


Polyfunctional response by ImmTAC (IMCgp100) redirected CD8⁺ and CD4⁺ T cells

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Summary

The success of immune system-based cancer therapies depends on a broad immune response engaging a range of effector cells and mechanisms. Immune mobilizing monoclonal T cell receptors (TCRs) against cancer (ImmTACTM molecules: fusion proteins consisting of a soluble, affinity enhanced TCR and an anti-CD3 scFv antibody) were previously shown to redirect CD8⁺ and CD4⁺ T cells against tumours. Here we present evidence that IMCgp100 (ImmTAC recognizing a peptide derived from the melanoma-specific protein, gp100, presented by HLA-A*0201) efficiently redirects and activates effector and memory cells from both CD8⁺ and CD4⁺ repertoires. Using isolated subpopulations of T cells, we find that both terminally differentiated and effector memory CD8⁺ T cells redirected by IMCgp100 are potent killers of melanoma cells. Furthermore, CD4⁺ effector memory T cells elicit potent cytotoxic activity leading to melanoma cell killing upon redirection by IMCgp100. The majority of T cell subsets belonging to both the CD8⁺ and CD4⁺ repertoires secrete key pro-inflammatory cytokines (tumour necrosis factor- α , interferon- γ , interleukin-6) and chemokines (macrophage inflammatory protein-1 α - β , interferon- γ -inducible protein-10, monocyte chemoattractant protein-1). At an individual cell level, IMCgp100-redirection T cells display a polyfunctional phenotype, which is a hallmark of a potent anti-cancer response. This study demonstrates that IMCgp100 induces broad immune responses that extend beyond the induction of CD8⁺ T cell-mediated cytotoxicity. These findings are of particular importance because IMCgp100 is currently undergoing clinical trials as a single agent or in combination with checkpoint inhibitors for patients with malignant melanoma.

Keywords: cancer; ImmTAC; immunotherapy; melanoma; T cells; T cell receptor.

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Introduction

The human immune system has an intrinsic ability to recognize and eliminate cancer; however, a combination of thymic negative selection and a multitude of immunosuppressive mechanisms employed by tumour cells result in an inefficient immune response.¹ Modes of enhancing the immune system's function within the tumour environment have been investigated for a number of years and have resulted in the emergence of promising immunotherapies.²

Clinical data have shown that a high density of CD3⁺ T cells, including CD8⁺ cytotoxic T cells (CTLs) and

CD45RO memory T cells, in the tumour microenvironment correlates with longer disease-free survival in many solid cancers.³ However, in many cases, even the presence of functional T cells does not lead to tumour regression. One of the reasons for this inefficient immune response is the ability of cancer cells to deactivate T cells by over-expressing ligands for inhibitory checkpoint molecules.⁴ Therapies using antibodies against checkpoint molecules or their ligands have generated positive outcomes in some cancer patients but the majority do not improve.⁵ The likely reason for this modest success rate is the lack of cancer-reactive T lymphocytes present in the body, which limits the number of patients that can be cured.

To overcome inadequate T cell responsiveness to cancer antigens, a new class of immune stimulatory reagents termed immune mobilizing monoclonal T cell receptors (TCRs) against cancer (ImmTAC) has been developed. ImmTAC molecules are bispecific reagents comprising an engineered monoclonal high affinity T cell receptor (mTCR) fused to an anti-CD3 single-chain antibody fragment (scFv).⁶ The mTCR specifically targets cells that present the epitope of interest (cancer-associated or cancer-specific antigen) whereas the anti-CD3 scFv engages polyclonal T cells. ImmTAC molecules have been demonstrated to successfully redirect and activate cytotoxic T cells to kill tumour cells with a high degree of potency: as few as five to ten epitopes are sufficient for an ImmTAC to mount successful clearance of target cells.⁷ *In vitro* studies carried out using CD8⁺ and CD4⁺ T cell populations have previously demonstrated that ImmTAC molecules induce a polyclonal response in these cells.⁷ In contrast to checkpoint inhibitory therapies, ImmTAC molecules engage any CD3⁺ T cells present in the environment, regardless of T cell specificity, so overcoming the need for cancer-specific T cells.

The phenotype of T cells influences the location of priming, the sensitivity of the T cell to the specific antigen and the quality of the T cell response. Terminally differentiated effector T (Temra) cells and effector memory T (Tem) cells circulate mainly in the peripheral tissue where they recognize antigens, mount a rapid cytokine response and deliver cytotoxic molecules to destroy antigen-positive cells.⁸ In contrast, naive and central memory T (Tcm) cells preferentially migrate through the secondary lymphoid tissues where they are primed by mature dendritic cells, expand and differentiate into effector and effector memory cells that will recirculate to the peripheral tissue.^{9,10} Recent studies have demonstrated that antigen presentation and naive T cell priming can also occur outside secondary lymphoid organs, within tumours in ectopic lymphoid-like structures, named tertiary lymphoid structures,¹¹ as an expedited mechanism for priming tumour-reactive T cells.

Engaging cytotoxic T cells is one of the major goals of cancer immunotherapy, primarily due to their direct killing ability; different subsets of these cells vary in the nature of their anti-tumour response. In both mouse and non-human primate studies, adoptively transferred CD8⁺ Tem cells rapidly developed into effector cells and efficiently killed tumour cells but only Tcm cells formed a persistent reservoir of functional T cells, occupied memory cell niches and provided a lasting anti-cancer immune response.^{12,13} Comparisons have also been made between the Tcm and naive CD8⁺ T cell repertoires and, although the findings are somewhat conflicting, both subpopulations bring a specific contribution to successful tumour elimination.^{14–16} Despite their crucial role in tumour clearance, CD8⁺ T cells on their own are not capable of

delivering a sustained cancer remission. This also requires CD4⁺ T cells, which not only enhance the effector functions of CD8⁺ T cells but also protect them from exhaustion.¹⁷

A reliable measure of T cell activation is acquisition of a polyfunctional phenotype in which an individual immune cell has the ability to exert several effector functions, as demonstrated for adoptive cell transfer therapy.¹⁸ Polyfunctionality of a T cell is reliant on the expression of key molecules including CD40 ligand, interferon- γ (IFN- γ), tumour necrosis factor- α (TNF- α) and interleukin-2 (IL-2).¹⁹ Polyfunctional T cells have been shown to be critical for mounting robust anti-tumour responses and acquisition of protective immunity against tumour recurrence.¹⁹

This study presents data that dissect the subsets of T lymphocytes involved in the inflammatory response induced by a specific ImmTAC, IMCgp100; a molecule that recognizes a gp100-derived peptide in complex with HLA-A*0201 on the surface of melanoma cells. IMCgp100 is currently being tested in Phase II clinical trials for the treatment of malignant melanoma. Using a range of *in vitro* assays, we investigated whether IMCgp100 can: (i) redirect and activate various CD8⁺ and CD4⁺ T cell subpopulations to directly kill melanoma cells including in the presence of regulatory T (Treg) cells; (ii) stimulate T cells to produce a range of key cytokines and chemokines; and (iii) induce individual T cells to exhibit a polyfunctional phenotype.

Materials and methods

ImmTAC engineering

IMCgp100, a gp100-specific ImmTAC, was prepared as previously described.⁷ Briefly, a high affinity TCR was generated from a wild-type gp100 TCR using directed molecular evolution and phage display selection.²⁰ The resulting high-affinity TCR β chain was fused to a humanized CD3-specific scFv via a flexible linker and the α - and β -chains of the resulting ImmTAC were expressed in *Escherichia coli* as inclusion bodies. ImmTAC molecules were then refolded and purified as previously described.^{7,21}

Cell lines and cell culture

Mel526 melanoma cells (HLA-A*0201⁺ and gp100⁺) were obtained from Thymed (Wendelsheim, Germany) and cultured in RPMI-1640 with 10% fetal calf serum, 1% penicillin/streptomycin and 1% glutamine (Life Technologies, Paisley, UK). A375 melanoma cells (HLA-A*0201⁺ and gp100⁻) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in R10 medium (RPMI-1640, 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine (Life

Technologies). Both cell lines were purchased in 2003, expanded in culture, frozen and stored in liquid nitrogen. The cell-line authentication and *Mycoplasma* testing were carried out every 4 months by the LGC Standards Cell Line Authentication Service (www.lgcstandards.com) and Mycoplasma Experience Ltd (www.mycoplasma-exp.com), respectively. The cells were not used beyond passage ten after thawing.

