio PBMC:Tregs = 1 : 1). Proliferation of αβ T cells was determined by assessing CFSE dilution on day 6 by three-colour staining additionally using anti-αβ TCR-PE and anti-CD3-PerCP.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). The two-sided Student *t*-test for independent samples was used to determine statistical significance of detected differences. $P < 0{\cdot}05$ was considered significant.

Results

Impaired phosphoantigen-induced γδ T-cell proliferation in cancer patients

We have recently shown that phosphoantigen-induced proliferation of γδ T cells *in vitro* is frequently impaired

in patients with lymphoid malignancies.[28] To confirm and extend this initial observation, we investigated the proliferative response of peripheral blood γδ T cells to phosphoantigen (BrHPP) and IL-2 (100 U/ml) from 336 consecutive patients with different types of cancer (MM, $n = 198$; B-NHL, $n = 65$; mRCC, $n = 22$; mMel, $n = 40$; mBC, $n = 4$; mCRC, $n = 7$). In an age-matched group of healthy donors ($n = 42$), 95% showed significant *in vitro* proliferation of γδ T cells in response to phosphoantigen/IL-2, but only 42% of cancer patients were responsive. Figure 1 shows representative cancer patients with maintained and impaired phosphoantigen-induced γδ T-cell proliferation, respectively (49% versus 1·1% γδ T cells after 1 week of culture). Cancer-associated impaired proliferative function of γδ T cells was observed in lymphoid malignancies (71% of MM and 42% of B-NHL) as well as in various advanced solid cancers (55% of mRCC, 27% of mMel, 50% of mBC and 29% of mCRC) (Table 1). Interestingly, individuals with MGUS ($n = 19$), a premalignant precursor of MM, also showed an increased frequency of impaired phosphoantigen-induced γδ T-cell proliferation compared with healthy individuals (47% versus 5%; data not shown). Impaired proliferative function of γδ T cells might therefore be a general phenomenon in patients with cancer and certain premalignant conditions.

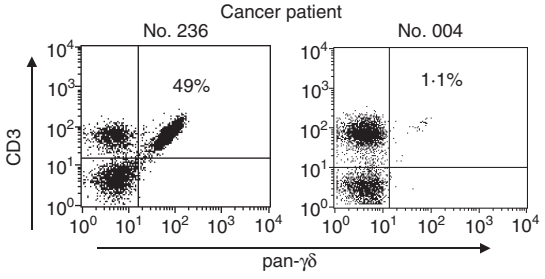

Figure 1. Proliferative response of γδ T cells to phosphoantigen/interleukin (IL)-2. Peripheral blood mononuclear cells (PBMC) from cancer patients were incubated with phosphoantigen (1 μM bromohydrinpyrophosphate) and IL-2 (100 U/ml), harvested on day 7, stained with anti-pan γδ T-cell receptor (TCR) fluorescein isothiocyanate (FITC) and anti-CD3 phycoerythrin (PE) and analysed by flow cytometry. Fluorescence-activated cell sorter (FACS) analysis of γδ T cells from one representative cancer patient (no. 236) with maintained and one representative cancer patient (no. 004) with impaired phosphoantigen-induced proliferation showed 49% versus 1·1% γδ T cells in $CD3^+$ T cells, respectively.

Table 1. *In vitro* proliferation of γδ T cells in response to phosphoantigen/interleukin (IL)-2

	+	−
Healthy donors	40/42 (95%)	2/42 (5%)
MM	57/198 (29%)	141/198 (71%)
B-NHL	38/65 (58%)	27/65 (42%)
mRCC	10/22 (45%)	12/22 (55%)
mMel	29/40 (73%)	11/40 (27%)
mBC	2/4 (50%)	2/4 (50%)
mCRC	5/7 (71%)	2/7 (29%)
All patients	141/336 (42%)	195/336 (58%)

MM, multiple myeloma; B-NHL, B-cell non-Hodgkin lymphoma; mRCC, metastatic renal cell cancer; mMel, metastatic malignant melanoma; mBC, metastatic breast cancer; mCRC, metastatic colorectal cancer. The amplification rate (A) was calculated as A = (% γδ day 7 × total cell number day 7)/(% γδ day 0 × total cell number day 0). A > 50-fold amplification rate was considered as positive (+) for phosphoantigen-induced γδ T-cell proliferation.

