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The cytotoxic molecule granulysin is capable of inducing either chemotaxis or fugetaxis in dendritic cells depending on maturation: a role for Vδ2$^+$ γδ T cells in the modulation of immune response to tumour?

Running title: Differential DC migration in response to granulysin

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Abbreviations

BCG: Bacillus Calmette Guérin

DC: Dendritic Cell

FSC: Forward Scatter

HLA-DR: Human Leukocyte Antigen–D Related

HMBPP: (E)-4-Hydroxy-3-Methyl-But-2-enyl Pyrophosphate

IPP: Isopentenyl Pyrophosphate

MACS: Magnetic Activated Cell Sorting

MEP: 2-C-Methyl-D-Erythritol-4-Phosphate

pAg: Phosphoantigen

PBMC: Peripheral Blood Mononuclear Cells

PMA/I: Phorbol Myristate Acetate and Ionomycin

SDF-1: Stromal Cell Derived Factor 1

SSC: Side Scatter

TCR: T Cell Receptor

ZA: Zoledronic Acid

Abstract

Release of granulysin by γδ T cells contributes to tumour cell killing. A cytolytic 9kDa isoform of granulysin kills tumour cells directly, while a 15kDa precursor has been hypothesised to cause both the maturation and migration of dendritic cell (DC) populations. Recruiting DC to a tumour is beneficial as these cells initiate adaptive immune responses, which contribute to the eradication of malignancies. In this study, Vδ2$^+$ γδ T cells were activated by stimulation of peripheral blood mononuclear cells (PBMC) with zoledronic acid (ZA) or *Bacillus Calmette Guérin* (BCG), or were isolated and cultured with tumour targets. While a large proportion of resting Vδ2$^+$ γδ T cells expressed 15kDa granulysin, 9kDa granulysin expression was induced only after stimulation with BCG. Increased levels of activation and granulysin secretion were also observed when Vδ2$^+$ γδ T cells were cultured with the human B cell lymphoma line Daudi. High concentrations of recombinant 15kDa granulysin caused migration and maturation of immature DC, and also initiated fugetaxis in mature DC. Conversely, low concentrations of recombinant 15kDa granulysin resulted in migration of mature DC, but not immature DC. Our data therefore support the hypothesis that Vδ2$^+$ γδ T cells can release granulysin, which may modulate recruitment of DC, initiating adaptive immune responses.

Introduction

A small subset of T cells possess a TCR composed of γ and δ chains rather than α and β, and these γδ T cells account for up to 5% of the T cells found within human peripheral blood(1). While the proportion of γδ T cells in the T cell population as a whole is low, this subset does not require processing and presentation of antigen to become activated, allowing a rapid response to infected or malignant target cells.

Previous research has shown evidence that γδ T cells bearing a Vδ2 chain, comprising approximately 80% of the γδ T cell population found in the peripheral blood of humans(2), are capable of recognising phosphoantigens (pAg) such as prenyl pyrophosphates. These are intermediates of isoprenoid synthesis pathways, present within both bacteria and eukaryotes. Within bacteria, the pAg (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) is produced in the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway, and its eukaryotic homologue isopentenyl pyrophosphate (IPP) is produced in the mevalonate pathway(3). Research has shown that $V\delta2^+$ γδ T cells are activated by cells that accumulate HMBPP and/or IPP(4). Although the exact mechanism by which these cells recognise pAg remains to be fully elucidated, the current hypothesis suggests that intracellular binding of pAg to the molecule butyrophilin 3A1 is involved(5–7). HMBPP has been found to be substantially more stimulatory than IPP to $V\delta2^+$ γδ T cells, allowing these cells to easily differentiate foreign bacteria from self cells(8). While the level of IPP within healthy eukaryotic cells is not usually sufficient to cause activation of $V\delta2^+$ γδ T cells, this molecule is overexpressed in some tumours in which the mevalonate pathway is dysregulated(9). Additionally, nitrogen-containing bisphosphonate drugs such as ZA can artificially elevate the level of IPP within cells, due to their inhibition of enzymes involved in the mevalonate pathway, resulting in an accumulation of IPP within the cell(10).

Granulysin is a cytotoxic effector molecule, used by several immune cell populations to kill pathogens, in addition to infected or transformed cells. γδ T cell expression of this molecule has been shown to be pivotal in the immune response to both *Mycobacterium tuberculosis* and *Plasmodium falciparum,* as well as several types of tumour(11–13). While the 9kDa isoform of granulysin has been shown to be directly cytotoxic, co-localising with other cytotoxic molecules such as granzymes(14,15), evidence suggests that the 15kDa full-length isoform, initially thought of as an inert precursor, could also have distinct immune functions. 15kDa granulysin localises to lysosome-related effector vesicles(15), and has recently been shown to cause the maturation of immature DC populations, and the migration of both immature and mature DC, in addition to monocytes, memory αβ T cells and NK cells(16–19).

In this paper, we show that Vδ2^{+} γδ T cells are capable of secreting granulysin in response to tumour. In addition, we show that recombinant 15kDa granulysin can cause the migration and maturation of DC, and propose that 15kDa granulysin has a dual migratory function; while we found immature DC migrate towards high concentrations of 15kDa granulysin, mature DC migrated only towards low concentrations of this molecule, and in fact migrated away from higher concentrations of 15kDa granulysin. This suggests the ability of 15kDa granulysin to induce both chemotaxis and fugetaxis of DC, in a concentration-dependent manner and depending on DC maturation status. We therefore propose that the degranulation of Vδ2^{+} γδ T cells in response to tumour can recruit and mature DC, leading to the initiation of an adaptive immune response to tumour antigens.

Materials and Methods

PBMC isolation

Whole blood samples taken from healthy donors were sourced from anonymised leukocyte blood cones supplied by the UK Blood Transfusion Service, London, UK or were collected from consenting healthy volunteers at St. George's, University of London, Tooting, UK. PBMC were isolated from whole blood samples by density adjusted centrifugation using Histopaque 1077 (Sigma-Aldrich, Dorset, UK). Residual red blood cells were removed through addition of Ammonium-Chloride-Potassium lysing buffer (Thermo Fisher Scientific, Massachusetts, USA), and contaminating platelets eliminated by three slow speed centrifugations (200g, 10 minutes), in RPMI 1640 medium (Sigma-Aldrich). Finally, PBMC were resuspended in freezing medium (composed of 45% (v/v) RPMI 1640 medium, 45% (v/v) FBS and 10% (v/v) DMSO (all Sigma-Aldrich)) and initially frozen at -80°C, before being transferred to liquid nitrogen for extended storage.

Cell isolations

$CD14^+$ monocytes and γδ T cells were isolated from PBMC using magnetic activated cell sorting (MACS). In order to isolate γδ T cells, non-γδ T cells (αβ T cells, NK cells, monocytes, B cells, DC, stem cells, granulocytes and erythroid cells) were depleted from PBMC using a $γ/δ^+$ T Cell Isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany). Monocytes were isolated through positive selection of $CD14^+$ cells using CD14 microbeads from Miltenyi Biotec. The purity of each isolated cell population was assessed by flow cytometry, and was >90% for γδ T cell populations and >95% for monocyte populations.

Cell cultures

All cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Sigma-Aldrich), 10,000U/ml penicillin and 10,000µg/ml streptomycin (Thermo Fisher Scientific).

For experiments involving PBMC, $1x10^6$ cells were seeded in 96-well round bottomed tissue culture plates (Corning, New York, USA) in a total volume of 200µl supplemented RPMI 1640 medium. The following reagents were used to stimulate cells as required: 10µg/ml BCG (Danish strain 1331; Statens Serum Institut, Denmark), 5µM ZA, 30ng/ml phorbol myristate acetate (PMA), and 1µg/ml ionomycin (all from Sigma-Aldrich).

For γδ T cell expansion experiments, $5x10^5$ PBMC were seeded in a total volume of 200µl supplemented RPMI 1640 medium in 96-well round bottomed tissue culture plates. 5µM ZA (Sigma-Aldrich) and 15ng/ml (315U/ml) IL-2 (R&D systems, Minnesota, USA) were added to the medium. Cells were then cultured for 9 days prior to isolation of the γδ T cell population by MACS, and fresh supplemented RPMI medium and IL-2 added every 2-3 days.

Daudi and Raji B cell lymphoma lines (European Collection of Authenticated Cell Cultures, Salisbury, UK) were used in experiments as γδ T cell susceptible and resistant target cells, respectively. Tumour cells were cultured in 75 cm^2 tissue culture flasks at a recommended density of $4x10^5$ cells/ml in supplemented RPMI 1640 medium (Sigma-Aldrich), and were passaged every 2-3 days to maintain the recommended cell density.

For co-culture experiments, $5x10^5$ expanded and isolated γδ T cells/ml and $5x10^5$ tumour cells/ml were added to a total volume of 200µl supplemented RPMI 1640 medium in 96-well round bottomed tissue culture plates, at a 1:1 ratio of target to effector cells. Cells were cultured for 24, 48 or 72 hours before being harvested.

DC differentiation and maturation

Isolated peripheral blood $CD14^+$ monocytes were seeded into 6-well flat-bottomed tissue culture plates at a density of $1x10^6$ cells/ml in a total volume of 3ml supplemented RPMI 1640 medium. 100ng/ml (2900U/ml) IL-4 and 50ng/ml (750U/ml) GM-CSF (both R&D systems) were added to the medium, and cells cultured for 7 days. Half of the total volume of medium was replaced every 2-3 days with fresh medium containing 100ng/ml (2900U/ml) IL-4 and 50ng/ml (750U/ml) GM-CSF. Following 7 days of culture, light microscopy and flow cytometry were used to confirm the differentiation of $CD14^+$ monocyte populations into immature DC. To test maturation, immature DC were treated for 24 hours with 100ng/ml recombinant LPS or 66nM recombinant 15kDa granulysin (both R&D systems). The purity of recombinant 15kDa granulysin used was determined by the manufacturer to be >95% by SDS-PAGE, and endotoxin contamination was assessed to be <1.0 EU per 1μg of the protein by the limulus amebocyte lysate method.