Purification of CD4⁺ and CD8⁺ T cell subpopulations

Human peripheral blood mononuclear cells (PBMC) were prepared from healthy donors by Ficoll-Hypaque (Robbins Scientific, San Diego, CA) density gradient centrifugation. CD8⁺ and CD4⁺ T cells were enriched from PBMC by negative selection using magnetic beads according to the manufacturer's protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then labelled with fluorescent mouse anti-human antibodies: CCR7-phycoerythrin (PE), CD45RA-allophycocyanin (APC) and CD4-peridinin chlorophyll protein (PerCP)-Cy5.5 or CD8-PE-Cy7 (BD Biosciences, San Diego, CA). Following addition of 7AAD (BD Biosciences) to eliminate dead cells, samples were acquired using FACS with FACSARIAII flow cytometer (BD Biosciences). Cell subsets were sorted according to the surface labelling and the purity was ensured by re-acquiring samples directly after the sort (> 95% pure). Electronic compensation was performed in all cases using antibody capture beads (BD Biosciences) stained separately with individual antibodies used in the experimental samples. Data were analysed with FLOWJO software (TreeStar Inc., Ashland, OR).

IFN- γ ELISpot assay

Assays were carried out according to the manufacturer's instructions (BD Biosciences). All assays were performed in triplicate in a maximum volume of 200 μ l. Each well contained 5×10^4 target cells (Mel526 or A375) and 1×10^4 effector cells (CD4⁺ or CD8⁺ subpopulations). IMCgp100 was added at final concentrations of 31, 82 or 131 pM. Where indicated, heteroclytic peptide for gp100_(280–288) (YLEPGPVTV) was added at 1 μ M. Plates were incubated overnight at 37°C in 5% CO₂ and the number of spots was quantified using an automated ELISpot reader (Immunospot Series 5 Analyzer; Cellular Technology Ltd., Shaker Heights, OH).

IncuCyte real-time quantification of cell killing and statistical analysis

Killing assays were carried out using the IncuCyte FLR-Platform (EssenBioScience, Ann Arbor, MI), as described previously.²² Target cells (Mel526 or A375) were plated at 1×10^4 cells per well of a 96-well plate in R10 and

incubated at 37°C in 5% CO₂. Effector cells (CD4⁺ or CD8⁺ T cell subpopulations) were washed twice and plated at 5×10^4 cells per well in R10. IMCgp100 was added at different concentrations (131, 82, 31 pM or no IMCgp100). 5 μ M NucView (EssenBioScience) was added to each well to allow detection of apoptosis. T cells obtained from each donor were tested in two replicates and a mean of the number of killed melanoma cells was taken at each time-point. Normalized values were created by removing the mean value of the 'no IMCgp100 control' from the 'IMCgp100 treated' value. The killing curves were then modelled using non-linear logistic growth curves:

$$\text{cells killed} = \frac{\text{Asym}}{1 + \exp\left(\frac{(\text{xmid} - \text{hours})}{\text{scale}}\right)}$$

A random effect was used to control donor-to-donor variability. Statistical differences between the killing curves were assessed by model comparison using analysis of variance. To build the non-linear mixed effects model the R library nlme was used. Plots and charts were made with the ggplot2 library. For the naive CD4⁺ T cells the values were low and did not fit a logistic growth model well, so in this case a linear mixed effects model using the lmer library was used to generate the line in Fig. 2(b).

Detection of granzyme B in CD4⁺ and CD8⁺ T cells

Target cells (Mel526) were detached, washed with R10 and plated at 5×10^5 cells per well in a flat-bottom 96-well plate. PBMC from a healthy donor were added at 2.5×10^5 cells per well. Selected wells were supplemented with IMCgp100 at 131, 82, 31 pM or 12.5 pM and no ImmTAC was added to the control wells. The cells were collected following 24 hr or 96 hr of incubation. Samples were labelled with fluorescently tagged mouse anti-human antibodies: anti-CD8-PE-Cy7 (BD Bioscience) and anti-CD4-APC-Cy7 (BD Bioscience). The cells were then fixed with Cytotfix/Cytoperm solutions (BD Bioscience) and intracellular labelling for human granzyme B-PE-Txr (Invitrogen, ThermoFisher Scientific, Waltham, MA) was performed according to the manufacturer's protocol. Data were acquired using FACS ARIAII (BD Bioscience) and analysed with FLOWJO V10 software (Tree Star Inc.).

Cytokine analysis

Cytokine and chemokine release [TNF- α , IL-6, IFN- γ -inducible protein-10 (IP-10), IL-10, monocyte chemoattractant protein 1 (MCP-1), IFN- γ , macrophage inflammatory protein 1 α/β (MIP-1 α/β), Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), IL-4, IL-2, monokine-induced by IFN- γ (MIG), IL-5 and IL-15] was measured by Luminex

immunoassay in accordance with the manufacturer's instructions (R&D Systems, Minneapolis, MN) using culture supernatants from the IncuCyte killing assays. The levels of cytokines and chemokines were determined with Luminex MAGPIX System (Merck Millipore, Billerica, MA).

Intracellular cytokine labelling and polyfunctional T cell assay

Freshly-isolated PBMC and Mel526 cells were resuspended in R10 and incubated overnight with a range of IMCgp100 concentrations (31, 82, 131 pM). Mel526 cells supplemented with 82 pM IMCgp100 pulsed with heteroclytic gp100 peptide were used as a positive control. Mel526 cells co-cultured with the effector cells without IMCgp100 served as a negative control. To evaluate cytotoxic degranulation activity of T cells, anti-CD107a-APC (BD Biosciences) was added into the culture medium. Brefeldin A (GolgiPlug; BD Biosciences) and monensin (GolgiStop; BD Biosciences) were added 2 hr after the addition of IMCgp100 to the assay. Following 14 hr of incubation, PBMC were incubated with a panel of antibodies to detect specific subpopulations of T cells: anti-CD3-PerCP-Cy5.5 (BioLegend, San Diego, CA), anti-CD4-APC-Cy7 (BioLegend), anti-CD8-BV500 (BD Biosciences), anti-CD45RA-BV711 and CCR7-PE (BD Biosciences). The labelled cells were permeabilized using Cytofix/Cytoperm kit (BD Biosciences) and then labelled with anti-IFN- γ -FITC (BD Biosciences), anti-TNF- α -BV421 (Biolegend), anti-IL-2-PE-Cy7 (BD Biosciences) and MIP-1 α -Alexa Fluor 700 (BD Biosciences). FACS analysis was performed using a FACS Aria II flow cytometer (BD Biosciences) and the data were analysed using FLOWJO software (Treestar). T cells positive for the various combinations of effector molecules were analysed and quantified using a Boolean gating function in FLOWJO software and analysed using PESTLE (freely available) and SPICE programs (<http://exon.niaid.nih.gov/spice/>). The results were presented as pie charts showing the percentage of cells displaying a combination of effector molecules.

Isolation of Treg cells and killing assay (IncuCyte)

Treg cells were obtained from two sources: (i) PBMC from two healthy donors were purified from blood in-house using Ficoll-Hypaque density gradient centrifugation (Robbins Scientific) and Treg cells were extracted using a CD4⁺ CD25⁺ CD127^{dim/-} Regulatory T Cell Isolation Kit II (Miltenyi Biotech Ltd.). Treg cell purity was assessed by labelling cells with anti-human antibodies: anti-CD3-FITC, anti-CD8-APC, anti-CD4-PE-Cy7, anti-CD25-BV-421 and anti-FoxP3-PE (BD Bioscience) and subsequent FACS analysis (Intellicyt iQplus platform and

ForeCyt software analysis), (ii) Treg cells were sourced from Tissue Solutions (Glasgow, UK). CD8 T were isolated by negative selection using CD8⁺ T Cell Isolation kit (Miltenyi Biotech) or provided by Tissue Solutions. A Treg cell killing suppression assay was carried out using the IncuCyte ZOOM-Platform (EssenBioScience, Ann Arbor, MI). Target cells (Mel526) were plated at 1.5×10^4 cells per well of a 96-well plate in R10 and incubated overnight at 37°C in 5% CO₂. Effector CD8⁺ T cells and Treg cells were added at 6×10^4 cells per well (at 1 : 1 CD8⁺ T cell/Treg cell ratio). IMCgp100 was added at 100 pM; 5 μ M NucView (EssenBioScience) was added into each well to allow detection of apoptosis.