Phosphoantigen-induced γδ T-cell proliferation inversely correlates with Treg frequencies in cancer patients

To investigate the potential role of Tregs in impaired γδ T-cell function in cancer patients, we first determined the frequencies of circulating $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs by flow cytometry in cancer patients ($n = 113$) and healthy controls ($n = 9$). Consistent with a series of recent reports, cancer patients showed significantly increased frequencies of Tregs compared with healthy controls (Fig. 2a,b). However, there was no statistically significant difference in Treg frequencies between cancer patients with impaired and maintained phosphoantigen-induced γδ T-cell proliferation (Fig. 2b). As using the ratio of Tregs to a given effector lymphocyte subset is a more comprehensive approach to determining the functional significance of Treg-mediated suppression, we compared the ratio of circulating Tregs to the phosphoantigen-sensitive $Vδ2^+$ subset of γδ T cells in the peripheral blood of both cohorts of cancer patients (impaired versus preserved phosphoantigen-induced γδ T-cell proliferation). In this analysis, the Treg:$Vδ2^+$ ratio was significantly higher in cancer patients with impaired phosphoantigen-induced proliferative response (mean Treg:$Vδ2^+$ ratio in cancer patients without γδ T-cell proliferation: 47·0; mean Treg:$Vδ2^+$ ratio in cancer patients with preserved γδ T-cell proliferation: 8·3; $P = 0{\cdot}000246$), suggesting that an inverse correlation exists between the Treg:$Vδ2^+$ ratio and phosphoantigen-induced γδ T-cell proliferation in cancer patients (Fig. 2c). Importantly, there was no statistically significant difference in relative or absolute numbers of $Vδ2^+$ T cells between cohorts, indicating that the impaired γδ T-cell proliferation in cancer patients is not attributable to a reduced frequency of this T-cell subset (data not shown). Hence, $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs might contribute to the impaired Vγ9Vδ2 T-cell proliferation in cancer patients and the Treg:$Vδ2^+$ ratio from peripheral blood can predict the capacity of γδ T cells to proliferate in response to phosphoantigens.

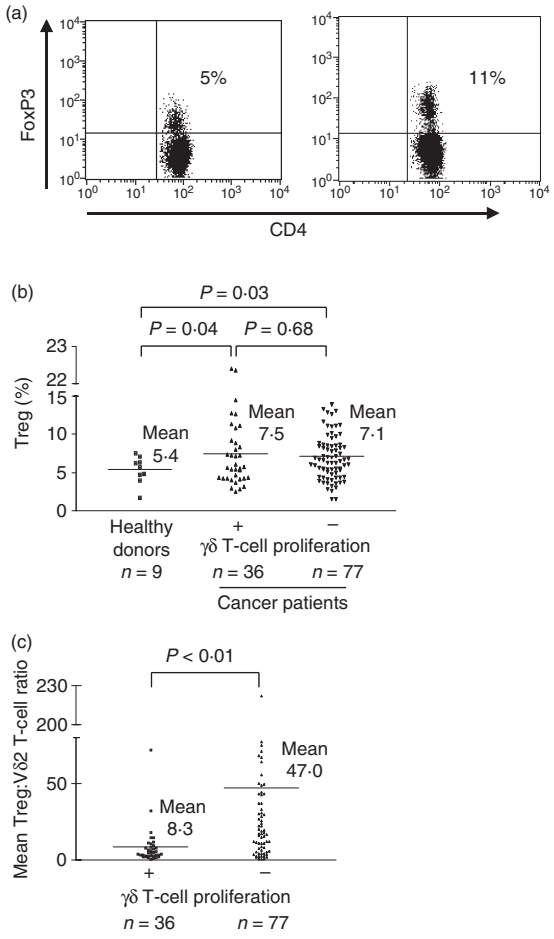

Figure 2. Comparison of circulating $CD4^+$ $FoxP3^+$ regulatory T cell (Treg) frequencies in healthy donors and cancer patients. Peripheral blood mononuclear cells (PBMC) from healthy donors and cancer patients were stained with anti-CD4 fluorescein isothiocyanate (FITC), anti-CD3 peridinin chlorophyll protein (PerCP) and anti-FoxP3 phycoerythrin (PE) and analysed by flow cytometry. To determine the frequencies of Tregs, CD4/CD3 double-positive cells were gated and further analysed for FoxP3 expression. (a) Representative fluorescence-activated cell sorter (FACS) analysis of Tregs from one healthy donor and one cancer patient, showing 5% versus 11% Tregs in PBMC, respectively. (b) Percentages of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs in $CD4^+$ T cells from nine healthy donors (mean ratio: 5·4) and 113 cancer patients, with maintained ($n = 36$; mean ratio: 7·5) and impaired ($n = 77$; mean ratio: 7·1) phosphoantigen-induced proliferation of γδ T cells. (c) Ratio of Tregs to the phosphoantigen-sensitive $Vδ2^+$ subset of γδ T cells in the peripheral blood of cancer patients with preserved (mean: 47·0) and impaired (mean: 8·3) phosphoantigen-induced γδ T-cell proliferation. Student's *t*-test was used to determine statistical significance of detected differences. $P < 0·05$ was considered significant.