Flow cytometry

Cells were washed in flow cytometry buffer (PBS supplemented with 1% (w/v) BSA, 0.1% (w/v) sodium azide and 0.5mM EDTA (all Sigma-Aldrich)), and stained with fluorochrome-conjugated antibodies according to manufacturer's instructions. Fc receptor blocking solution was added to flow cytometry buffer at a ratio of 1:20 prior to staining, to prevent non-specific binding (Biolegend, San Diego, USA). Following staining, cells were washed three times in flow cytometry buffer, before being fixed with 4% (w/v) paraformaldehyde (BD biosciences). For experiments involving intracellular staining, 3.5μM brefeldin A (Sigma-Aldrich) was added for the final three hours of culture to block protein trafficking. Following any required surface staining, cells were simultaneously fixed and permeabilised using 4% (w/v) paraformaldehyde and 0.1% (v/v) saponin (Cytofix/Cytoperm kit, BD biosciences, Oxford, UK) prior to staining with fluorochrome-conjugated antibodies, according to manufacturer's

instructions. The following antibodies were used: Alexa fluor 488-15+9kDa granulysin(RB1), PE-CD56(B159), FITC-CD8(RPA-T8) (all BD biosciences), Alexa fluor 647-9kDa granulysin(DH2), PE-dazzle-CCR5(J418F1), FITC-CCR7(G043H7), FITC-CD107a(H4A3), FITC/APC-CD27(M-T271), PerCP-CD3(OKT3), FITC/APC-CD45RA(HI100), FITC/APC-CD69(FN50), PerCP-Cy5.5-CD80(2D-10), Alexa fluor 647-human leukocyte antigen-D related(HLA-DR)(L243) (all Biolegend), PE-CD14(TÜK4), APC-Granzyme B(REA226), PE-Vδ1(REA173), PE-Vδ2(REA771), PE-γδTCR(11F2) (all Miltenyi Biotec). For all experiments, matched isotype controls were used to determine levels of non-specific binding.

To measure degranulation, anti-CD107a antibodies and 1μM monensin (Sigma-Aldrich) were added for the final four hours of culture, prior to harvesting cells for staining. To measure tumour cell death, a live/dead discrimination dye (Thermo Fisher Scientific) was used, allowing quantification of dead Raji or Daudi cells by flow cytometry. The dye was diluted 100-fold in FACS buffer containing cells to be stained. The cells were then incubated for 30 minutes at room temperature before being stained with a FITC-conjugated antibody specific for CD19, allowing identification of tumour cells. Fluorescent peaks representing live and dead cells were established prior to commencement of experiments using viable and heat-killed tumour cells, respectively, and tumour cell death calculated as the percentage fluorescence observed within each condition, which was above that previously established to represent live cells.

Stained cells were run on an LSRII flow cytometer (BD biosciences), and data analysed using FACSdiva (BD Biosciences) or FlowJo (FlowJo LLC, Oregon, USA) software.

ELISA

The concentrations of granulysin, granzyme B and IFNγ that were present within co-culture supernatants was determined by ELISA (R&D systems). The commercial granulysin ELISA could not distinguish between the 15 and 9 kDa isoforms of granulysin, and as such, data reflects the concentration of total granulysin only. Briefly, plates were coated with a mouse anti-human antibody specific for each protein of interest. A two-fold, seven-point serial dilution of each protein was performed in order to generate a standard curve, and samples of unknown concentration added to the plate. Biotinylated mouse anti-human antibodies specific for each protein were added, followed by streptavidin-horseradish peroxidase. Finally, a 1:1 solution of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine was added to plates to induce a colour change, and the reaction stopped by addition of 2M sulphuric acid. Between each step, plates were washed three times in wash buffer (0.05% Tween-20 diluted in PBS; Sigma-Aldrich). The final absorbance of each sample was read at 450nm and protein concentrations interpolated from the standard curve using a four-parameter logistic model provided by Graphpad Prism.

Ibidi μ-migration assays

Migration of DC populations was assessed using Ibidi μ-migration assays, performed according to manufacturer's instructions (Ibidi, Martinsried, Germany). In brief, immature or mature DC were diluted to a concentration of $3x10^6$ cells/ml in collagen gel, and added to the cell chamber of a μ-migration slide (Ibidi). Unsupplemented medium was added to each chemoattractant chamber of the μ-migration slide, and chemoattractants of interest (500ng/ml RANTES, 2ng/ml CCL19, 10nM or 66nM recombinant 15kDa granulysin (all R&D systems)) were added to one chemoattractant chamber in order to produce a concentration gradient across the slide. Migration was monitored using a time-lapse microscope (Olympus IX70 inverted system, Olympus Corporation, Tokyo, Japan) equipped with a Hamamatsu C4742-95 digital

camera and a motorised stage controlled by Image Pro-Plus software (Media Cybernetics, USA). The microscope and stage were enclosed within a heated (37°C) humidified chamber (Solent Scientific, UK) at 5% CO_2. Images were captured every 15 minutes over a period of 24 hours and were then used to analyse the migration of cells towards each chemoattractant, using ImageJ software (NIH, Maryland, USA).

Statistical analyses

All statistical analyses were carried out using Graphpad Prism software (Prism 7, Graphpad Software, California, USA). Significance was determined using either one-way or two-way ANOVA or paired *t*-tests, assuming Gaussian distribution in all cases. Unless otherwise stated, data is presented as mean ± standard deviation (SD). Statistical differences with *P*-values <0.05 are reported in the figures. *, **, *** and **** are used to report *P*-values of <0.05, <0.01, <0.001 and <0.0001, respectively.

Results

Granulysin is expressed in Vδ1$^+$ and Vδ2$^+$ γδ T cell populations

We first sought to confirm previous evidence that γδ T cells express granulysin when in a resting state(20). We assessed the intracellular expression of granulysin within this cell population and compared it to that observed within NK cells and CD8$^+$ αβ T cells, previously shown to express granulysin constitutively and following an activation signal, respectively(21,22). We then further separated the peripheral blood γδ T cell population into Vδ1$^+$ and Vδ2$^+$ subpopulations, and assessed the resting state expression of granulysin in each subpopulation.

Flow cytometry was used to identify each immune cell population within PBMC preparations (figure 1A) and to determine the frequency of granulysin expression within these cells. Two antibodies were used to distinguish between total granulysin (hereafter referred to as 15+9kDa granulysin) expression, and 9kDa granulysin expression (figure 1B). While the 9kDa isoform of granulysin is produced through cleavage of the 15kDa precursor and therefore exhibits identical epitopes, the antibody used here to identify 9kDa granulysin has been previously cited in the literature to have higher affinity for the cleaved 9kDa granulysin isoform, as compared to the full-length 15kDa precursor(19,23,24).

NK cell populations had the highest percentage of cells expressing both isoforms of granulysin, and CD8$^+$ αβ T cells the lowest (figures 1C and D). Expression of granulysin, and granzyme B within γδ T cell populations was most similar to that observed within NK cell populations (figure 1E). The percentage of Vδ2$^+$ γδ T cells found to be expressing either isoform of granulysin was analogous to that observed within γδ T cell populations as a whole, while very few Vδ1$^+$ γδ T cells were observed to express either isoform of granulysin (figures 1F-1H).

Taken together, these results show that peripheral blood γδ T cells, and in particular those cells expressing a Vδ2 chain, express both isoforms of granulysin when in a resting state, in a manner most comparable to NK cells of the innate immune system.

γδ T cell stimuli can increase the expression of intracellular 9kDa granulysin.

We and others have shown that Vδ2$^+$ γδ T cells within PBMC preparations can be activated in response to short-term treatment with ZA and BCG(25,26). However, the ability of these stimuli to cause changes to the intracellular expression of granulysin within this cell population remains to be determined. We therefore conducted experiments to investigate whether 24 hours of stimulation with these reagents could cause an increase in the intracellular expression of granulysin in Vδ2$^+$ γδ T cell populations present within PBMC preparations.

Twenty-four hours of stimulation with ZA or BCG did not cause any marked expansion of Vδ2$^+$ γδ T cells within the PBMC population (not shown). However, an increase in the expression of activation marker CD69 on this cell population was observed, and was comparable to that seen following stimulation with phorbol myristate acetate and ionomycin (PMA/I), known to cause activation of this cell type (Figure 2A). Activation seen in response to either ZA or BCG was not observed within populations of CD8$^+$ T cells or NK cells (Figure 2A).

The percentage of Vδ2$^+$ γδ T cells expressing either 15+9kDa granulysin or granzyme B was not changed in response to ZA or BCG stimulation (Figures 2B and 2C). Interestingly, we observed a statistically significant increase in the percentage of Vδ2$^+$ γδ T cells expressing 9kDa granulysin when PBMC were treated with BCG for 24 hours (Figure 2D). A small increase in expression of this isoform was also observed following stimulation with ZA, although this was not statistically significant. This increase in 9kDa granulysin expression on BCG treatment was not seen within populations of NK cells or CD8$^+$ αβ T cells (Figure 2D) or when isolated populations of Vδ2$^+$ γδ T cells were stimulated with BCG (Figure 2E). This suggests that this response is specific to Vδ2$^+$ γδ T cell populations, and furthermore confirms

previous literature which suggests that the involvement of additional immune cell populations is crucial to BCG-induced stimulation of Vδ2$^+$ γδ T cell populations[26].

Taken together, these data show that Vδ2$^+$ γδ T cells present within PBMC populations are activated by ZA or BCG, but only BCG stimulation causes a change in the expression of intracellular granulysin within this cell population.

Granulysin is released from Vδ2$^+$ γδ T cells in response to tumour

We next designed experiments to investigate the release of granulysin from Vδ2$^+$ γδ T cells following culture with tumour cells. Vδ2$^+$ γδ T cells were isolated from PBMC pre-treated for 9 days with IL-2 and ZA in order to induce expansion of the Vδ2$^+$ γδ T cell population. Isolation of the Vδ2$^+$ γδ T cell population was achieved using MACS, and purity determined by flow cytometry (Figure S1). As expansion of Vδ2$^+$ γδ T cells requires prior activation of this cell type through treatment with ZA, cells were tested for markers of exhaustion throughout the expansion period in order to determine if they would be feasible for use in subsequent co-culture studies. While we found an increase in markers PD-1 and Lag-3 during the expansion process, Vδ2$^+$ γδ T cells were still capable of secreting granulysin following the 9 day expansion period, and so were determined viable for use in co-culture studies (Figure 3A).

Vδ2$^+$ γδ T cells were cultured with the B cell lymphoma lines Daudi and Raji, known to be sensitive and resistant to Vδ2$^+$ γδ T cell killing, respectively(9,27). In addition, Raji cells were pre-treated for 24 hours with ZA and subsequently washed prior to co-culture, in order to render them more susceptible to Vδ2$^+$ γδ T cell killing(28). Preceding co-culture, the ability of Daudi cells, Raji cells and Raji cells pre-treated with ZA to produce the cytotoxic molecules investigated within this set of experiments was determined, and intracellular staining showed no expression of 15+9kDa granulysin, 9kDa granulysin, granzyme B or IFNγ within these cell populations (not shown). Following 24, 48 and 72 hours of culture, Vδ2$^+$ γδ T cells and co-culture supernatants were harvested and used in flow cytometry and ELISA experiments, respectively.

Our data show that culture with Daudi cells, Raji cells and Raji cells pre-treated with ZA caused a comparable increase in the expression of CD69 on Vδ2$^+$ γδ T cells when compared to that seen on Vδ2$^+$ γδ T cells cultured alone. This observation was seen regardless of the tumour cell

line tested, suggesting that Vδ2$^+$ γδ T cells are activated by both Daudi and Raji tumour cell lines (Figure 3B). Of note, we found that the percentage of Vδ2$^+$ γδ T cells expressing CD69 was approximately 60% lower than that observed when these cells were within a PBMC preparation (Figure 2A). This is presumably due to the fact that isolated Vδ2$^+$ γδ T cells had been previously activated by ZA, during expansion of this cell population within PBMC preparations prior to isolation. Interestingly, although we observed activation of Vδ2$^+$ γδ T cells in response to all three tumour cell lines, we found only very small concentrations of IFNγ within supernatants taken from co-culture of Vδ2$^+$ γδ T cells with all tumour cell lines tested (Figure 3C).