Results

Redirection of CD8⁺ T cell subpopulations against melanoma cell lines using IMCgp100

The high affinity ImmTAC, IMCgp100, has been previously shown to redirect unstimulated CD8⁺ T cells to antigen-positive tumour cells where they then elicit effector functions (cytokine production and lytic activity).⁷ In this study, defined T cell subsets were tested for their anti-tumour activity when recruited by IMCgp100. Using flow cytometry, a total pool of CD8⁺ T cells (all T cells expressing CD8 receptor) obtained from healthy donors was sorted into: naive (Naive, CD45RA⁺ CCR7⁺), central memory (Tcm, CD45RA⁻ CCR7⁺), effector memory (Tem, CD45RA⁻ CCR7⁻) and CD45RA⁺ effector memory (Temra, CD45RA⁺ CCR7⁻) cells (Fig. 1a). The capacity of each T cell subpopulation to kill gp100-expressing melanoma cells was tested by incubation with Mel526 cells in the presence of IMCgp100 at a clinically relevant concentration of 82 pM. The rate of killing was monitored by detecting caspase 3/7 activation for 40 hours or 44 hours.

Fig. S1 (see Supplementary material) shows the donor-by-donor variation in the killing curves for the CD8 subpopulations. Given that the purpose of this experiment was to evaluate the T cell killing capacity following IMCgp100 stimulation, only donors whose ImmTAC-treated T cells induced apoptosis beyond that of non-stimulated control cells were included in the analysis (one out of eight donors, Donor 4, did not reach these data inclusion criteria due to high background killing observed in the absence of ImmTAC). To assess the overall differences between T cell subsets, the data for all donors were pooled and non-linear mixed effects models using a logistic growth framework were used to model the cell killing; a random effect was used to model the donor-to-donor variation. Figure 1(b) displays data points obtained from all donors and the best model as determined by maximum likelihood for each cellular subset. Figure 1(c) shows the model parameters that were fitted for each subpopulation. The most rapid killing was achieved by the

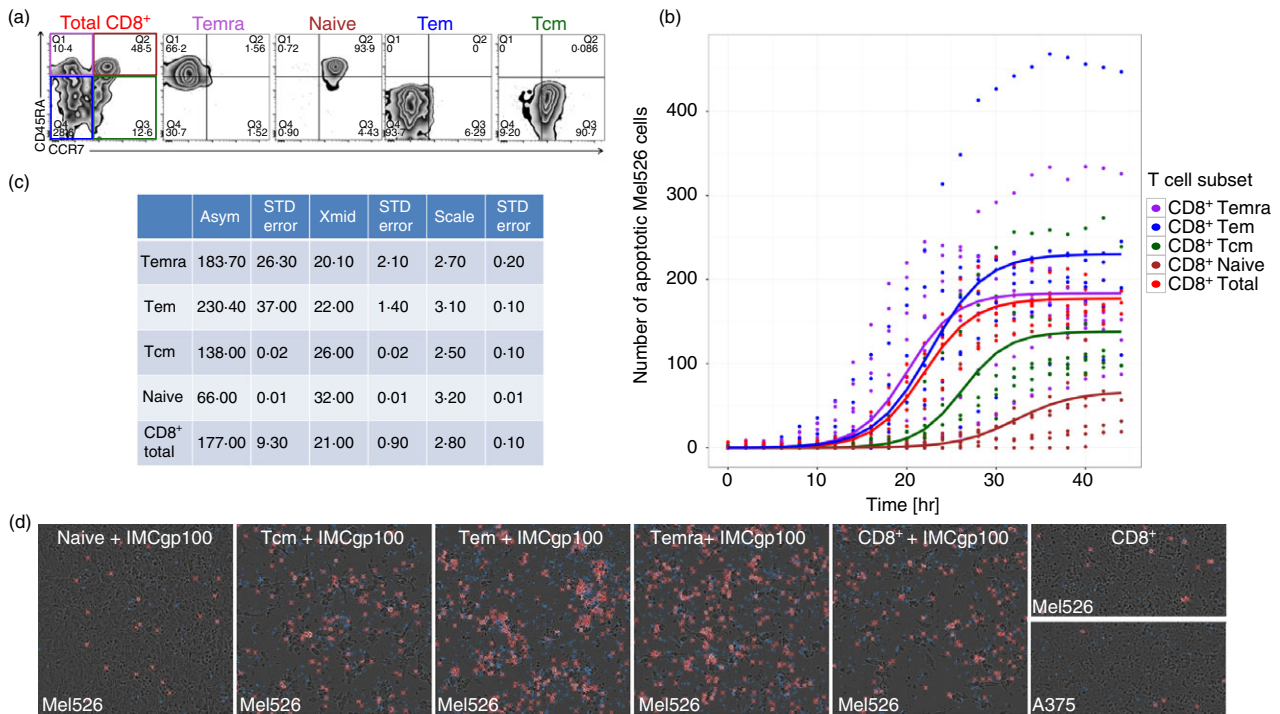


Figure 1. IMCgp100 induced killing capacity of different CD8⁺ T cell subpopulations. (a) Isolated CD8⁺ T cells (here obtained from Donor 8) were FACS sorted, according to cell surface labelling of CD45RA and CCR7 markers, into four subpopulations: naive, effector memory (Tem), CD45RA effector memory (Temra) and central memory (Tcm). Melanoma target cells (Mel526 cell line: HLA-A2+ve, gp100+ve) were incubated with each of the CD8⁺ T cell subpopulations (Naive, Tem, Temra and Tcm) as well as the total pool of CD8⁺ T cells with or without addition of IMCgp100 at 82 pM. The A375 cell line (HLA-A2+ve, gp100–ve) was used as a negative control. The number of apoptotic cells/area (in mm²) was determined every 2 hr by the InCuCyte imaging system. (b) The killing curves produced for the individual CD8⁺ T cell subsets from seven donors using non-linear logistic growth mixed effects model. (c) Fitted values and standard errors for the model fitted to each cell population. Asym representing Asymptote (the maximum level of the curve), Xmid (the time taken to reach half the level of the Asymptote) and Scale (controls the angle of the curve) (d) Representative images obtained from Donor 8 at the end point (44 hr) of the IcuCyte assay. Red crosses and blue dots depict apoptotic cancer cells and apoptotic T cells, respectively.

Temra subpopulation as demonstrated by the lowest Xmid value (the time required to reach half the maximum level of the curve). Tem cells also killed melanoma cells rapidly, with Xmid 2 hr higher than that of Temra. The Tcm subset was slower to kill. Statistical comparisons were made between the Temra subpopulation and the Tcm, and also between the Tem and the Tcm. The differences in both of those cases were statistically significant with $P < 0.001$ (model comparison analysis of variance). The naive cells displayed markedly lower killing levels when compared with all the other subpopulations ($P < 0.001$). Notably, naive cells' ability to induce a meaningful level of killing above the control level was only demonstrated by Donor 7 cells (see Supplementary material, Fig. S1). It is possible that the naive cells, having been subjected to experimental conditions for a substantial amount of time, became activated and lost their naive properties. The differences in the cytotoxic ability between the T cell subpopulations were also observed by visualization of the wells at the end of the killing time-course assay as presented for Donor 8 (Fig. 1d).

Importantly, incubation of Mel526 cells with the total pool of CD8⁺ T cells from the same donor but in the absence of IMCgp100 did not induce any apoptosis, and the total pool of CD8⁺ lymphocytes was not activated to kill gp100-negative A375 cells in the presence of the ImmTAC, as expected. A few other donors showed elevated background level of killing in the controls (see Supplementary material, Fig. S1) which probably stems from the presence of melanoma-reactive T cells in the donors' blood.

IMCgp100 triggers cytolysis of melanoma cells by CD4⁺ T cell subpopulations

To determine whether IMCgp100 is able to trigger a T helper response and directly induce melanoma cell death, the same experiments as described earlier were repeated with CD4⁺ T cells.