Tregs inhibit phosphoantigen-induced γδ T-cell proliferation *in vitro*

To directly assess the capacity of Tregs to interfere with γδ T-cell proliferation, $CD4^+$ $CD25^+$ Tregs were purified by cell sorting or magnetic cell separation from healthy donors (purity ~90%) and co-cultured at different ratios with either freshly isolated PBMC or purified γδ T-cell lines from the same donor (Fig. 3a). In contrast to $CD4^+$ $CD25^-$ T cells, purified $CD4^+$ $CD25^+$ Tregs exhibited a cell dose-dependent inhibition of phosphoantigen-induced γδ T-cell proliferation, as shown by CFSE assay and by assessment of γδ T-cell proliferation after 1 week of culture (Fig. 3b–d). Consistent with the findings in cancer patients, at lower Treg:PBMC cell ratios than 1 : 1 the suppressive effect of Tregs began to diminish. The inhibitory effect of Tregs on γδ T-cell proliferation could not be overcome by higher doses of cytokines important for γδ T-cell proliferation, such IL-2 (up to 1000 U/ml) or IL-15, indicating that competitive consumption of cytokines is not responsible for Treg-mediated inhibition of γδ T-cell proliferation (data not shown). Titration experiments demonstrated that the suppressive effect of Tregs on γδ T-cell proliferation could be enhanced by using more stimulated Tregs (Tregs stimulated with PHA + IL-2 instead of IL-2 alone) (Fig. 3e). To exclude indirect effects of Tregs on γδ T-cell proliferation via a third cell population within PBMC, γδ T cell-enriched PBMC after depletion of αβ T cells, B cells, NK cells or monocytes were co-cultured with Tregs. The results of these experiments demonstrated that inhibition of phosphoantigen-induced proliferation of γδ T cells by Tregs is maintained after depletion of these PBMC subpopulations and suppression occurs at real γδ T-cell:Treg ratios of 1 : 1 to 1 : 5 (Fig. 3f). Similar ratios have been reported in the literature for Treg-mediated inhibition of other effector lymphocyte subsets.[10–12]

In contrast with the broad inhibitory effects of Tregs described for other lymphocyte subsets, the suppressive effect of Tregs on γδ T cells was restricted to proliferation. As shown in Fig. 4, neither pre-activated Tregs themselves nor supernatant from pre-activated Tregs (data not shown) significantly affected phosphoantigen-induced up-regulation of an activation marker (CD69) (Fig. 4a) or cytokine production (IFN-γ) (Fig. 4b) of naïve γδ T cells. The functional capacity of non-naïve γδ T cells (BrHPP-induced γδ T-cell lines) to kill the γδ T cell-sensitive tumour cell line Daudi (Fig. 4c) was also not significantly reduced by Tregs.

To confirm the inhibitory effects of Tregs on phosphoantigen-induced γδ T-cell proliferation in cancer patients, we depleted Tregs *in vitro* using anti-CD25 antibodies. As shown for a representative cancer patient who showed impaired γδ T-cell proliferation, depletion of Tregs restored the ability of γδ T cells to proliferate in the presence of phosphoantigen and IL-2 (Fig. 5). In summary, these data strongly support the ability of Tregs to suppress phosphoantigen-induced proliferation of γδ T cells.