Culture of Vδ2$^+$ γδ T cells with tumour cell lines caused an increase in the percentage of cells expressing CD107a (Figure 3D). While the percentage of Vδ2$^+$ γδ T cells expressing CD107a following culture with Daudi cells and Raji cells pre-treated with ZA was comparable, CD107a expression on Vδ2$^+$ γδ T cells cultured with untreated Raji cells did not increase significantly above that observed in untreated cells, suggesting a lack of degranulation in response to this tumour cell type. Culture of Vδ2$^+$ γδ T cells with Daudi tumour cells caused the highest concentrations of granulysin released into co-culture supernatants (Figure 3E). However, contrary to our expectations, we found that the concentration of granzyme B within co-culture supernatants did not follow this pattern. Instead, the concentrations of granzyme B found within co-culture supernatants were notably lower than the concentrations of granulysin observed, and in fact, culture of Vδ2$^+$ γδ T cells with Daudi cells actually produced the lowest concentrations of granzyme B present within co-culture supernatants (Figure 3F). The peak in granulysin release from Vδ2$^+$ γδ T cells cultured with Daudi cells was observed following 48 hours of culture, and correlated with the time point at which the maximal killing of Daudi cells (79.3±13.3%) was observed (Figure 3G). Culture of Vδ2$^+$ γδ T cells with untreated Raji cells did not induce any secretion of granulysin above that produced by Vδ2$^+$ γδ T cells cultured

alone, and also did not result in any marked increase in tumour cell death. Interestingly, while culture of Vδ2$^+$ γδ T cells with Raji cells pre-treated with ZA did not appear to result in increased granulysin secretion in comparison to culture with untreated Raji cells, there was a substantial increase in killing of Raji cells pre-treated with ZA following 24 hours of culture. This suggests that treating this cell line with ZA did result in some sensitisation to Vδ2$^+$ γδ T cell killing.

Taken together, these data suggest that Vδ2$^+$ γδ T cells are activated by tumour cells, subsequently resulting in degranulation, granulysin release, and cell death. However, this appears to occur without concomitant IFNγ and granzyme B release.

Recombinant 15kDa granulysin can induce a mature phenotype in DC.

Previous studies have shown the ability of recombinant 15kDa granulysin to cause maturation of immature DC(17,19), and we sought here to replicate these findings. Immature DC were differentiated from isolated populations of peripheral blood $CD14^+$ monocytes (Figure S2), before being cultured for 24 hours in the presence of recombinant 15kDa granulysin. Culture of immature DC with LPS was used as a positive control of maturation, while medium alone was used as a negative control, and had no effect on maturation (Figure 4). Flow cytometry and light microscopy were used to confirm the maturation of DC in response to each reagent tested.

Results showed that recombinant 15kDa granulysin was capable of maturation of immature DC (Figure 4). Expression of CD80 and human leukocyte antigen-D related (HLA-DR), classical markers of maturation, increased significantly on cells following culture in the presence of recombinant 15kDa granulysin, and the MFI observed was comparable to that seen following culture with the positive control of LPS. Additionally, expression of chemokine receptor CCR5, often expressed by immature, and not mature, DC, decreased following culture with both reagents. Conversely, expression of CCR7, the lymph node homing chemokine associated with maturation, increased. The expression of CD14 and CCR2 on DC prior to and following maturation was also determined, and did not alter (not shown).

Taken together, these findings suggest that recombinant 15kDa granulysin is capable of causing maturation of DC.

Recombinant 15kDa granulysin can induce concentration-dependent migration of immature and mature DC.

As we had determined that 15kDa granulysin was capable of causing the maturation of immature DC, we next investigated if 15kDa granulysin could also cause migration of immature or mature DC. Previous literature has shown that 10nM recombinant 15kDa granulysin can cause the migration of several immune cell populations, including DC(16,17). We therefore sought to replicate DC migration in response to 10nM recombinant 15kDa granulysin, and additionally tested migration in response to a higher concentration of 66nM recombinant 15kDa granulysin. Ibidi µ-migration assays and time-lapse microscopy were used to follow the migration patterns of cells in response to each stimulus for 24 hours. Figure S4 provides detailed methodology for determination of percentage migration in response to a stimulus. Recombinant RANTES was used as a positive control of migration for immature DC, and recombinant CCL19 was used as a positive control of migration for mature DC. Concentrations used for positive controls were based on manufacturer's recommendation.

In keeping with results obtained by Deng *et al.*(16), we found that while immature DC did not migrate in response to 10nM recombinant 15kDa granulysin, this concentration of granulysin caused marked migration of mature DC (Figures 5A and 5B). Interestingly, when the concentration of recombinant 15kDa granulysin was increased to 66nM, mature DC no longer migrated towards this reagent, and in fact the percentage migration of these cells was determined to be less than that seen in response to the negative control of medium alone, indicating a movement away from this concentration of granulysin (Figure 5C). In contrast, immature DC were found to migrate towards 66nM recombinant 15kDa granulysin (Figure 5A), and percentage migration was found to be comparable to the positive control of recombinant RANTES.

Taken together, these data suggest that recombinant 15kDa granulysin can cause the migration of immature and mature DC. In addition, results suggest that granulysin may differentially cause both migration and repulsion of matured DC, dependent on the concentration of granulysin.

Discussion

The remit of this study was to determine whether Vδ2$^+$ γδ T cells released granulysin in response to a tumour target, and to investigate the functional consequences of this response. In this paper, we present evidence that Vδ2$^+$ γδ T cells express granulysin intracellularly in a constitutive manner, and release this molecule on culture with the tumour cell lines Daudi and Raji, albeit to differing degrees. In addition, we show that 15kDa granulysin can cause the maturation of immature DC, and further propose that 15kDa granulysin may have a dual capacity to cause both the chemotaxis of immature DC and the fugetaxis of mature DC, in a concentration-dependent manner.

Often referred to as 'the bridge between the innate and adaptive immune systems', it has been established that Vδ2$^+$ γδ T cells possess properties of both innate and adaptive immune cells. Despite being a relatively small immune cell subset, Vδ2$^+$ γδ T cells have been shown to respond rapidly to tumour, due to their ability to recognise targets without prior antigen processing and presentation. In this way, Vδ2$^+$ γδ T cells make a crucial contribution to the immune response to cancer. In fact, research by Gentles *et al.* into the association of infiltrating immune cell subsets with prognostic outcomes showed that γδ T cells were ranked as the highest indicator of a favourable outcome for 25 different malignancies and 14 solid tumours(29). Additionally, several studies have cited the involvement of Vδ2$^+$ γδ T cells activated with BCG in the regression of tumour. For example, Takeuchi and colleagues

determined that production of IL-17 by γδ T cells following BCG inoculation in bladder cancer was responsible for the subsequent recruitment of neutrophils required for an antitumour response(30). More recently, substantial Vδ2$^+$ γδ T cell infiltration has been identified in metastatic melanoma lesions following intralesional injection of BCG. Yang *et al.* observed an increase in CXCL9, 10 and 11, in addition to increased expression of butyrophilin 3A1 in these lesions following treatment, which they hypothesised resulted in the attraction and subsequent activation of intralesional Vδ2$^+$ γδ T cells(31). Interestingly, injection of these lesions with BCG led to a 50% regression in tumour size, which may be linked to the increased number of responding Vδ2$^+$ γδ T cells(31). This emerging correlation between positive cancer outcomes and the presence of activated Vδ2$^+$ γδ T cells led us to further investigate the ways in which this cell population could be activated to release cytotoxic molecules, and the functional effects of this with regard to tumour cell killing.

In this study, we show that γδ T cells do not require a stimulatory signal to express 15kDa granulysin intracellularly, and only express 9kDa granulysin to a high level, at least in our hands, following BCG stimulation. These findings show similarities of γδ T cells with both NK cells of the innate immune system, and CD8$^+$ αβ T cells of the adaptive immune system. NK cells have been shown to express both isoforms of granulysin constitutively, while CD8$^+$ αβ T cells have been shown to express granulysin only 3-4 days after activation(22,32). We also found that the percentage of Vδ2$^+$ γδ T cells expressing each isoform of granulysin was comparable to that observed within the γδ T cell population as a whole. The presence of intracellular granulysin within this subpopulation prior to recognition of tumour and subsequent activation may allow a more rapid release of granulysin into the surrounding environment when activation of the cell does occur.

Our findings within this study have confirmed previous evidence that Vδ2$^+$ γδ T cells can be activated by both ZA and BCG, as long as additional immune cell populations are

present(25,26). However, only activation of PBMC with BCG was able to cause a change in the percentage of Vδ2$^+$ γδ T cells expressing granulysin. It is not surprising that stimulation of Vδ2$^+$ γδ T cells caused an increase in the expression of 9kDa granulysin only; this is the cytotoxic isoform of granulysin which has been shown to increase within other T cell populations following activation(32). Interestingly, previous studies have shown an increase in the expression of granulysin within populations of CD8$^+$ αβ T cells and CD4$^+$ αβ T cells following BCG vaccination of neonates, so it is perhaps not unexpected that this effect is also seen in populations of Vδ2$^+$ γδ T cells(33). However, a significant increase in intracellular 9kDa granulysin expression was not observed following stimulation of Vδ2$^+$ γδ T cells with ZA. This could be due to differences in the efficacy of IPP produced in response to ZA, and HMBPP produced by BCG infection(8). Alternatively, the stimulation of PBMC with BCG has been shown to cause activation of other populations of immune cells(34,35). This activation may deliver a co-stimulatory signal to Vδ2$^+$ γδ T cells which is necessary for the upregulation of 9kDa granulysin within these cells, and which is not delivered following stimulation with ZA, and highlights the requirement for other immune cell populations in the activation of Vδ2$^+$ γδ T cells in response to these reagents.

For this reason, we sought to confirm that isolated Vδ2$^+$ γδ T cells could release granulysin in response to tumour without additional activation. We showed that *in vitro,* Vδ2$^+$ γδ T cells released substantial amounts of granulysin in response to culture with Daudi tumour cells, known to be sensitive to Vδ2$^+$ γδ T cell killing, and that they could also release this molecule, albeit to a lesser degree, in response to Raji cells (resistant to Vδ2$^+$ γδ T cell killing) and Raji cells pre-treated with ZA. It is interesting to note that while co-culture caused an increase in the production of the cytotoxic molecule granulysin by Vδ2$^+$ γδ T cells, production of the classical cytotoxic cytokine IFNγ remained low over all conditions. This is in contrast to previous evidence, which shows Vδ2$^+$ γδ T cells to express IFNγ following activation, and that

Vδ2$^+$ γδ T cells deficient in IFNγ are less likely to be able to kill tumour(36,37). A potential explanation for this finding is that both Daudi and Raji cells express receptors for IFNγ, suggesting that any IFNγ released by Vδ2$^+$ γδ T cells in response to tumour may be taken up by the tumour cells themselves, and as such will not be present within supernatants to be detected by ELISA(38,39). The fact that the highest concentrations of IFNγ detected by ELISA were found within supernatants taken from the co-cultures of Vδ2$^+$ γδ T cells with Daudi cells suggests an excess of IFNγ produced by Vδ2$^+$ γδ T cells that cannot be taken up by the Daudi cells.