CD4⁺ T cells were FACS sorted into naive, Tcm and Tem subpopulations using antibodies specific for CD45RA and CCR7 markers (the Temra T cell

population is absent from the CD4⁺ repertoire, as previously reported²³ and employed in the killing assay (Fig. 2a). The kinetics of killing for the total pool of CD4⁺ T cells as well as for the naive and Tcm subpopulations did not reach a plateau during the time-course of the assay (48–80 hr; see Supplementary material, Fig. S2). The data were analysed as described for CD8⁺ T cell subpopulations and all eight donors fulfilled the data inclusion criteria. Although CD4⁺ T cells are not typically considered cytotoxic, they were redirected by IMCgp100 and induced apoptosis of Mel526 melanoma cells (Fig. 2b). The speed of the total CD4⁺ T cell-mediated melanoma cell killing was lower than that of total CD8⁺ T cells (Xmid of 42 hr versus 32 hr) (Fig. 2c). Out of all CD4⁺ subpopulations, Tem cells killed the fastest, followed by Tcm cells. The differences in killing ability between Tem and Tcm cells were statistically significant ($P < 0.001$, model comparison analysis of variance).

Naive CD4⁺ T cells were unable to induce any cell death of the melanoma line in the time-frame of the experiment and therefore a linear mixed effects model had to be fitted for this subpopulation (Fig. 2b). Images of the wells acquired at the end of the assay from Donor 16 illustrate the ability of CD4⁺ T lymphocytes to directly induce apoptosis of cancer cells, with the Tem cell subpopulation being the most efficient (Fig. 2d). To investigate the possible mechanism mediating target cell death, we measured the protein level of granzyme B, a lytic enzyme responsible for activating caspase-dependent cell death, in CD8⁺ and CD4⁺ T cell populations following incubation with a target cell line (Mel526) and increasing concentrations of IMCgp100 (see Supplementary material, Fig. S3). FACS analysis revealed that granzyme B levels were altered in both T cell subpopulations at 24 and 96 hr time-points. The relative mean fluorescence intensity (relative MFI), was used to compare the average amount of granzyme B

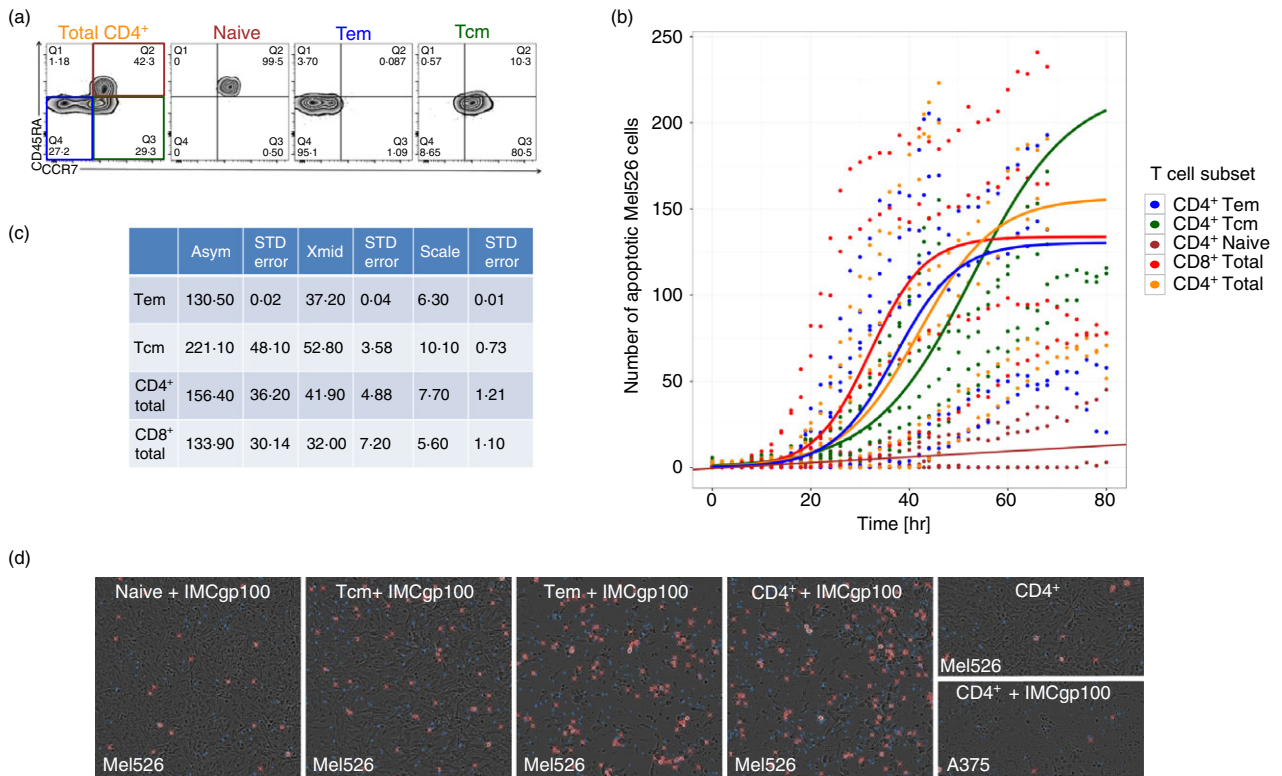


Figure 2. ICMgp100-induced killing capacity of different CD4⁺ T cell subpopulations. (a) Magnetically enriched CD4⁺ T cells (here obtained from Donor 16) were FACS sorted, according to cell surface labelling of CD45RA and CCR7 markers, into four subpopulations, including: naive, effector memory (Tem) and central memory (Tcm). Melanoma target cells (Mel526 cell line: HLA-A2+ve, gp100+ve) were incubated with each of the CD8⁺ T cell subpopulations (naive, Tem and Tcm) as well as the total pool of CD4⁺ T cells with or without addition of IMCgp100 at 82 pM. The A375 cell line (HLA-A2+ve, gp100–ve) was used as a negative control. The number of apoptotic cells/area (in mm²) was determined every 2 hr by the Incucyte imaging system. (b) The killing curves produced for the individual CD4⁺ T cell subsets from eight donors using non-linear logistic growth mixed effects model, except naive subpopulation, which was fixed with a linear mixed effects model due to the lack of killing; the intercept: –0.51, standard error: 1.96; slope: 0.165, standard error: 0.1. (c) Fitted values and standard errors for the model fitted to each cell population. Asym representing asymptote (the maximum level of the curve), Xmid (the time taken to reach half the level of the asymptote) and Scale (controls the angle of the curve). (d) Representative images obtained from Donor 16 at the end point (44 hr) of the IcuCyte assay. Red crosses and blue dots depict apoptotic cancer cells and apoptotic T cells, respectively.

in the labelled cells. Relative MFI doubled or tripled between CD8⁺ T cells untreated and treated with 131 pM IMCgp100 at 24 and 96 hr respectively. In addition, the proportion of cells containing granzyme B increased over twofold following the 96 hr incubation. Similar to CD8⁺ T cells, the content of granzyme B in CD4⁺ T cells increased in an ImmTAC dose-dependent manner. At the highest concentration of IMCgp100, relative MFI increased twofold and fourfold at 24 and 96 hr, respectively. In contrast to CD8⁺ cells, numbers of CD4⁺ cells producing granzyme B increased marginally throughout the experimental conditions.

IMCgp100 mediates CD8⁺ T cell killing despite the presence of Treg cells

Tumour microenvironments can contain a population of Treg cells that harbour the ability to suppress effector T cells and limit the efficacy of antigen-specific anti-tumour responses. To determine whether Treg cells exert a suppressive effect on the IMCgp100-driven cytotoxic T cell response, CD4⁺ CD25⁺ CD127dim⁻, FoxP3⁺ cells were tested with CD8⁺ T cells in a killing assay (Fig. 3). Autologous CD8⁺ T cells and Treg cells were incubated at a 1 : 1 ratio in the presence of Mel526 cells and IMCgp100 at 100 pM and the rate of killing was monitored by detecting caspase 3/7 activation over a 50-hr time-course. The kinetics and extent of melanoma cell killing carried out by CD8⁺ T cells from four donors were equal whether or not Treg cells were present in the assay (Fig. 3a, b). This demonstrated that Treg cells mixed with effector T cells in equal proportion did not suppress the ImmTAC-mediated killing properties of the latter.