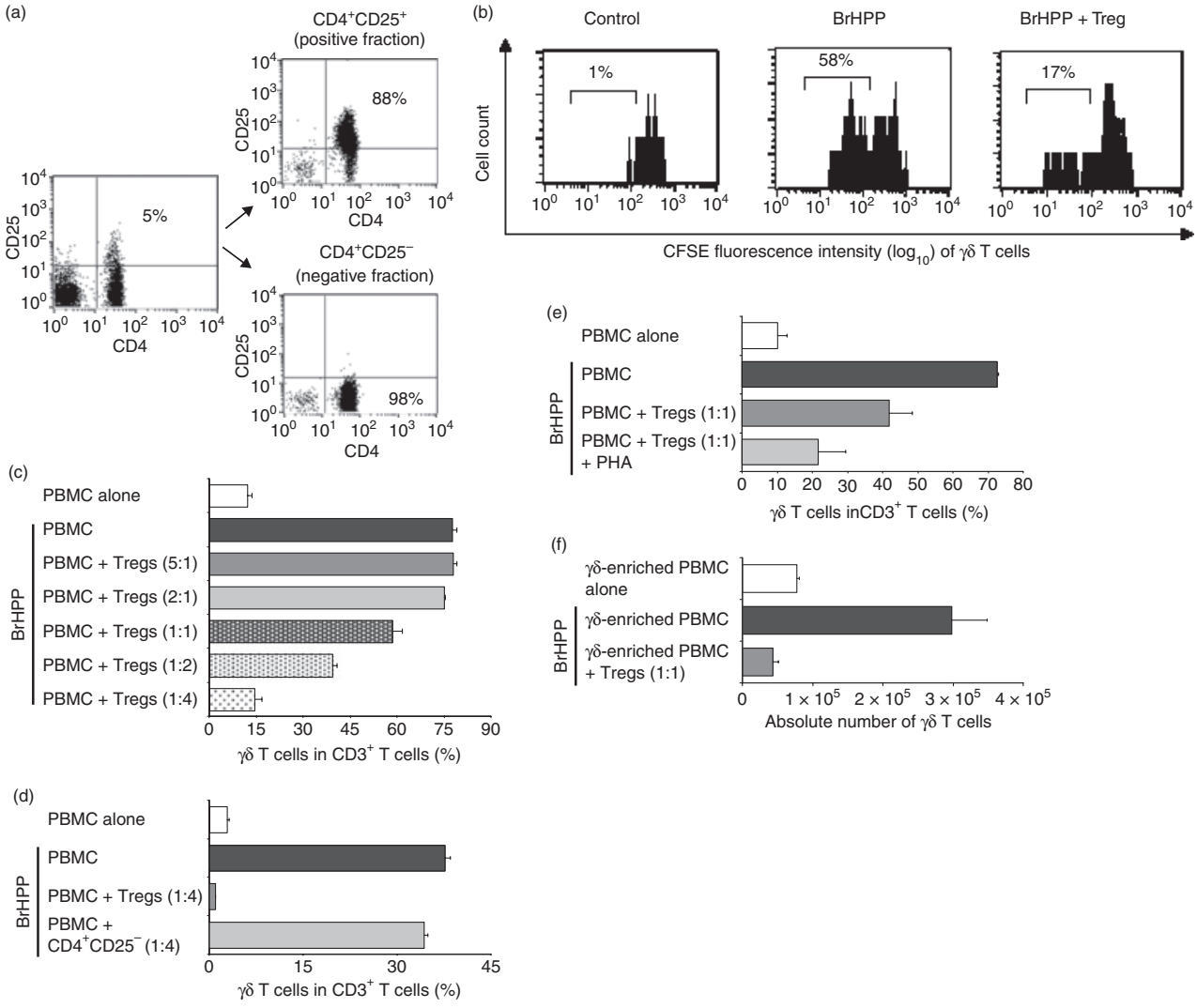

Figure 3. Inhibitory effects of purified regulatory T cells (Tregs) on phosphoantigen-induced γδ T-cell proliferation. (a) $CD4^+$ $CD25^+$ Tregs were isolated from peripheral blood mononuclear cells (PBMC) by using a FACSVantage cell sorter cytometer. After sorting, the $CD4^+$ $CD25^+$ fraction and the $CD4^+$ $CD25^-$ fraction were reanalysed to confirm enrichment and purity (88% versus 98%, respectively) as shown for one representative donor. (b) After depletion of αβ T cells a carboxyfluorescein succinimidyl ester (CFSE)-labelled γδ T-cell line was incubated with medium alone or bromohydrinpyrophosphate (BrHPP, 1 μM) and co-cultured with autologous purified Tregs at a ratio of 1 : 2·5. On day 6, cells were analysed by flow cytometry. γδ T-cell receptor (TCR)/CD3 double-positive T cells were gated, and loss of CFSE represented proliferation of gated cells. The percentage of proliferating cells is indicated for one representative donor. (c, d) PBMC were incubated with medium alone or BrHPP (1 μM) and co-cultured (c) with autologous purified Tregs at the indicated ratios or (d) with the $CD4^+$ $CD25^-$ T-cell fraction. On day 7, proliferation of γδ T cells was analysed by flow cytometry. (e) Stimulation of Tregs with phytohaemagglutinin (PHA; 2·5 μg/ml). (f) Preserved inhibition of phosphoantigen-induced γδ T-cell proliferation by Tregs after depletion of other PBMC populations [αβ T cells, B cells, natural killer (NK) cells and monocytes]. Representative results are shown after depletion of αβ T cells within PBMC. Similar results were obtained after depletion of B cells, NK cells or monocytes. After depletion of αβ T cells, 2×10^4 γδ T cell-enriched PBMC (25% γδ T cells in PBMC) were incubated with medium alone or BrHPP (1 μM) and co-cultured with autologous Tregs at a ratio of 1 : 1, which corresponds to a γδ T-cell:Treg ratio of 1 : 4. On day 7, proliferation of γδ T cells was analysed by flow cytometry, and absolute γδ T-cell numbers are shown. Each bar represents mean values ± standard deviation of triplicate cultures from one representative of at least three independent healthy donors.