It is interesting that the pre-treatment of Raji cells with ZA did not increase the release of granulysin to a level comparable to that seen following culture of Vδ2$^+$ γδ T cells with Daudi cells, despite increasing the amount of tumour cell death. The addition of ZA to Raji cells has been previously shown to cause an increase in IPP expression, and thus an increase in Vδ2$^+$ γδ T cell cytotoxicity. It is possible that too low a concentration of ZA was used within experiments detailed here. While we used 5μM ZA to induce sensitisation of Raji cells to Vδ2$^+$ γδ T cell killing, previous evidence by Idrees *et al.* has shown that inhibition of FPP synthase was not observed within Raji cells until a concentration of 1mM ZA was added to cells *in vitro*. In addition, cytotoxicity of Vδ2$^+$ γδ T cells, as characterised by production of TNFα, was also not observed below this concentration of ZA(40). Despite this, our data suggest that granulysin may be released by Vδ2$^+$ γδ T cells that infiltrate and recognise tumours *in vivo,* which are sensitive to killing by this cell type.

We next investigated the role of granulysin in the maturation of DC, and showed that recombinant 15kDa granulysin was capable of causing the maturation of immature DC in a manner similar to that seen in response to recombinant LPS. Granulysin has been described as an immune alarmin[17], and several other alarmins have been previously shown to cause the maturation of immature DC. For example, research by Dumitriu *et al.* showed that high

mobility group box 1 caused maturation of DC, characterised by an increase in expression of CCR7(41). We found that high concentrations of recombinant 15kDa granulysin were also able to cause migration of immature DC. This is of interest as it suggests that in a physiological setting, granulysin released by Vδ2$^+$ γδ T cells in response to a tumour target may contribute to the influx of immature DC to the tumour site. However, perhaps more noteworthy is the observation that recombinant 15kDa granulysin appears to cause both the chemotaxis and fugetaxis of matured DC dependent on concentration. Low concentrations of granulysin were found to cause a marked migration of mature DC, while high concentrations of granulysin induced a movement of these cells away from this molecule. Research has shown evidence of this phenomenon previously. Tharp determined that whether a neutrophil migrated towards or away from IL-8 was dependent on the absolute concentration of this molecule(42). Using microfluidic linear gradient generators and time-lapse microscopy, results showed that at concentrations of 120nM, neutrophils were seen to migrate towards IL-8, while at concentrations of 1.2μM, neutrophils displayed potent fugetaxis(42). A similar phenomenon has been shown for the ability of stromal cell derived factor (SDF)-1 to cause the attraction and repulsion of T cells. At 100ng/ml, SDF-1 caused chemoattraction of both naïve and memory CD4$^+$ and CD8$^+$ αβ T cells, while higher concentrations of 10μg/ml SDF-1 caused repulsion of these cells(43).

In order to further assess the contribution of granulysin released by Vδ2$^+$ γδ T cells in response to tumour in the maturation and migration of DC, it would be important to next determine whether we could replicate our findings using supernatants taken from the co-culture of these cells with tumour targets. We have conducted preliminary experiments, and observed that supernatants taken from the co-culture of Vδ2$^+$ γδ T cells and Daudi cells could effectively induce both maturation of immature DC, and the migration of these cells towards the supernatant source (Figures S4 and S5A). Interestingly, we also were able to replicate the

differential migration of mature DC towards or away from granulysin, depending on the concentration of this molecule present in the supernatants tested. As can be seen from figure S5B, mature DC migrated towards supernatants taken from the co-culture of Vδ2$^+$ γδ T cells with Raji cells, which contained low concentrations of granulysin (an average of 4.85ng/ml). However, these cells migrated away from supernatants taken from the co-culture of Vδ2$^+$ γδ T cells with Daudi cells, containing higher concentrations of this molecule (an average of 28.60ng/ml). The inclusion of a granulysin blocking antibody, prior to addition of the supernatants to cultures of DC, would allow definitive confirmation of the involvement of 15kDa granulysin in the effects observed, and this would form the basis of future work.

Our data suggests that Vδ2$^+$ γδ T cells, through the production and release of granulysin, may be involved in orchestrating adaptive immunity against tumour. Through the release of granulysin, this cell population may contribute to the arrival of immature DC populations to a site of tumour, and may then further contribute to the migration of matured DC away from the tumour site, and towards lymph nodes in order to activate the adaptive immune response. We believe this to be an interesting facet of the anti-tumour response of Vδ2$^+$ γδ T cells, and therefore worthy of further investigation.

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Conflict of Interest

The authors declare no financial or commercial conflicts of interest.

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Figure legends

Figure 1: Resting γδ T cells express granulysin.

*A) Representative flow cytometry plots depicting the gating strategy used to identify cell populations of interest from PBMC preparations. Lymphocytes were gated (G1) according to size (forward scatter; FSC) and granularity (side scatter; SSC). Within gate G1, $CD8^+$ αβ T cells, NK cells and γδ T cells were further gated on using established lineage markers for these cells (G2-G4). B) Following identification of each immune cell population, the percentage of cells expressing 15+9kDa granulysin or 9kDa granulysin was determined by flow cytometry (expression within γδ T cell populations depicted). C-E) Percentage expression of 15+9kDa granulysin, 9kDa granulysin, and granzyme B within populations of $CD8^+$ αβ T cells, NK cells and γδ T cells, as determined by flow cytometry. F) Gating strategy used to differentiate between $Vδ1^+$ γδ T cells and $Vδ2^+$ γδ T cells. G-H) Percentage expression of 15+9kDa and 9kDa granulysin within populations of $Vδ1^+$ γδ T cells and $Vδ2^+$ γδ T cells as determined by flow cytometry. Data shown are obtained from between 6 and 10 independent experiments using PBMC from 10 individual donors, with error bars (SD). Differences between groups were assessed by one-way ANOVA. *=p<0.05. ***=p<0.001 ****=p<0.0001.*

Figure 2: Stimulation of $Vδ2^+$ γδ T cells with BCG causes an increase in the intracellular expression of 9kDa granulysin.

*A-D) The percentage of $Vδ2^+$ γδ T cells, NK cells and $CD8^+$ T cells expressing early activation marker CD69, 15+9kDa granulysin, granzyme B, and 9kDa granulysin following 24 hours of PBMC stimulation with ZA, BCG or PMA/I, as determined by flow cytometry. E) The percentage of $Vδ2^+$ γδ T cells expressing 9kDa granulysin following either 24 hours of PBMC stimulation with BCG, or following isolation of this cell population from unstimulated PBMC, and subsequent stimulation of these isolated $Vδ2^+$ γδ T cells for 24 hours. Data shown are mean values obtained from 6 independent experiments using PBMC from 6 individual donors, with error bars (SD). Statistics refer to the differences between treatment group and untreated group, and were assessed by one-way ANOVA. **=p<0.01. ***=p<0.001.*

Figure 3:Vδ2⁺ γδ T cells release granulysin in response to tumour.

*A) The expression of exhaustion markers PD-1 and Lag-3 on, and the secretion of granulysin by Vδ2⁺ γδ T cells during the 9 day expansion process. B) The percentage of Vδ2⁺ γδ T cells to express early activation marker CD69 following 24, 48 or 72 hours of culture with Daudi cells, Raji cells or Raji cells pre-treated for 24 hours with 5μM ZA, as determined by flow cytometry. C) The concentration of IFNγ found within supernatants taken from 24, 48 or 72 hour co-culture of Vδ2⁺ γδ T cells with tumour cell lines, as determined by ELISA. D) The percentage of Vδ2⁺ γδ T cells to express degranulation marker CD107a following 24, 48 or 72 hours of culture with Daudi cells, Raji cells or Raji cells pre-treated for 24 hours with 5μM ZA, as determined by flow cytometry. E) The concentration of granulysin found within supernatants taken from 24, 48 or 72 hour co-culture of Vδ2⁺ γδ T cells with tumour cell lines, as determined by ELISA. F) The concentration of granzyme B found within supernatants taken from 24, 48 or 72 hour co-culture of Vδ2⁺ γδ T cells with tumour cell lines, as determined by ELISA. G) Percentage killing of tumour cells by Vδ2⁺ γδ T cells following 24, 48 and 72 hours of culture, as determined by flow cytometry. Data shown is from 6 independent experiments, using Vδ2⁺ γδ T cells from 6 individual donors, with error bars (SD). Differences between groups were assessed by two-way ANOVA comparing negative control (Vδ2⁺ γδ T cells alone) with all other groups. *=p<0.05. **=p<0.01. ***=p<0.001. ****=p<0.0001.*

Figure 4: Granulysin can cause maturation of immature DC

*Changes in MFI of cell surface markers CD80, CCR7, CCR5 and HLA-DR on monocyte-derived immature DC following culture with 100ng/ml LPS or 66nM recombinant 15kDa granulysin), as determined by flow cytometry. Treatment of cells with medium alone was included as a negative control. Data shown is the average taken from 6 individual donors. Differences between groups were assessed by two-way ANOVA comparing negative controls (pre-maturation and medium alone) with all other groups. *=p<0.05. **=p<0.01.*

Figure 5: Recombinant 15kDa granulysin causes differential migration of immature and mature DC

A) The migration of immature DC in response to 10nM or 66nM recombinant 15kDa granulysin, as determined by Ibidi μ-migration assays. 500ng/ml recombinant RANTES was used as a positive control of immature DC migration, while 2ng/ml recombinant CCL19 was used as a negative control. B) The migration of mature DC in response to 10nM or 66nM recombinant 15kDa granulysin, as determined by Ibidi μ-migration assays. 500ng/ml recombinant RANTES was used as a negative control of mature DC migration, while 2ng/ml recombinant CCL19 was used as a positive control. Concentrations used for positive and negative controls were based on

Differential DC migration in response to granulysin

*manufacturer's recommendation. Data shown is from 6 independent experiments using immature and LPS-matured DC differentiated from the monocytes of 6 individual donors, with error bars (SD). Differences between groups were assessed using one-way ANOVAs *=p<0.05. **=p<0.01. ***=p<0.001. UN = untreated. GNLY = 15kDa granulysin.*