IFN- γ as a measure of redirected CD8⁺ and CD4⁺ T cell subpopulations against melanoma cells

To further investigate the activation status of CD8⁺ and CD4⁺ T cells, individual subpopulations were co-cultured with Mel526 cells in the presence of decreasing concentrations of IMCgp100 (131, 82, 31, 12 and 1 pM). The highest number of IFN- γ -secreting cells was found in the Temra subpopulation (Fig. 4a). The maximum number of responding cells was seen for 31 pM of IMCgp100, implying that these cells are readily activated and require a very low concentration of ImmTAC to switch on the production and secretion of this cytokine. Tem cells also demonstrated a high degree of activation, with only slightly lower number of cells releasing IFN- γ at the maximum dose of 131 pM of ImmTAC, when compared with the Temra subpopulation. However, the number of IFN- γ -secreting Tem cells declined dramatically below 31 pM of ImmTAC concentration, in line with their weaker cytotoxic properties. As expected from the killing assay, Tcm lymphocytes were less responsive in producing IFN- γ than Temra or Tem, whereas few naive cells produced detectable levels of this cytokine. With the exception of Temra, which demonstrated complete activation at 31 pM IMCgp100, all of the cells released IFN- γ in an ImmTAC dose-dependent manner (Fig. 4a).

In contrast to CD8⁺ T cells, within the CD4⁺ repertoire mainly the CD4⁺ Tem subpopulation secreted IFN- γ following IMCgp100 stimulation with only a few CD4⁺ Tcm cells secreting this cytokine and only at the highest concentrations (Fig. 4b). The number of IFN- γ -secreting cells for the CD4⁺ Tem cell subpopulation at the highest ImmTAC concentration was lower than for CD8⁺ Tem cells and comparable to that of CD8⁺ Tcm cells. None of the naive cells produced any detectable IFN- γ .

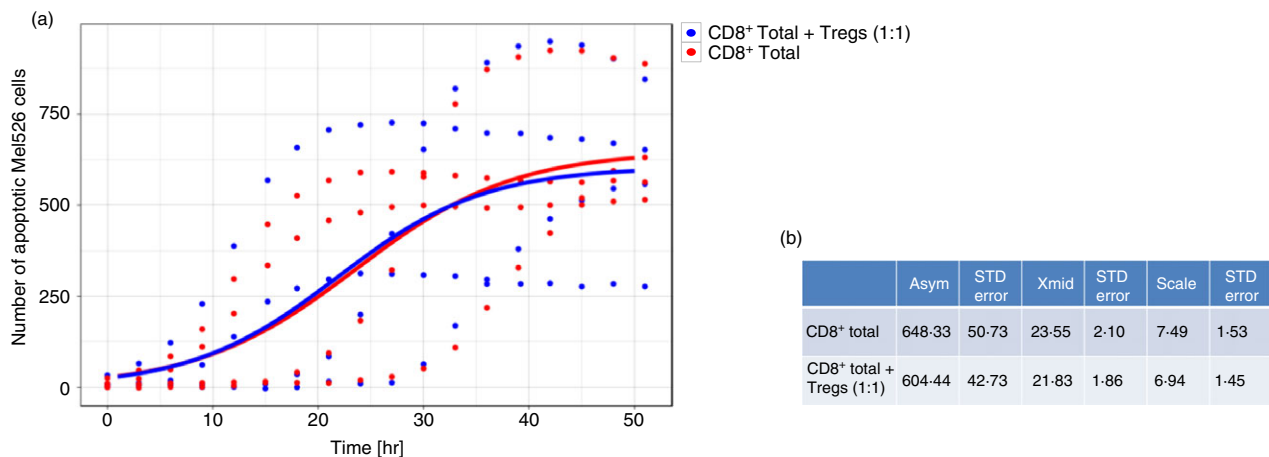


Figure 3. Cytotoxic T cell killing is not impacted by regulatory T (Treg) cells. (a) The killing curves for total CD8⁺ T cell population and a mixture of CD8⁺ T cells and Treg cells at 1 : 1 ratio obtained from four donors were generated using a non-linear mixed effects model. (b) Fitted values and standard errors for the model are presented in the table. Asym representing Asymptote (the maximum level of the curve), Xmid (the time taken to reach half the level of the Asymptote) and Scale (controls the angle of the curve).

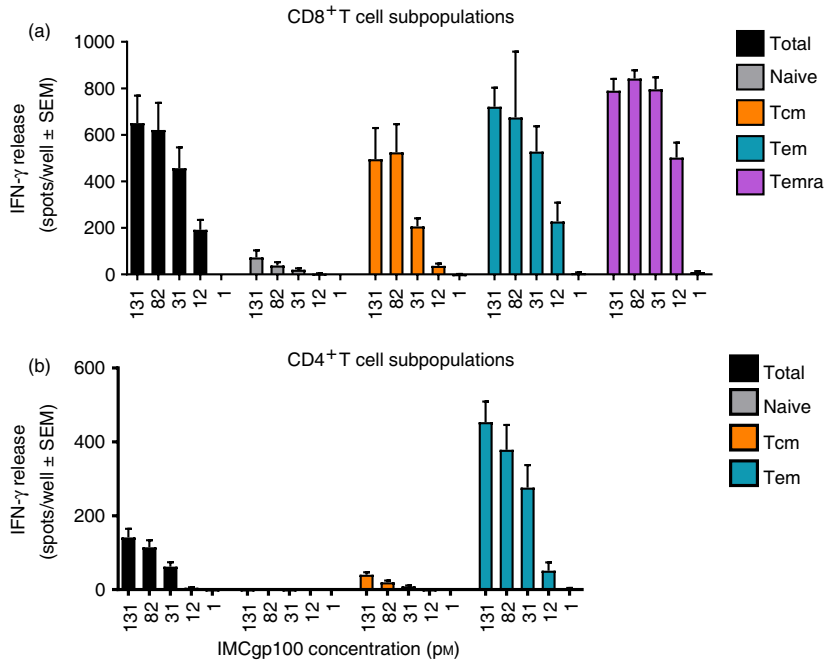


Figure 4. Activation status of the individual CD8⁺ and CD4⁺ T cell subsets upon IMCgp100 redirection. (a) Mel526 cells were incubated with naive, effector memory (Tem), CD45RA effector memory (Temra) and central memory (Tcm). CD8⁺ T cells along with IMCgp100 at decreasing concentrations (131, 82, 31, 12 and 1 pM). Interferon- γ (IFN- γ) release was measured using ELISpot and represented as a number of spots. Means obtained from four donors are presented. (b) The same assay was carried out using the individual CD4⁺ T cell subpopulations (naive, Tcm and Tem). SEM; standard error of the mean.

These findings support and complement the killing assay results obtained for both CD8⁺ and CD4⁺ T cell subsets. Indeed, the cells with the greatest capacity to induce melanoma cell death also possess the greatest ability to produce IFN- γ , serving as a marker of immunological activity.

IMCgp100 induces broad cytokine and chemokine release

A robust and durable immune response is driven and maintained by cytokines and chemokines. To assess the ability of IMCgp100 to induce a variety of mechanisms beyond T cell cytolytic activity and IFN- γ release, we evaluated supernatant obtained from IMCgp100-supplemented cultures of Mel526 and different subpopulations of CD4⁺ or CD8⁺ T cells. The concentrations of a range of cytokines and chemokines were measured following 44 hr (CD8⁺ T cells) or 48 hr (CD4⁺ T cells) of incubation.

These data are presented in Fig. 5(a). All T cell subsets secreted comparable levels of the chemokines tested, with the exception of MIP-1 α/β of which naive T cells produced around tenfold less than Temra, Tem and Tcm cells. More variation was observed in the cytokine production levels. Similar amounts of TNF- α and IFN- γ were secreted by Temra, Tem and Tcm subsets, whereas all of the subpopulations secreted comparable levels of IL-2 and IL-6. Both IL-15 and IL-10 were detected at very low levels in the supernatant from the CD8⁺ T cell populations. Overall, the Tcm subpopulation produced and

secreted the highest amounts of the pro-inflammatory molecules. Naive T cells were able to neither contribute to the killing of melanoma cells (Fig. 1b) nor initiate IFN- γ -driven responses but they did secrete a range of chemokines following IMCgp100 treatment. CD4⁺ Tem and Tcm cells produced approximately equal levels of the chemokines RANTES, MIG, IP-10 and MCP whereas MIP-1 α/β was only secreted by Tem cells. Of all the cytokines assessed, IL-6 was the most abundantly produced by both Tem and Tcm, closely followed by IL-2 and TNF- α . Very low levels of IFN- γ , IL-15, IL-10 or IL-4 were detected for all of the CD4⁺ T cell subsets. CD4⁺ naive T cells produced the lowest amounts of the cytokines and chemokines tested.