Treg suppression of phosphoantigen-induced γδ T-cell proliferation is cell–cell contact independent and mediated by a non-proteinaceous soluble factor

To analyse the mechanisms of Treg-mediated suppression of phosphoantigen-induced γδ T-cell proliferation, we first separated Tregs from γδ T cells by a semi-permeable membrane (Transwell experiments). As shown in Fig. 6(a), the suppressive effect of Tregs on γδ T-cell proliferation by separation of Tregs from γδ T cells was almost completely maintained, suggesting that direct cell-to-cell contacts did not significantly contribute to

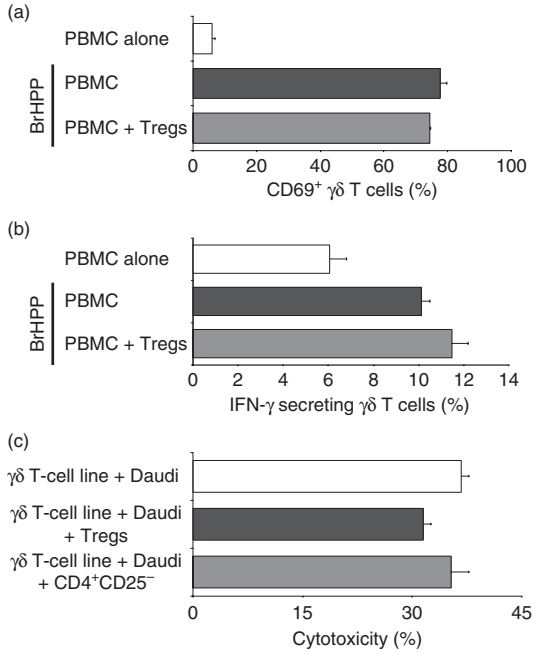

Figure 4. Effects of purified regulatory T cells (Tregs) on γδ T-cell function. Fresh peripheral blood mononuclear cells (PBMC) were incubated with bromohydrinpyrophosphate (BrHPP, 1 μM) or medium alone and co-cultured with autologous purified interleukin (IL)-2-activated Tregs at ratio 1 : 1 for 24 hr and further analysed for (a) expression of activation marker CD69 or (b) interferon (IFN)-γ secretion. Each bar represents mean values ± standard deviation (SD) of triplicate cultures from one healthy donor and is representative of two independent experiments. (c) In a standard cytotoxicity assay, a BrHPP-induced γδ effector T-cell line was incubated with the Burkitt lymphoma cell line Daudi alone [effector: target (E:T) ratio = 25 : 1] or together with purified Tregs, or as a control with the $CD4^+$ $CD25^-$ fraction of PBMC (Treg or $CD4^+$ $CD25^-$ to PBMC ratio = 3 : 1 and E:T ratio = 25 : 1). The percentage cytotoxicity is expressed as percentage specific lysis ± SD of triplicate cultures from one representative donor.

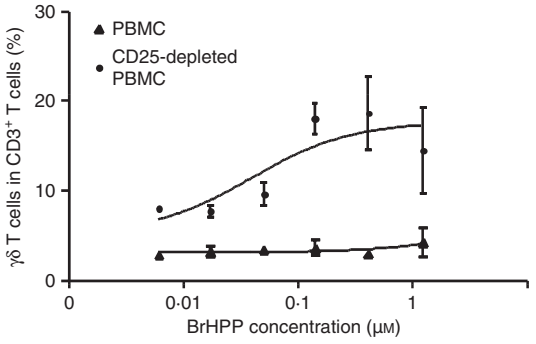

Figure 5. Depletion of $CD25^+$ cells restores the capacity of γδ T cells to respond to phosphoantigen. Peripheral blood mononuclear cells (PBMC) from cancer patients were stimulated with bromohydrinpyrophosphate (BrHPP; 0·014; 0·041; 0·123; 0·37 and 1·11 μM) and interleukin (IL)-2 (100 U/ml) before and after *in vitro* depletion of $CD25^+$ T cells and proliferation of γδ T cells was analysed on day 7 by flow cytometry. Data shown represent mean values ± standard deviation of triplicate cultures from one donor representative of three cancer patients.

suppression. Importantly, attempts to transfer suppression of γδ T-cell proliferation using supernatants of purified Tregs were successful, and this suppression could be enhanced by additional stimulation of Tregs using PHA (data not shown), suggesting that a soluble factor secreted by Tregs contributes to suppression (Fig. 6a).

As the suppressive activity was clearly demonstrable in supernatants of Tregs, we started to characterize the biochemical nature of the soluble factor secreted by Tregs. As shown in Fig. 6(b), the inhibitory effect of the Treg supernatant was maintained even after heat treatment (56° and 99°) and was resistant to protease treatment, suggesting that a non-proteinaceous soluble factor secreted by Tregs is responsible for Treg-mediated suppression of γδ T-cell proliferation. Thus, these data argue against immunosuppressive cytokines such as TGF-β and IL-10 being mediators of the suppressive activity in Treg supernatants. In fact, neutralization of neither TGF-β (by anti-TGF-β mAb, TGF-β soluble receptor II or LAP) nor IL-10 (by anti-IL-10 mAb) restored phosphoantigen-induced γδ T-cell proliferation in the presence of Tregs (data not shown).