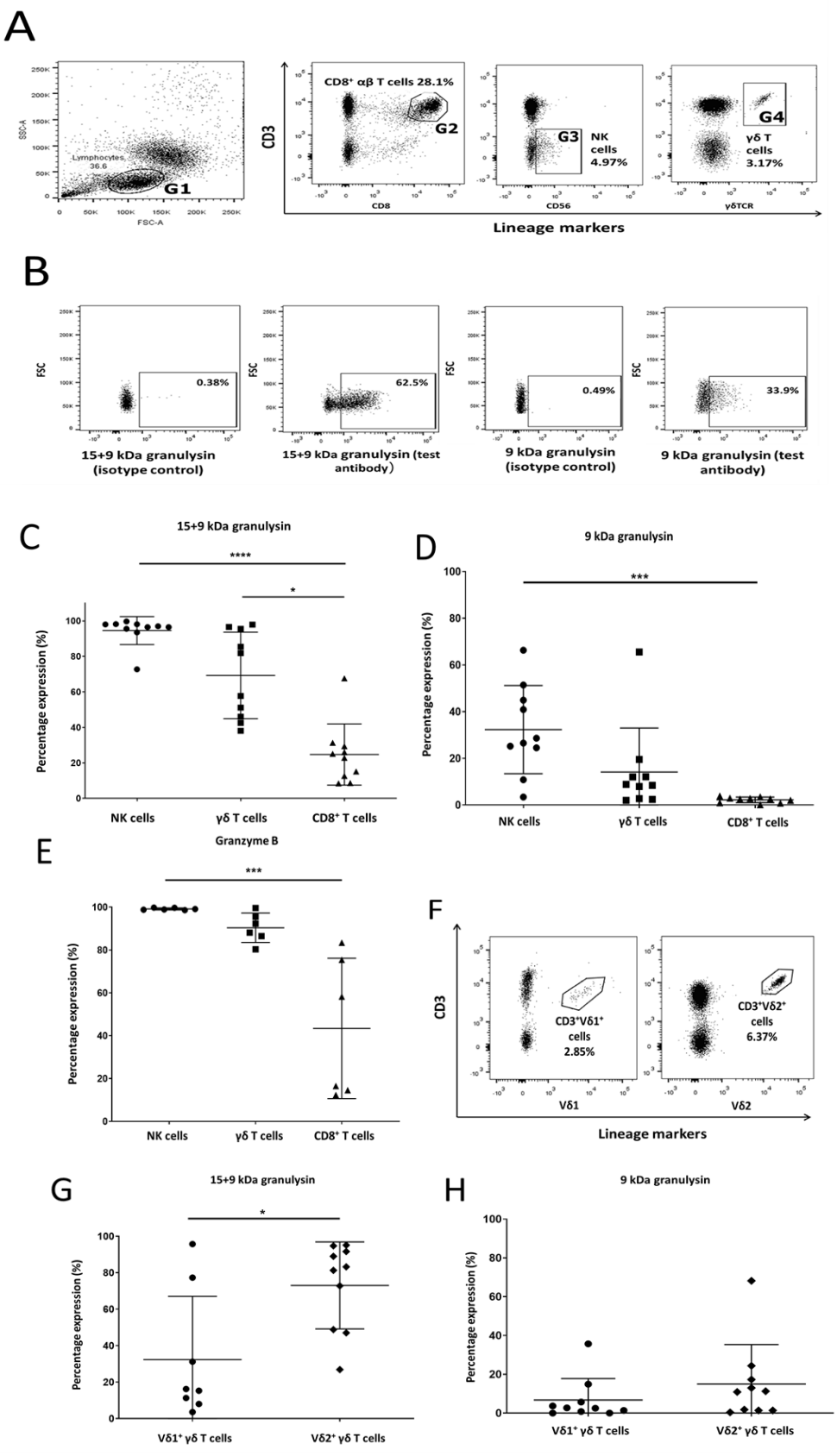

A
SSC-A
FSC-A
Lymphocytes
36.6
G1
CD3
CD8
CD8⁺ αβ T cells 28.1%
G2
G3
NK
cells
4.97%
CD56
G4
γδ T
cells
3.17%
γδTCR
Lineage markers
B
FSC
0.38%
15+9 kDa granulysin
(isotype control)
62.5%
15+9 kDa granulysin (test
antibody)
0.49%
9 kDa granulysin
(isotype control)
33.9%
9 kDa granulysin (test
antibody)
C
15+9 kDa granulysin
****
*
Percentage expression (%)
NK cells
γδ T cells
CD8⁺ T cells
D
9 kDa granulysin
***
Percentage expression (%)
NK cells
γδ T cells
CD8⁺ T cells
E
Granzyme B
***
Percentage expression (%)
NK cells
γδ T cells
CD8⁺ T cells
F
CD3
CD3⁺Vδ1⁺
cells
2.85%
CD3⁺Vδ2⁺
cells
6.37%
Vδ1
Vδ2
Lineage markers
G
15+9 kDa granulysin
*
Percentage expression (%)
Vδ1⁺ γδ T cells
Vδ2⁺ γδ T cells
H
9 kDa granulysin
Percentage expression (%)
Vδ1⁺ γδ T cells
Vδ2⁺ γδ T cells

FIGURE 2

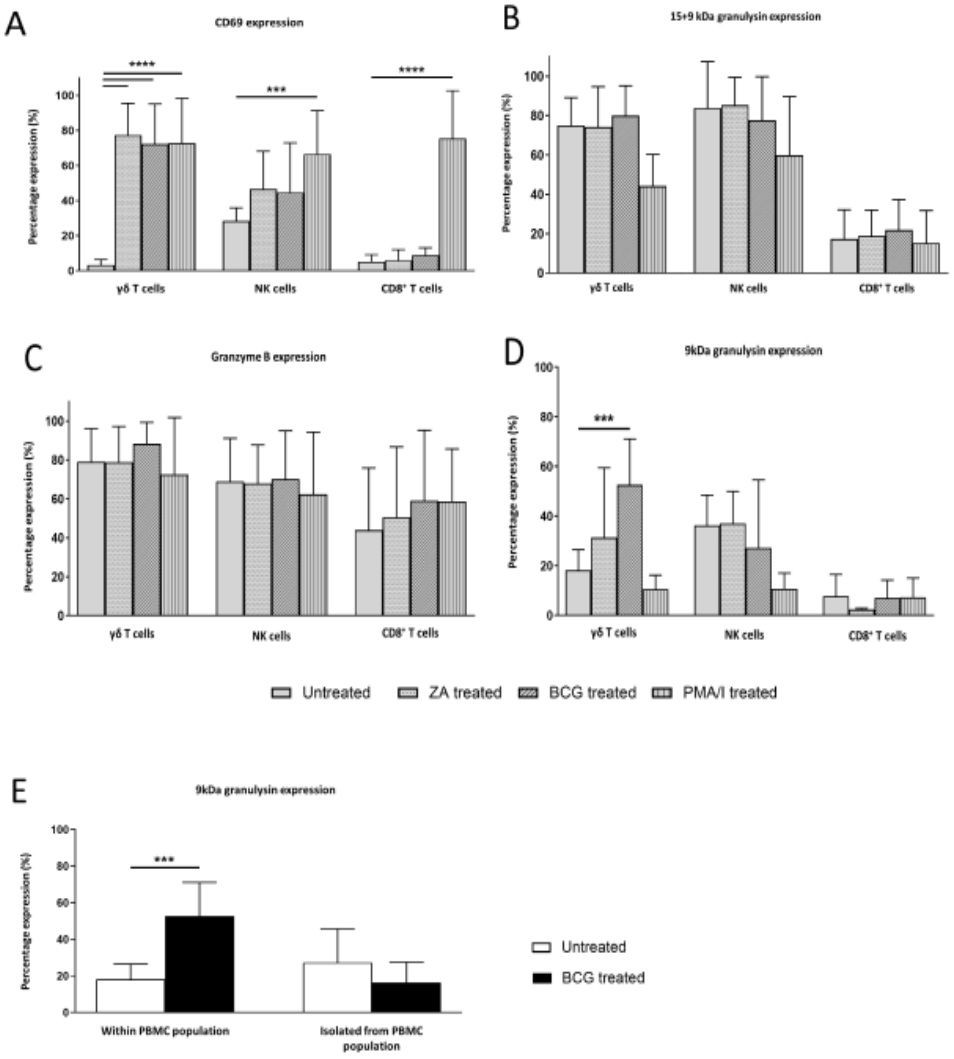

A
CD69 expression
Percentage expression (%)
γδ T cells
NK cells
CD8⁺ T cells
B
15+9 kDa granulysin expression
C
Granzyme B expression
D
9kDa granulysin expression
Untreated
ZA treated
BCG treated
PMA/I treated
E
9kDa granulysin expression
Within PBMC population
Isolated from PBMC population
Untreated
BCG treated