None of the CD8⁺ or CD4⁺ T cell populations secreted substantial levels of IFN- γ in this assay, though IFN- γ was readily detected using the ELISpot assay (Fig. 4a, b). This probably reflects the different sensitivities and timescales of the two experiments: ELISpot measured IFN- γ released by individual cells following 24 hr incubation with ImmTAC, whereas the Luminex assay measured IFN- γ secretion into the supernatant by the cell population at 44–48 hr post-stimulation. Interferon- γ is expressed early, degrades quickly and can be sequestered from the cellular growth medium by binding to its ligand on the melanoma cells.

These experiments demonstrate that both CD8⁺ and CD4⁺ T cells produce a range of cytokines and chemokines as a consequence of IMCgp100 stimulation with different T cell subsets varying in type and level of lymphokines released.

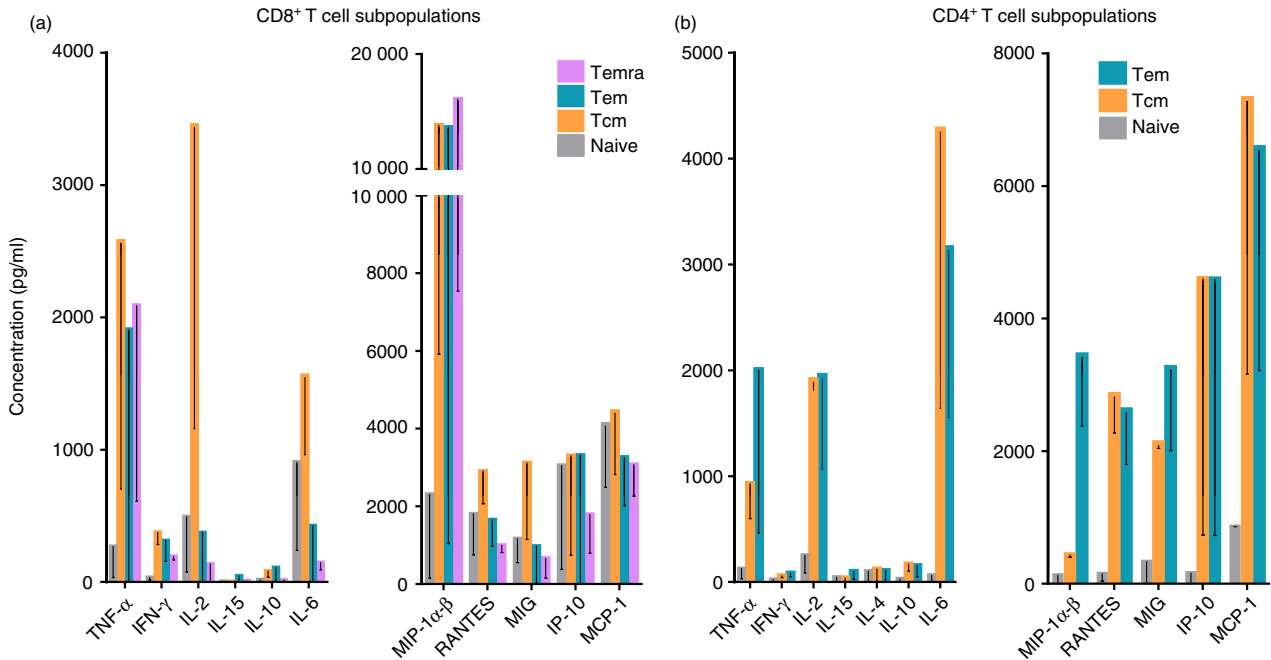


Figure 5. IMCgp100-induced cytokine and chemokine secretion by CD8⁺ (a) and CD4⁺ (b) T cell subsets. Mel526 cells were incubated with specific T cell subpopulations and supplemented with IMCgp100 at 82 pM for 44 hr (CD8⁺ culture) or 48 hr (CD4⁺ culture). Selected cytokines [tumour necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin-2 (IL-2), IL-15, IL-10, IL-6, IL-4] and chemokines [macrophage inflammatory protein-1 α/β (MIP-1 α/β), Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES), monocyte induced by IFN- γ (MIG), IFN- γ -inducible protein-10 (IP-10), monocyte chemoattractant protein 1 (MCP-1)] were measured in the culture medium using Luminex assay. Interleukin-4 was only measured in the culture medium from CD4⁺ cells as CD8⁺ T cells do not produce it. Cytokines and chemokines were measured in duplicates from three (a) or two (b) donors.

IMCgp100 drives a strong polyfunctional response of individual CD8⁺ and CD4⁺ cells

To mount a strong and efficient response against cancer or viral infection a T cell must initiate a range of effector functions including proliferation, mobilization of adaptive and innate immune responses (by secreting cytokines and chemokines) and direct killing of tumour or virally infected cells through a cytolytic mechanism (release of lytic granules). Preliminary investigation using total pool of CD8⁺ and CD4⁺ lymphocytes has shown some evidence that ImmTAC biologics are capable of inducing such polyfunctional responses against cancer cells.⁷ To assess this capability further, at a single-cell level, PBMC from healthy donors were incubated with Mel526 cells and supplemented with a range of concentrations of IMCgp100 (31, 82 and 131 pM) or with a positive control (Mel526 cells pulsed with the gp100 peptide) supplemented with 82 pM IMCgp100. The CD8⁺ and CD4⁺ T cell subpopulations were individually tested for the ability to produce pro-inflammatory cytokines TNF- α and IFN- γ , IL-2 (a promoter of proliferation), MIP1 β (a key chemokine), and to up-regulate CD107a on the cell surface (a marker of lytic granules degranulation).

Incubation with IMCgp100 induced polyfunctionality in all of the CD8⁺ T cell subsets but marked differences in the proportion of polyfunctional cells were observed between the groups (Fig. 6). Naive T cells were the weakest responders regardless of the ImmTAC concentration. Only around 5% of naive T cells demonstrated two or more effector functions and this proportion was maintained even in the peptide-pulsed positive control. The Tem and Tcm subpopulations both responded in an IMCgp100 dose-dependent manner but the degree of polyfunctionality among Tcm cells was dramatically lower than among the Tem counterparts. Thirteen per cent of Tem cells demonstrated four effector functions in comparison with < 5% of the Tcm subpopulation and these differences were even greater in the positive control: 28 and 5%, respectively. Temra reached a maximum response at 31 pM of IMCgp100 with over 40% of cells demonstrating at least one effector function, but higher concentrations of ImmTAC or incubating with peptide-pulsed melanoma cells had no further effect. Temra cells subjected to the different treatments showed between 31 and 37% of cells exhibiting two or more effector functions. Overall, the Temra and Tem subsets were the strongest responders to IMCgp100 under all treatment