Given that non-proteinaceous soluble factors interacting with amino acid metabolism or iNOS activity have been reported to be able to mediate suppressive effects on T-cell proliferation, we next examined whether these factors might be responsible for the cell–cell contact-independent suppression of phosphoantigen-induced γδ T-cell proliferation. However, Tregs maintained their inhibitory effect on γδ T-cell proliferation in the presence of the IDO inhibitor 1-methyl-L-tryptophan, the arginase inhibitor nor-NOHA and the iNOS inhibitor L-NMMA, suggesting that neither depletion of tryptophan nor depletion of arginine nor induction of NO contributed to Treg-mediated suppression of γδ T-cell proliferation (Fig. 6c). Thus, these data argue for a novel cell–cell contact-independent suppressive activity of Tregs on γδ T cells, which is neither dependent on cytokines nor dependent on known non-proteinaceous soluble factors.

Role of Treg-induced soluble factors in inhibition of αβ T-cell proliferation

It is widely accepted that Tregs exert their regulatory effect on ($CD4^+$ and $CD8^+$) αβ T cells via cell–cell contact mechanisms. To analyse the effect of Tregs on αβ T cells, polyclonal alloreactive (MLR) and antigen-specific (CMV pp65-specific) proliferation of αβ T cells in the presence of Tregs and Treg supernatant, respectively, was examined. As shown in Fig. 7(a), co-culture of Tregs suppressed the proliferation of CMV pp65-specific $CD8^+$ αβ T cells. However, the supernatant of purified Tregs inhibited neither the proliferation of CMV pp65-specific $CD8^+$

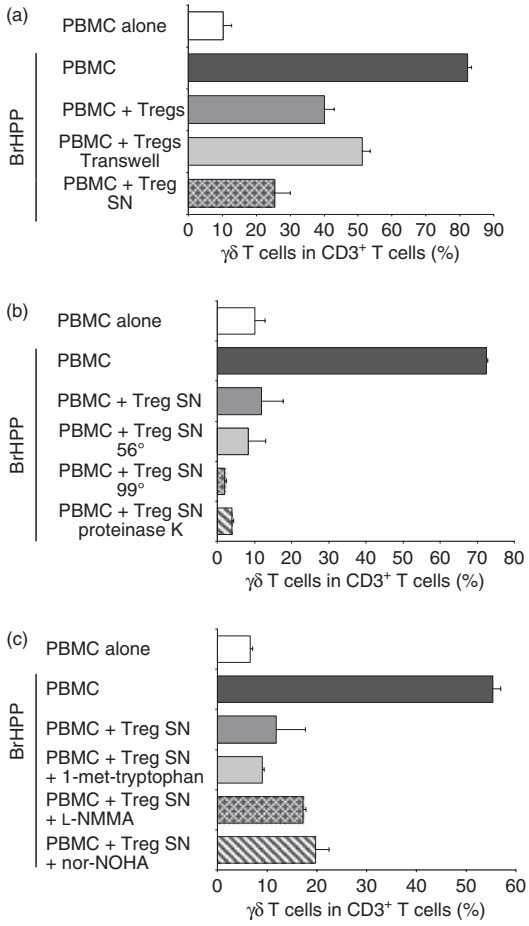

Figure 6. Mechanisms of regulatory T cell (Treg)-mediated suppression of γδ T-cell proliferation. (a) Peripheral blood mononuclear cells (PBMC) were incubated with bromohydrinpyrophosphate (BrHPP, 1 μM) or medium alone and co-cultured with autologous purified Tregs separated by semi-permeable membranes (Transwells) or with supernatant (SN) from overnight-cultivated and interleukin (IL)-2-stimulated Tregs. (b) Supernatants were heated for 15 min at 56° or 99°, or treated with proteinase K (2 hr at 37°). (c) PBMC incubated with BrHPP were co-cultured with supernatant from overnight-cultivated Tregs alone or treated with 1-methyl-L-tryptophan (1·5 mM), N^G-monomethyl-L-arginine (L-NMMA; 2 mM) or N^ω-hydroxy-nor-L-arginine (nor-NOHA; 0·5 mM) at ratio 1 : 1 and proliferation of γδ T cells was analysed on day 7 by flow cytometry. Each bar represents mean values ± standard deviation of triplicate cultures from one healthy donor and is representative of four independent experiments.