FIGURE 3

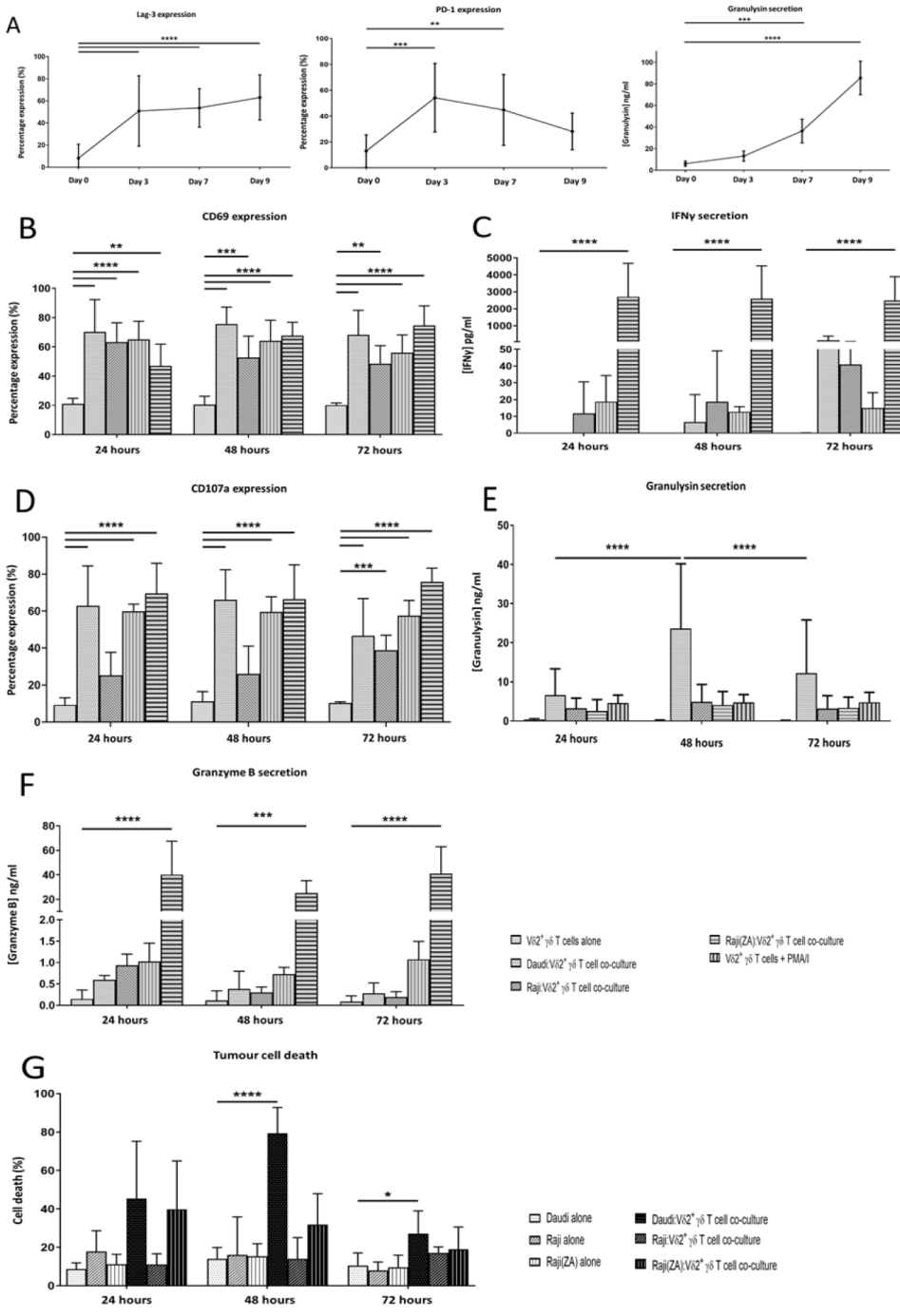

A
Lag-3 expression
PD-1 expression
Granulysin secretion
Percentage expression (%)
[Granulysin] ng/ml
Day 0
Day 3
Day 7
Day 9
B
CD69 expression
C
IFNγ secretion
[IFNγ] pg/ml
24 hours
48 hours
72 hours
D
CD107a expression
E
Granulysin secretion
F
Granzyme B secretion
[Granzyme B] ng/ml
Vδ2+ γδ T cells alone
Daudi:Vδ2+ γδ T cell co-culture
Raji:Vδ2+ γδ T cell co-culture
Raji(ZA):Vδ2+ γδ T cell co-culture
Vδ2+ γδ T cells + PMA/I
G
Tumour cell death
Cell death (%)
Daudi alone
Raji alone
Raji(ZA) alone
Daudi:Vδ2+ γδ T cell co-culture
Raji:Vδ2+ γδ T cell co-culture
Raji(ZA):Vδ2+ γδ T cell co-culture

FIGURE 4

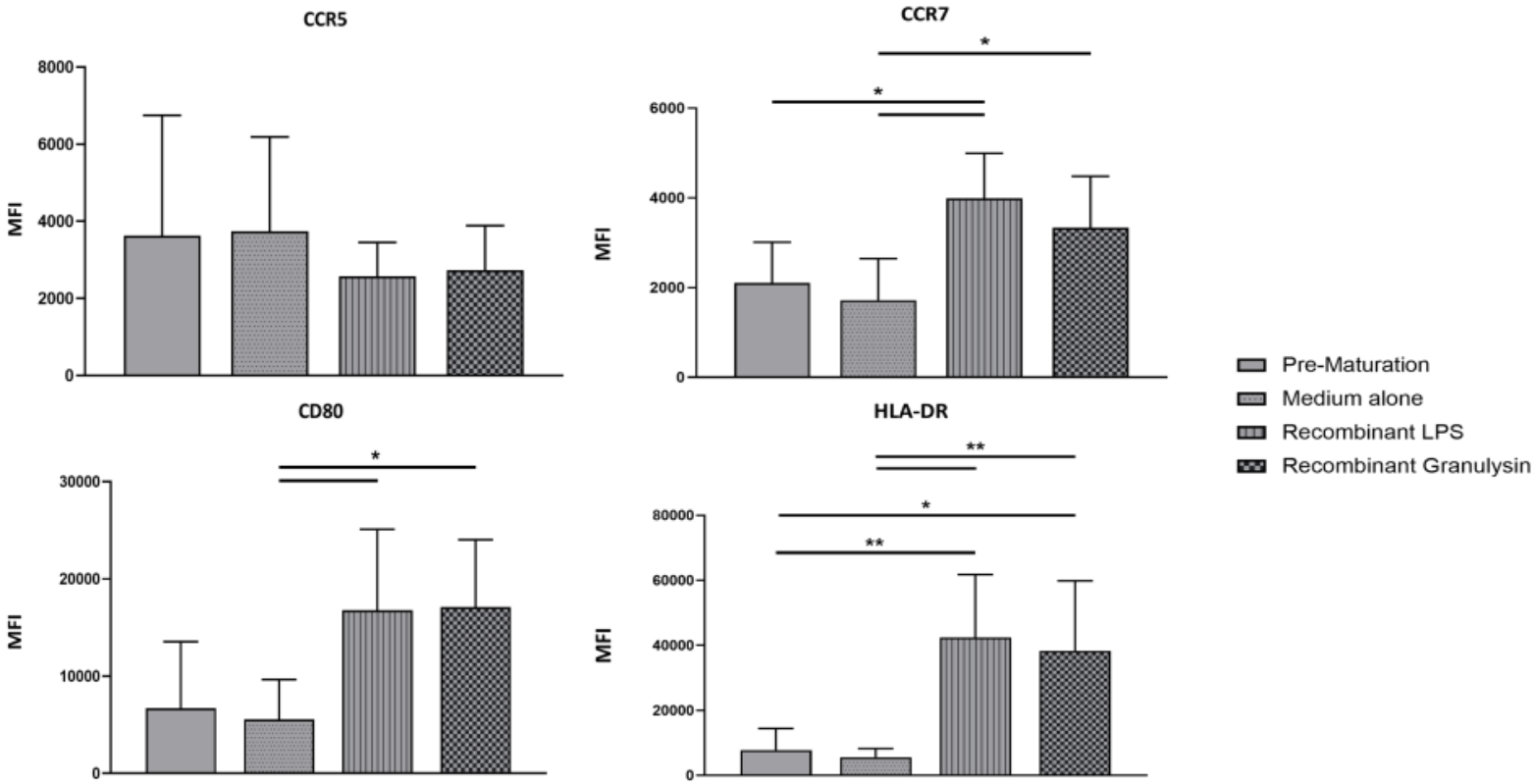
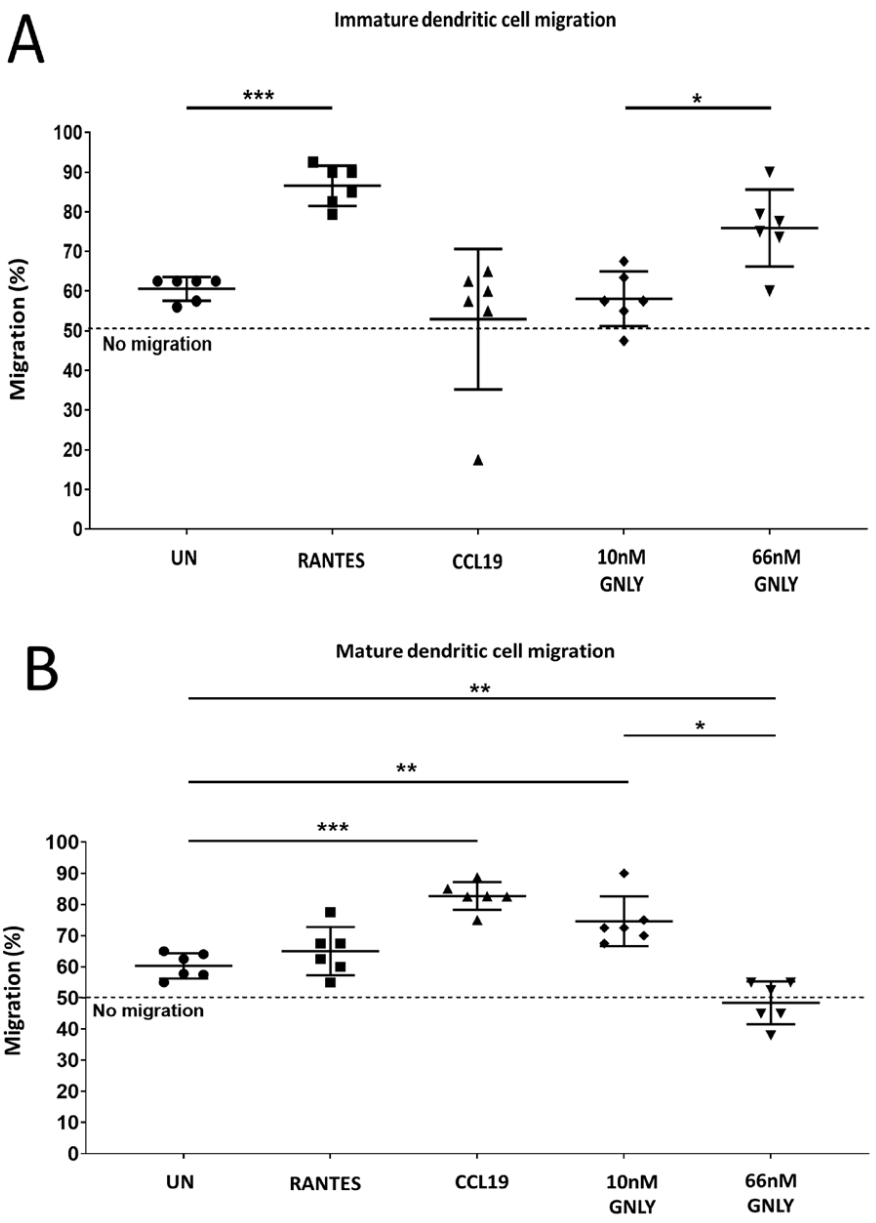

CCR5
MFI
8000
6000
4000
2000
0
CCR7
*
*
6000
4000
2000
0
CD80
*
30000
20000
10000
0
HLA-DR
**
*
**
80000
60000
40000
20000
0
Pre-Maturation
Medium alone
Recombinant LPS
Recombinant Granulysin

FIGURE 5


A
Immature dendritic cell migration
***
*
Migration (%)
No migration
UN
RANTES
CCL19
10nM GNLY
66nM GNLY
B
Mature dendritic cell migration
**
*
**
***
Migration (%)
No migration
UN
RANTES
CCL19
10nM GNLY
66nM GNLY