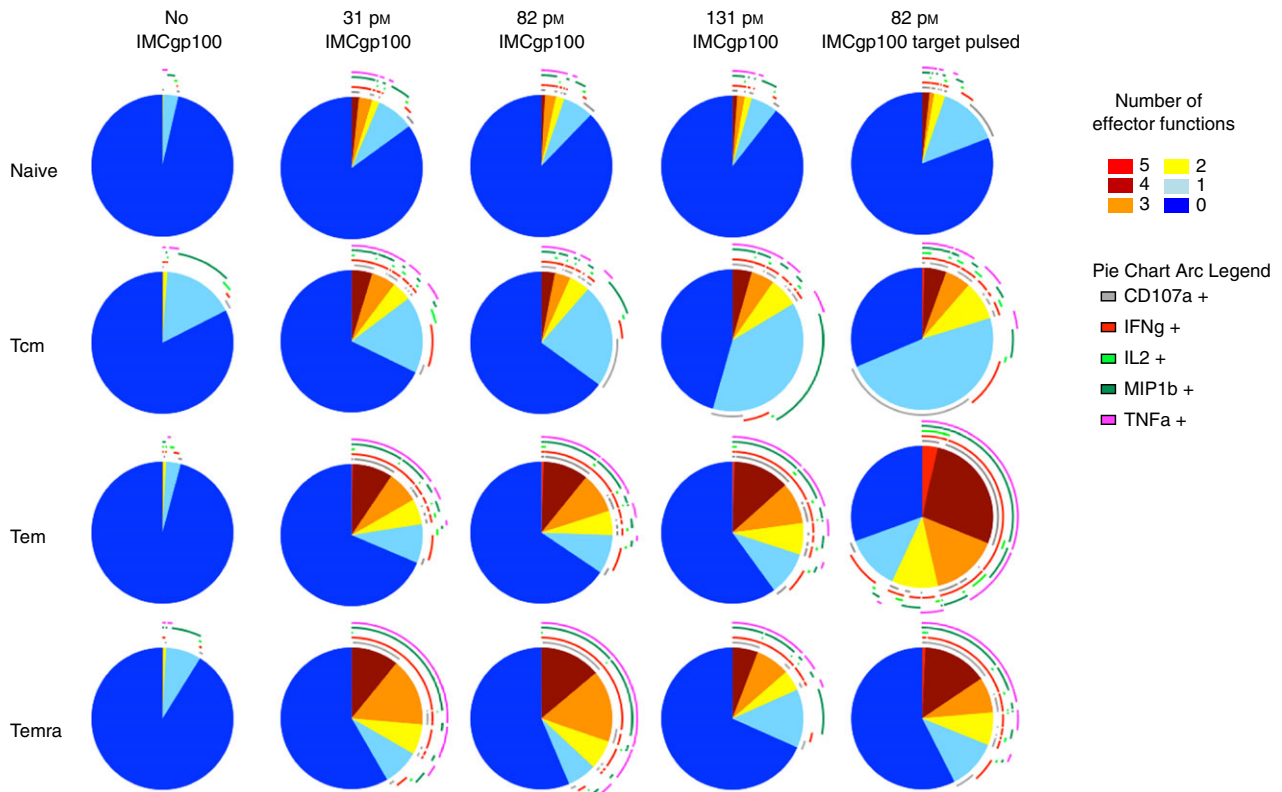


Figure 6. Polyfunctionality of different CD8⁺ T cell subpopulations induced by IMCgp100 stimulation. Peripheral blood mononuclear cells (PBMC) were incubated with the Mel526 cell line in the presence of increasing concentrations of IMCgp100 (31, 82 and 131 pM) for 16 hr. PBMC incubated with melanoma cells pulsed with gp100 peptide at 1 μ M served as a positive control, whereas PBMC incubated with Mel526 cells in the absence of IMCgp100 served as a negative control. T cell subpopulations were identified using CCR7 and CD45RA markers and the amounts of selected proteins [CD107, interferon- γ (IFN- γ), interleukin-2 (IL-2), macrophage inflammatory protein 1 β (MIP1 β) and tumour necrosis factor- α (TNF- α)] were measured in each subpopulation using FACS. The pie charts show the proportion of cells expressing between one and five of the markers tested (mean values from three donors). Blue, light blue, yellow, orange, brown and red slices refer to a proportion of cells expressing none, one, two, three, four or all five of the tested markers, respectively. The pie chart arcs indicate the specific proteins being produced.

conditions and this was manifested in the highest proportion of polyfunctional T cells displaying three, four or five effector functions. The majority of the polyfunctional cells produced TNF- α and IFN- γ , followed by MIP-1 β and CD107 α . Production of IL-2 was the least frequent in the time-frame of the experiment.

CD4⁺ T cells had markedly lower capacity to display a polyfunctional phenotype (Fig. 7). Similar to CD8⁺ T cells, the naive CD4⁺ subpopulation responded poorly to IMCgp100 stimulation, reaching a maximum of 2% of cells with two or more functions. The Tcm subpopulation acquired a higher proportion of polyfunctional cells but no dose-dependent increase was noted. The highest proportion of Tcm cells exhibiting two or more effector functions of around 3% was reported for the 131 pM treatment. The most potent IMCgp100 dose-dependent polyfunctional response in the CD4⁺ T cell group was demonstrated by the Tem subpopulation. The proportion of cells displaying two or more effector functions

reached 7% at 131 pM ImmTAC treatment. This proportion was further elevated to 30% when the antigen presentation was artificially increased upon peptide pulsing. The polyfunctional CD4⁺ T cells produced TNF- α , IFN- γ and MIP-1 β most frequently, whereas IL-2 and CD107 α were less abundant. In summary, both CD8⁺ and CD4⁺ repertoires demonstrated a capacity for a polyfunctional response to IMCgp100 but the proportion of polyfunctional cells differed between individual subpopulations.

Discussion

Exploiting the immune system to eradicate cancer has been a key focus of many new therapies over recent years; however, limited efficacy has been observed. A fundamental step for the elimination of tumour is recognition of cancer cells by specific T lymphocytes that will lead to cell death, release of antigens, cross-

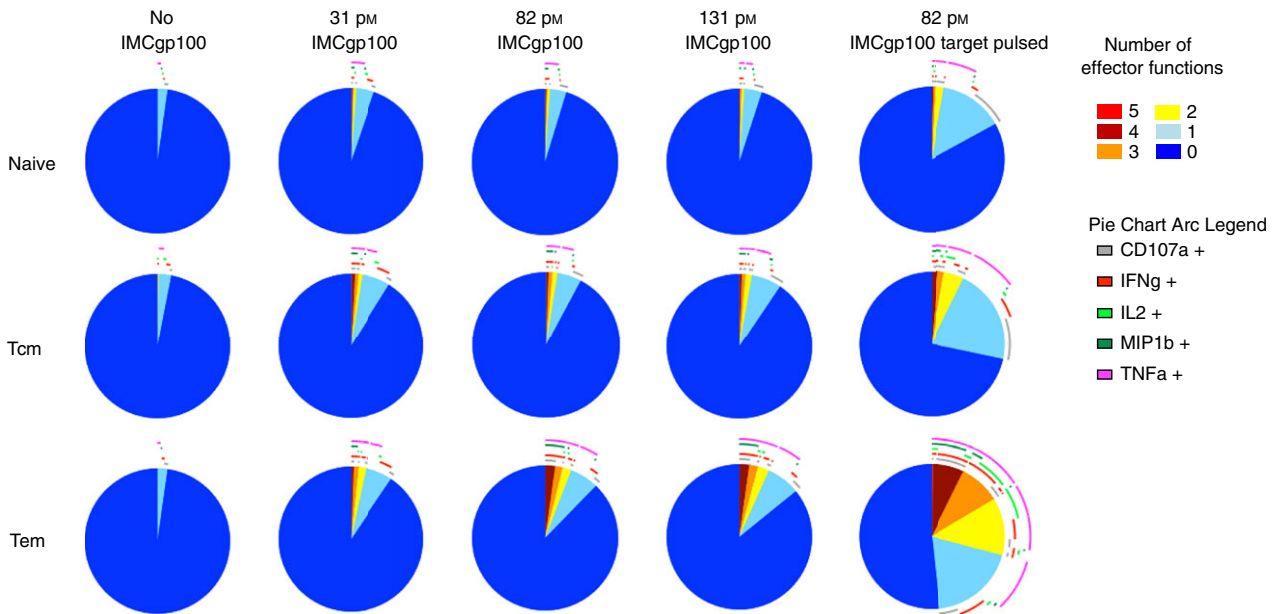


Figure 7. Polyfunctionality of different CD4⁺ T cell subpopulations induced by IMCgp100 treatment. Peripheral blood mononuclear cells (PBMC) were incubated with the Mel526 cell line in the presence of increasing concentrations of IMCgp100 (31, 82 and 131 pM) for 16 hr. PBMC incubated with melanoma cells pulsed with gp100 peptide at 1 μM served as a positive control, whereas PBMC incubated with Mel526 cells in the absence of IMCgp100 served as a negative control. T cell subpopulations were identified using CCR7 and CD45RA markers and the amounts of selected proteins (CD107, interferon-γ (IFN-γ), interleukin-2 (IL-2), macrophage inflammatory protein 1β (MIP1β) and tumour necrosis factor-α (TNF-α)) were measured in each subpopulation using FACS. The pie charts show the proportion of cells expressing between one and five of the markers tested (mean values from three donors). Blue, light blue, yellow, orange, brown and red slices refer to a proportion of cells expressing none, one, two, three, four or all five of the tested markers respectively. The pie chart arcs indicate the specific proteins being produced.

presentation of these antigens to a wider range of T cells and generation of a multi-targeted immune response. Engagement of a broad spectrum of immune cells, including members of the innate immune system, maximizes the likelihood of a durable anti-cancer environment.¹

In previous studies, we have shown that ImmTAC molecules successfully redirect cytotoxic CD8⁺ T cells, which execute apoptotic cancer cell death.⁷ In this study we characterize, which specific subpopulations of CD8⁺ T cells are involved in this process. Temra cells demonstrate the highest capacity to kill, rapidly followed by Tem, whereas Tcm and naive subpopulations are inefficient at inducing cell death. This is not unexpected given that the primary role of both terminally differentiated and effector memory cytotoxic T cells is to facilitate direct target cell destruction or rapid generation of effector killer cells, respectively. Both subpopulations contain abundant lytic granules harbouring granzymes and perforins, but Temra T cells have been reported to harbour the highest amounts.²⁴ In this study, we show that granzyme B is present in CD8⁺ T cells with levels rising with increasing concentration of ImmTAC.