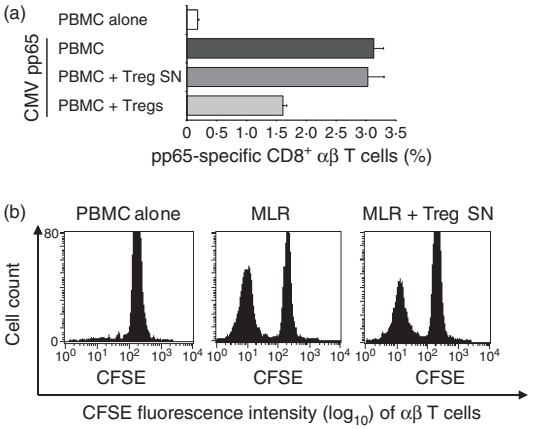

Figure 7. Effects of purified regulatory T cells (Tregs) on αβ T-cell proliferation. (a) Peripheral blood mononuclear cells (PBMC) from cytomegalovirus (CMV)-seropositive healthy donors were re-stimulated with CMV pp65-loaded mature dendritic cells (DCs) alone or co-cultured with autologous purified Tregs (ratio PBMC: Treg = 1 : 4) or supernatant (SN) from overnight-cultivated Tregs (ratio PBMC:Treg supernatant = 1 : 1). After 1 week, proliferation of CMV pp65-specific $CD8^+$ αβ T cells was determined by flow cytometry. Each bar represents mean values ± standard deviation of triplicate cultures from one representative donor. (b) In a mixed lymphocyte reaction (MLR), carboxyfluorescein succinimidyl ester (CFSE)-labelled PBMC were incubated with allogeneic immature dendritic cells (ratio 1 : 10) alone or together with supernatant from overnight-cultivated autologous purified Tregs (ratio PBMC:Treg supernatant = 1 : 1). On day 6, cells were analysed by flow cytometry. αβ T-cell receptor (TCR)/CD3 double-positive T cells were gated, and loss of CFSE represents proliferation of gated cells. One representative donor of three is shown.

αβ T cells (Fig. 7a) nor the proliferation of alloreactive αβ T cells (MLR) (Fig. 7b), indicating that the suppressive effects of Tregs on αβ T cells and γδ T cells are mediated by different mechanisms.

Discussion

Tregs have a crucial role in impeding immune surveillance against cancer and hampering the development of effective anti-tumour immunity.[3] Increasing evidence supports the existence of increased frequencies of Tregs in both peripheral blood and the tumour microenvironment of solid tumours and haematological malignancies.[33] Thus, our results are consistent with a series of recent reports demonstrating increased frequencies of Tregs (characterized as $CD4^+$ $CD25^+$ $FoxP3^+$ T cells) in cancer patients and patients with certain premalignant conditions such as MGUS as compared with healthy individuals.[33,34] However, recent data have shown that the ratio of Tregs to effector T cells, rather than the Treg frequency in peripheral blood or tumour specimens, predicts more precisely the inhibitory effects of Tregs on a target cell population (especially for rare effector populations such as γδ T cells) and is a more promising independent prognostic factor for tumour recurrence and survival than the Treg frequency alone.[35–37] Our data confirm the predictive value of Treg:effector T-cell ratios, because we found that the Treg:γδ T-cell ratio, unlike the absolute or relative number of circulating Tregs, was negatively correlated with phosphoantigen-induced γδ T-cell proliferation in cancer patients. In addition to this indirect evidence, this study demonstrates for the first time the capacity of Tregs to directly inhibit γδ T-cell

proliferation *in vitro*. Our findings therefore might explain the frequent proliferative anergy of γδ T cells in cancer patients. Although other factors might also play a role in suppression of γδ T-cell function, we have defined Treg-mediated suppression as one major mechanism for impaired γδ T-cell proliferation in cancer patients.[38] Therefore, a combination of depletion or attenuation of Tregs and concomitant stimulation of γδ T cells may overcome Treg-mediated suppression and enhance γδ T cell-mediated immunotherapy in cancer patients.

Impaired phosphoantigen-induced proliferation of peripheral γδ T cells has been reported not only in cancer but also in the course of certain chronic infections (HIV, tuberculosis and toxoplasmosis).[28,30,39] Several groups demonstrated qualitative and quantitative alterations of the peripheral Vγ9Vδ2 T-cell subset of chronically infected persons including a polyclonal decrease in the absolute number of Vγ9Vδ2 T cells, decreased cytokine (TNF-α and IFN-γ) production and proliferative anergy after stimulation with phosphoantigens in spite of the presence of IL-2.[30,40] Although earlier studies suggested a potential role of cytokine dysfunction, increased sensitivity to apoptosis as a consequence of chronic antigenic stimulation, a dysbalance in the expression of killer inhibitory receptors (KIRs) or shedding of NKG2D ligands in down-regulating γδ T-cell effector functions in chronic infection and cancer, our results indicate that Tregs play a major role in the proliferative anergy of Vγ9Vδ2 T cells.[38,40–43] In fact, increased frequencies of Tregs were recently observed in patients with chronic active infections, supporting the role of Treg-mediated suppression of Vγ9Vδ2 T cells in chronically infected persons.[44,45] Although the role of Tregs in various chronic infections is currently highly controversial for various reasons, a high perforin:Treg ratio in lymphoid tissues of HIV-infected patients has been shown to correlate with non-progressive disease, suggesting that the immune control of virus replication represents a balance between cell-mediated immune response and Treg-mediated counter regulation.[46] However, the relevance of Tregs in infection-associated γδ T-cell anergy remains to be established.