Supplemental figures

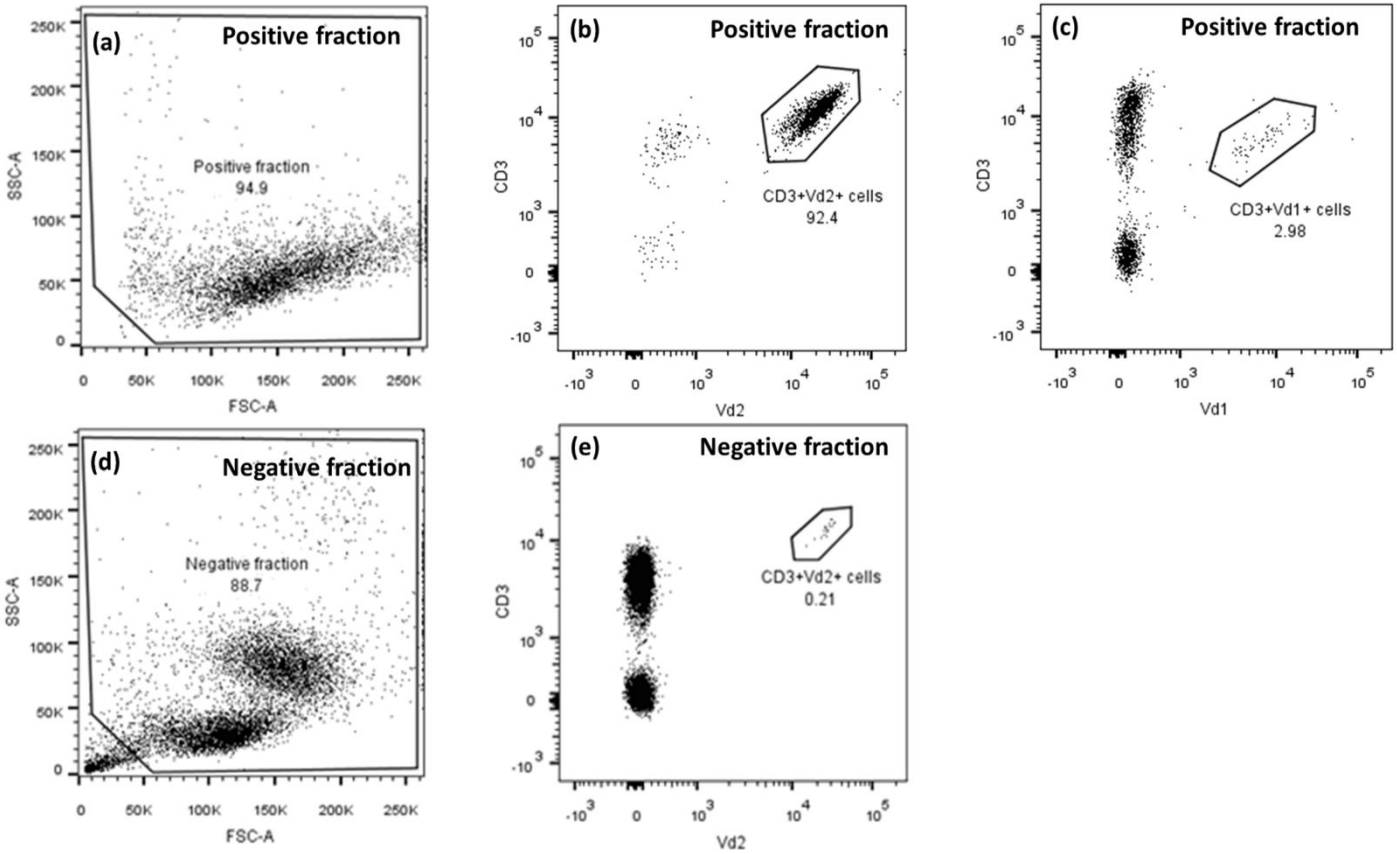


Figure S1: Representative gating strategy used to determine purity of isolated Vδ2⁺ γδ T cells.

PBMC were isolated from whole blood samples taken from healthy volunteers, and treated with 5μM ZA and 15ng/ml (315U/ml) IL-2 for 9 days in order to expand populations of Vδ2⁺ γδ T cells. Following expansion culture, γδ T cells were isolated using MACS and negative selection, and the purity of γδ T cell populations following isolation determined by flow cytometry. The percentage of Vδ2⁺ γδ T cells within positive (a-c) and negative fractions (d-e) was established. Cells were plotted based on FSC and SSC, and a gate placed around all cells ((a) and (d)). Fluorochrome-conjugated antibodies specific for cell surface markers CD3 and Vδ2 were used to identify populations of Vδ2⁺ γδ T cells within each fraction ((b) and (e)). Within the positive fraction, the percentage of Vδ1⁺ γδ T cells was also identified using a fluorochrome-conjugated antibody specific for Vδ1, as the isolation process is not specific for Vδ2⁺ γδ T cells (c). If the combined percentage of Vδ1⁺ and Vδ2+ γδ T cells was over 90%, cells were used in subsequent co-culture experiments.

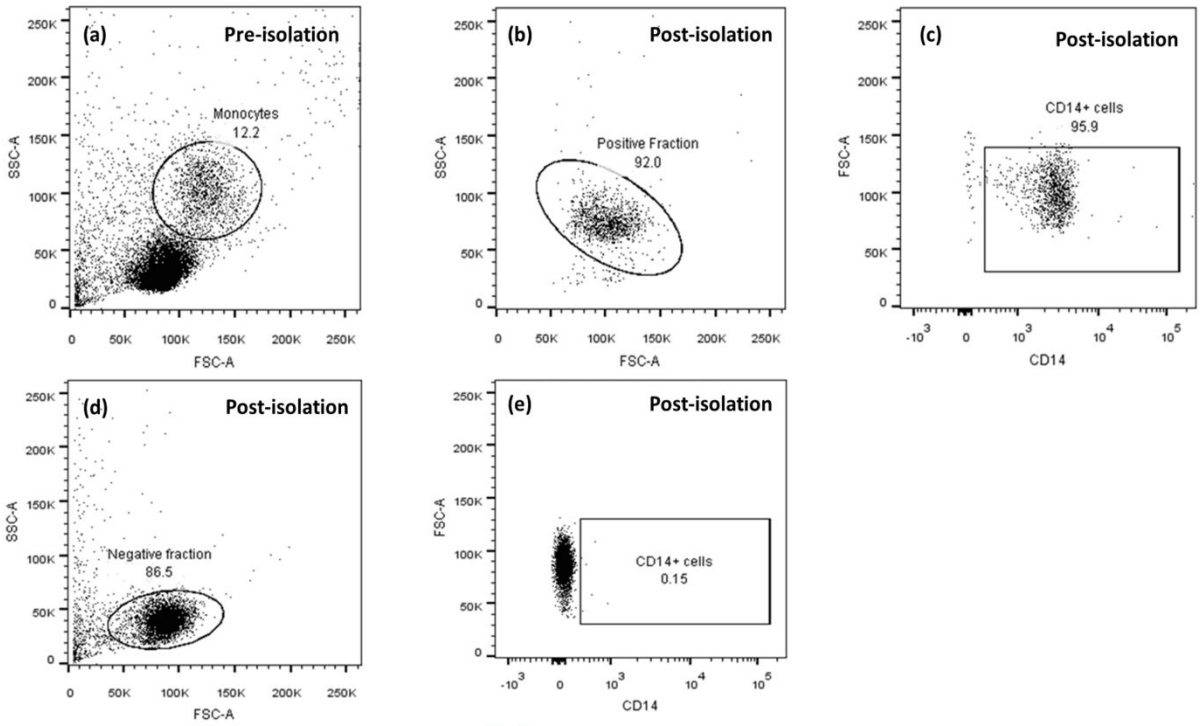
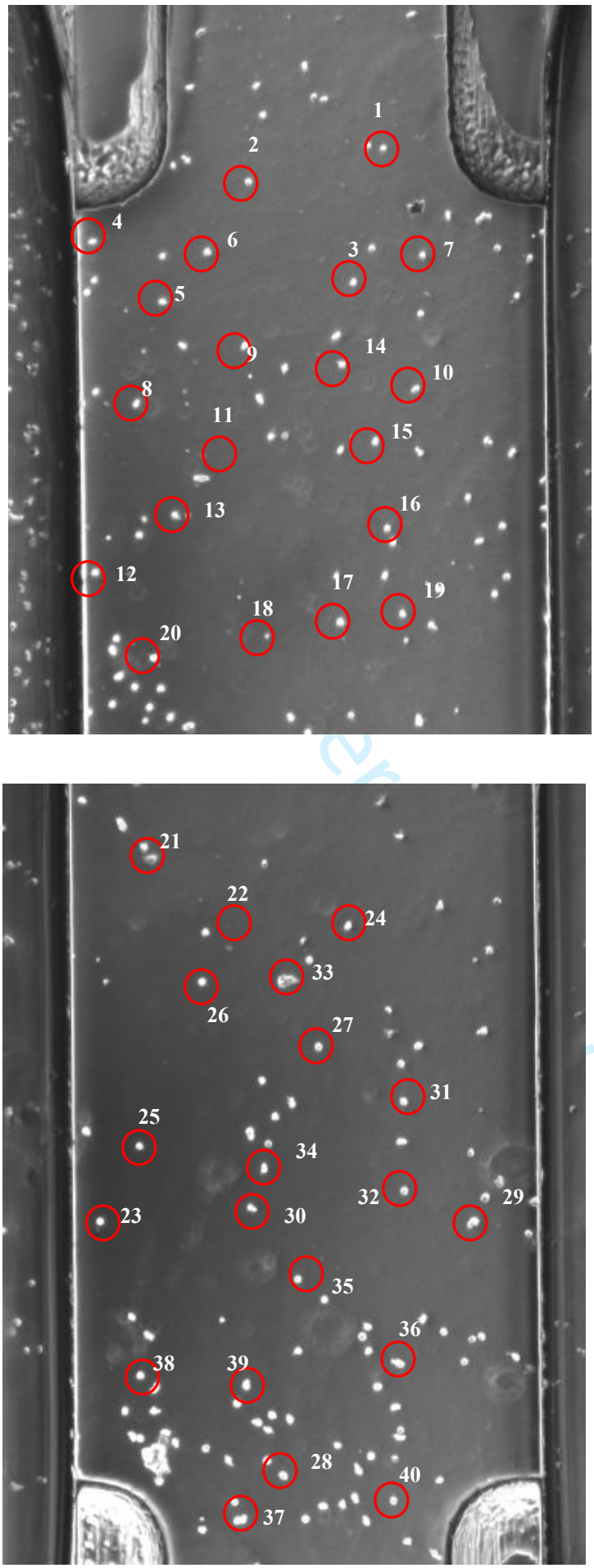


Figure S2: Representative gating strategy used to establish purity of CD14⁺ monocytes following isolation.

Populations of CD14⁺ monocytes were identified within PBMC preparations prior to isolation (a). Following isolation via positive selection and through MACS, cells present in the positive fraction were gated on (b), and the percentage of CD14⁺ cells within this fraction determined (c). In addition, the negative fraction (d) was also subjected to flow cytometry, and the percentage of CD14⁺ cells present within this fraction determined (e), in order to establish the efficiency of the isolation procedure.