Out of the CD4⁺ T lymphocytes activated by IMCgp100 Tem cells are potent killers of melanoma cells,

albeit with a lower efficiency compared with effector CD8⁺ T cells. CD4⁺ T lymphocytes are not typically recognized for their cytotoxic functions but there are reports demonstrating that a subset adopts a cytotoxic phenotype, such as in chronic infection.²⁵ Consistent with these reports, we demonstrate that the killing of target cells is at least in part dependent on granzyme B release since it is detected in CD4⁺ T cells following ImmTAC stimulation. The ability of IMCgp100 to activate CD4⁺ T cells is extremely important as these cells have been demonstrated as having a vital role in maintaining a persistent anti-cancer response in patients.²⁶ A combined transfer of CD8⁺ and CD4⁺ T lymphocytes results in a higher efficacy in eradicating cancer than the transfer of CD8⁺ T cells alone.¹⁷ Melanoma-specific naive CD4⁺ T cells transferred to a lymphopenic host have been shown to differentiate into Th1 phenotype, release IFN-γ, granzyme and perforin, and lead to regression of an established tumour.²⁷

Apart from cytotoxicity, we show that IMCgp100 redirected lymphocytes secrete a number of key cytokines and chemokines, of which CD8⁺ and CD4⁺ Tcm cells produce the highest amounts. Evidence of the engagement of this subpopulation of T cells is critical as Tcm cells have been shown to be indispensable in developing a prolonged

anti-tumour defence in a number of studies of adoptive immunotherapy.¹⁶ Both memory and effector T cells secrete high levels of TNF- α , IL-2, IL-6 and IFN- γ . Both TNF- α and IFN- γ are strong pro-inflammatory agents, which promote apoptosis of cancer cells, induce maturation of dendritic cells and induce inflammation of endothelial cells, resulting in enhanced adhesion and extravasation of immune cells attracted to the tumour environment.^{28–31} T cell subpopulations secrete very low levels of IL-10, a potent terminator of inflammatory responses, implying that IMCgp100 triggers a pro-inflammatory trajectory with some anti-inflammatory properties that may serve to ensure appropriate moderation of the response. Interferon- γ also induces secretion of chemokines and RANTES, MIG (CXCL9), IP-10 (CXCL10) and MCP-1 (CCL2) are abundantly produced by both CD4⁺ and CD8⁺ effector and memory T cells. As some of these chemokines are reported to be associated with trafficking of T cells into tumours and other anti-tumour effects these *in vitro* data demonstrate further mechanisms induced by IMCgp100 that may benefit patients treated with the drug.³² It has also been reported that IFN- γ and MIG were significantly higher in melanoma patients who had a response to anti-programmed death-ligand 1 (PDL-1) treatment versus those with progressive disease.³³ MIP-1 is produced in large quantities by cytotoxic T cells and only modestly by CD4⁺ Tem lymphocytes, in line with its role in inducing tumoricidal activity and promoting the T helper type 1 phenotype.³⁴ Although naive T cells from both CD8⁺ and CD4⁺ pool do not secrete cytokines tested upon IMCgp100 redirection, CD8⁺ naive T cells contribute to chemokine production suggesting that they play a role in attracting other immune cells to the tumour site.

Further to cytokine and chemokine release by the specific subsets of T cells, we demonstrate that single isolated cells adopt a polyfunctional phenotype following IMCgp100 redirection. These individual T cells produce between two and five different effector functions, which is important as multiple effector functions are deployed by the most effective T cell responses, as demonstrated in infection and cancer.³⁵ Polyfunctional T cells have also been shown to be imperative for a vaccination to provide persistent immunity against a pathogen.³⁶ This study shows that all T cell subpopulations develop a proportion of cells with a polyfunctional phenotype as a consequence of IMCgp100 treatment. The CD8⁺ pool responds much more strongly to the ImmTAC stimulation than the CD4⁺ pool, which manifests with a higher percentage of T cells producing multiple effector molecules. The level of polyfunctionality achieved by the T cells is not only correlated with the concentration of IMCgp100 but also with the abundance of antigen presented on the target cell surface, as demonstrated using peptide-pulsed melanoma cells. The majority of T cell

subpopulations respond to ImmTAC in a dose-dependent manner but the CD8⁺ Temra subpopulation is an exception. Temra T cells appear to reach a maximum capacity for inducing polyfunctionality at a lower IMCgp100 concentration, in agreement with their activation status measured by IFN- γ release. This has important implications for treatment of patients and confirms that clinically relevant doses are sufficient to induce a strong polyfunctional response in redirected T cells. Additionally, the cytokines and chemokines released by IMCgp100 redirected T cells are strong attractants of a multitude of other immune cells. The density of tumour-infiltrating lymphocytes has been shown to be predictive of patient survival for a number of different tumours.^{37–40}

An additional mechanism that is considered important for the induction of a self-sustaining, durable anti-tumour response is epitope spreading. The capacity of dendritic cells to present additional epitopes *in vitro*, other than the target cancer peptide, has already been described for ImmTAC molecules.⁴¹ Epitope spreading plays an essential role in successful therapy as demonstrated in numerous studies on vaccines.⁴² Improved clinical outcomes for patients who showed a broadened immune response to epitopes not incorporated in the vaccine have been reported.⁴³ ImmTAC-induced antigen spreading may be enhanced in the cancer environment due to the presence of tertiary lymphoid structures which, along with secondary lymphoid organs, contain a pool of naive and memory T cells. Tertiary lymphoid structures have been found in many cancers,⁴⁴ their presence in the tumour niche correlates significantly with a favourable patient outcome and serves as a predictive factor of a successful immunotherapy.⁴⁵ It is likely that dendritic cells loaded with cancer antigens will present them to cancer-specific naive T cells, residing in tertiary lymphoid structures, with the possibility of the priming event being boosted by the presence of the ImmTAC; these naive T cells may then overcome the threshold of activation and clonally expand to augment the ImmTAC-mediated polyclonal T cell response.

Mobilization of effector and memory T cell subsets is an important aspect of a successful immunotherapy.⁴ ImmTAC-mediated therapy provides a benefit of such mobilization and other therapies that promote the generation of effector T cells or prevent T cell anergy could be complementary. Indeed, substantial PD-L1 protein levels and FoxP3⁺ cells have been detected in tumour biopsies from patients. In the majority of cancers, Treg cells demonstrate a marked negative effect on patient prognosis. High FoxP3⁺ Treg infiltration is strongly associated with a shorter overall survival in patients with melanoma whereas in other indications the impact of Treg cells is less clear.⁴⁶ In this study,

we demonstrate in an *in vitro* killing assay, that Treg cells used at an equal proportion with cytotoxic T cells fail to affect IMCgp100-mediated target cell apoptosis.

This study has demonstrated that IMCgp100 redirects and activates both CD8⁺ and CD4⁺ T cell subsets; these T cells can kill cancers, release a broad array of immune effectors and display a polyfunctional phenotype. Treg cells, which can be present in the tumour microenvironment, appear ineffective to IMCgp100 driven cancer cell apoptosis in the conditions used *in vitro*. Collectively, these data illustrate the breadth and polyfunctionality of immune responses induced by IMCgp100, providing further support for its therapeutic potential and continued clinical development.

Disclosures

All authors are employees of Immunocore Ltd. IMCgp100 biologic was produced by Immunocore Ltd and the study was entirely funded by this organization.

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Supporting Information

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

Figure S1. IncuCyte killing curves generated for CD8⁺ T cell subpopulations from eight healthy donors.

Figure S2. IncuCyte killing curves generated for CD4⁺ T cell subpopulations from eight healthy donors.

Figure S3. IMCgp100 induces increase of protein level of granzyme B in T cells.