Although previous studies have demonstrated a role of Tregs in suppressing other innate lymphocyte subsets such as NK cells and NKT cells *in vitro*,[11,12,47,48] this is the first study to provide mechanistic insights into Treg–γδ T-cell interactions and to formally demonstrate that Tregs display an immunoregulatory effect on human γδ T cells. In contrast to the reported cell–cell contact-dependent suppressive effect of Tregs on αβ T cells, our data show that the inhibitory effect of Tregs on γδ T cells is cell–cell contact independent and restricted to proliferation. Furthermore, Transwell and supernatant experiments suggest that Tregs secrete non-proteinaceous soluble factors which potently inhibit phosphoantigen-induced γδ T-cell proliferation. However, in our experimental system, neither the soluble factors TGF-β, IL-10 and NO, nor IDO- or arginase-dependent immunosuppressive mechanisms appeared to be involved in the Treg-mediated suppressive effect on γδ T-cell proliferation. Further analyses are required to characterize the soluble non-proteinaceous factor secreted by Tregs and experiments to determine the biochemical properties of this factor are currently underway in our laboratory.

There is accumulating evidence that Tregs suppress multiple types of immunocompetent cells and activated Tregs can inhibit a wide range of immune responses through bystander suppression.[15] However, our results indicate that Tregs may not only control immune responses via mechanisms targeting various types of immune cells (i.e. cell–cell contact-dependent or TGF-β-mediated mechanisms), but can also mediate suppressive effects through cell type-specific mechanisms such as the inhibition of γδ T-cell proliferation by a soluble non-proteinaceous factor reported here. Thus, our analysis has uncovered a new suppressive function of Tregs that does not affect all lymphocyte subsets and underscores the existence of cell–cell contact-independent and strictly cell–cell contact-dependent suppressive activities of Tregs.

Recent studies have demonstrated that IL-2 signalling is required for thymic development, peripheral expansion, and suppressive activity of Tregs.[49] In patients with melanoma and mRCC, the frequency of Tregs was significantly increased after IL-2 treatment, which might explain in part the limited anti-tumour activity of IL-2 administration in cancer patients.[50] IL-2 has also been used in human and preclinical non-human primate studies to expand γδ T cells in response to phosphoantigens.[28,29,51] However, repetitive applications of phosphoantigen in combination with IL-2 induced less vigorous expansion of γδ T cells *in vivo*, suggesting a progressive exhaustion of the γδ T-cell proliferative response.[28,51] Our findings might provide an explanation for this reduced proliferative response of γδ T cells after successive treatment with phosphoantigen and IL-2, because IL-2-mediated induction of Tregs might counteract the expansion and potential anti-tumour effects of γδ T cells. This emphasizes a major potential disadvantage of IL-2 in clinical γδ T cell-based immunotherapy approaches and therefore has major implications for the design of future clinical trials.

In conclusion, this study demonstrates the capacity of Tregs to inhibit γδ T-cell proliferation *in vitro* via a cell–cell contact-independent mechanism and thus increased Treg:γδ T-cell ratios may contribute to the frequently impaired γδ T-cell proliferative response in cancer patients. Our results extend recent findings concerning various cellular and molecular events to explain how Tregs suppress immune responses and suggest that immunopharmacological manipulations to deplete Tregs might enhance the effectiveness of γδ T cell-mediated tumour immunotherapy.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Preserved inhibition of phosphoantigen-induced γδ T-cell proliferation by regulatory T cells (Tregs) after depletion of different peripheral blood mononuclear cell (PBMC) populations [B cells, natural killer (NK) cells and monocytes]. After depletion of different PBMC subsets, 2×10^4 γδ T cell-enriched PBMC (20–30% γδ T cells within PBMC) were incubated with medium alone or with bromohydrinpyrophosphate (BrHPP 1 μM) and co-cultured with autologous Tregs at a ratio of 1 : 1, which corresponds to γδ T-cell/Treg ratios from 1 : 3 to 1 : 5. On day 7, the proliferation of γδ T cells was analysed by flow cytometry, and absolute γδ T-cell numbers are shown. Each value represents mean values ± standard deviation of triplicate cultures from one donor representative of at least three independent healthy donors.

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