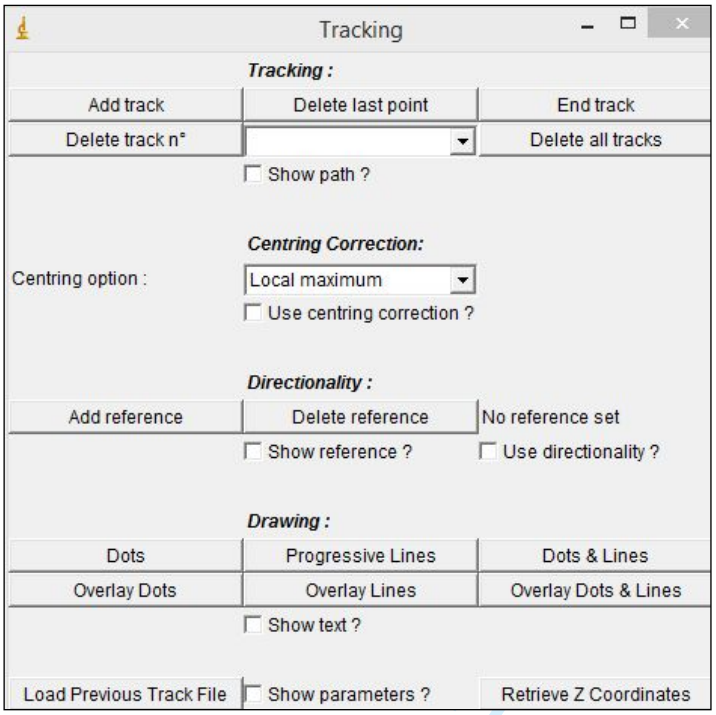
S3: Example µ-migration assay analysis.

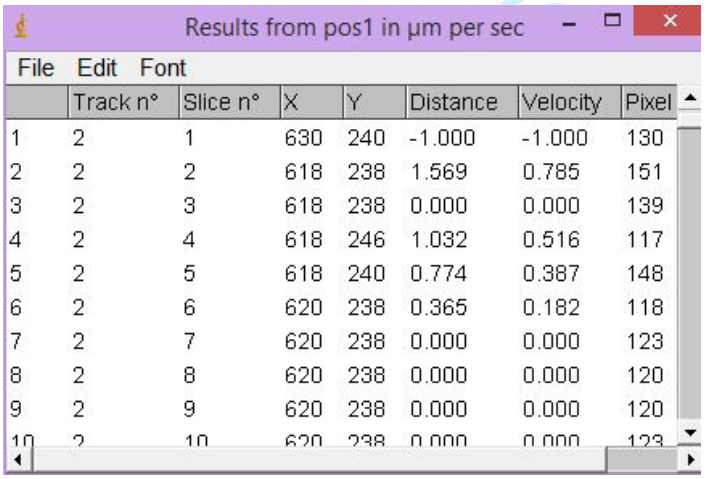
Migration of immature and mature DC in response to granulysin was measured using Ibidi µ-migration assays. Photographs of cells present within each chemoattractant chamber were taken every 15 minutes during a 24 hour time period, using a time-lapse microscope. Following the end of the assay, images of the cells prior to commencement of the assay were used to choose 40 cells for tracking (S3.1).



S3.1: Representative photograph taken of one cell chamber of a µ-migration slide prior to commencement of the assay, and the 40 cells chosen for tracking.

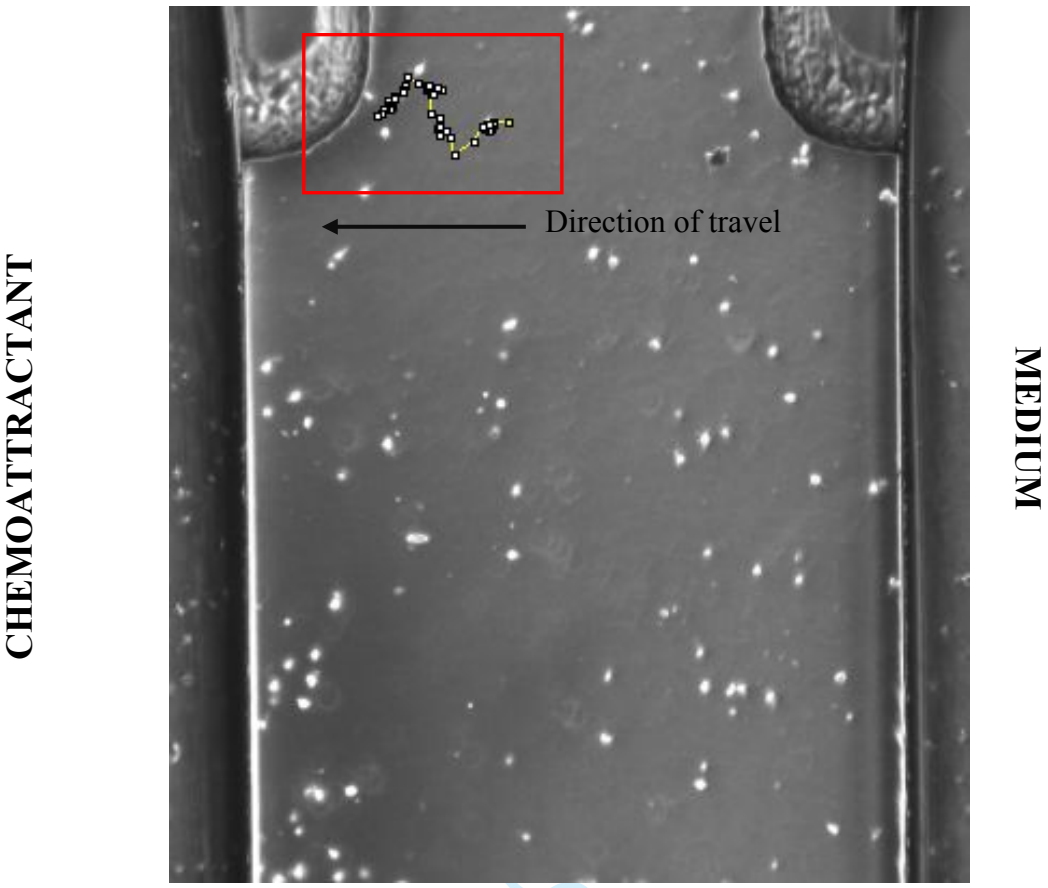
Once cells had been chosen, ImageJ software was used to manually track the migratory path of each selected cell. This was conducted through importing all images taken of each part of a chamber within a 24 hour run, and manually clicking through each image following the path of the selected cell. A plugin for ImageJ called manual tracking (S3.2) recorded the position of the cell on each image as images were clicked through. This was repeated for each of the 40 selected cells. A proportion of experiments were additionally tracked by an independent researcher, in order to ensure the validity of results obtained.





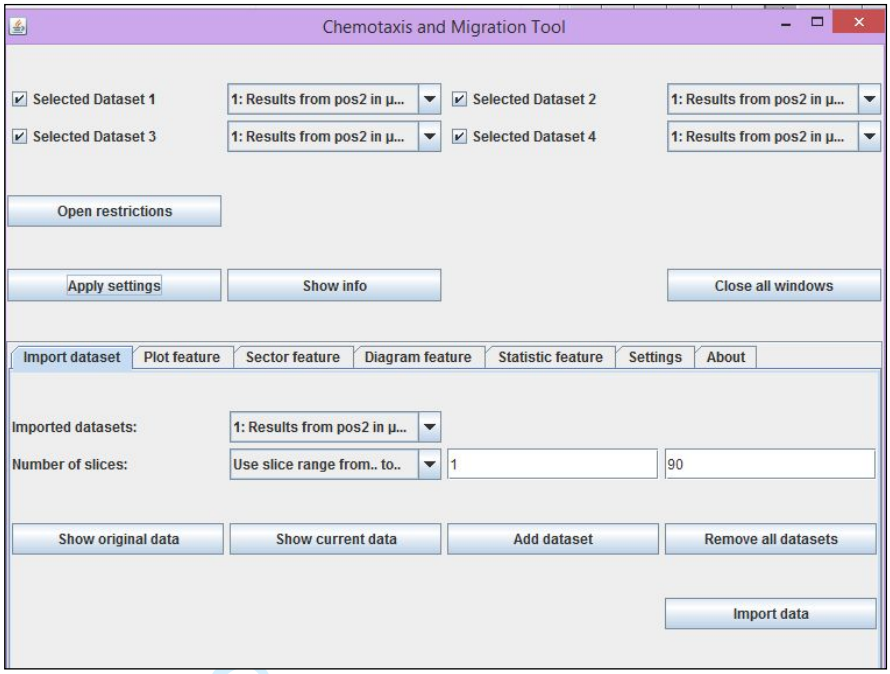
	Track n°	Slice n°	X	Y	Distance	Velocity	Pixel
1	2	1	630	240	-1.000	-1.000	130
2	2	2	618	238	1.569	0.785	151
3	2	3	618	238	0.000	0.000	139
4	2	4	618	246	1.032	0.516	117
5	2	5	618	240	0.774	0.387	148
6	2	6	620	238	0.365	0.182	118
7	2	7	620	238	0.000	0.000	123
8	2	8	620	238	0.000	0.000	120
9	2	9	620	238	0.000	0.000	120
10	2	10	620	238	0.000	0.000	123

S3.2: 'Manual tracking' plugin within ImageJ software used for tracking migratory path of cells.

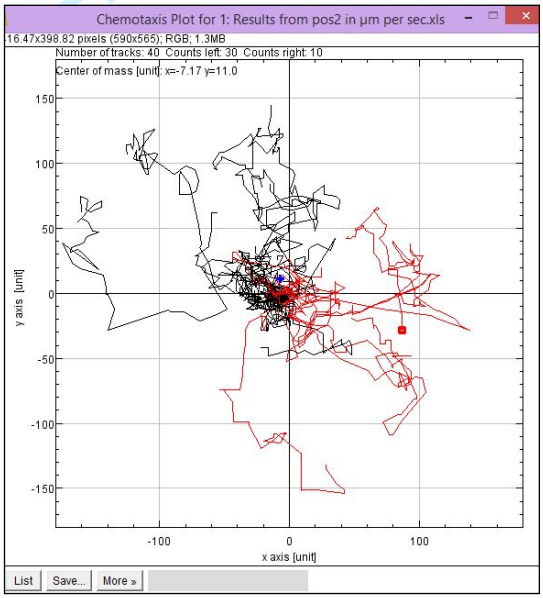


S3.3: Representative image showing a single migratory path of a cell manually tracked using the 'manual tracking' plugin within ImageJ software.

Once all 40 cells had been tracked, a second plugin called 'chemotaxis tool' was utilised to determine the number of cells which had migrated towards the chemoattractant (S3.4).



71



72

73 **S3.4: Representative graph plotted using the 'chemotaxis tool' plugin within ImageJ software, showing the**
74 **migratory tracks of 40 cells, and the number of cells which had moved towards or away from the**
75 **chemoattractant.**

76

77 In this case, 30 cells out of a total of 40 were determined to have moved towards the chemoattractant (black lines),
78 while 10 cells had moved away from it (red lines). Therefore, a percentage migration of 75% was achieved in
79 response to this chemoattractant.

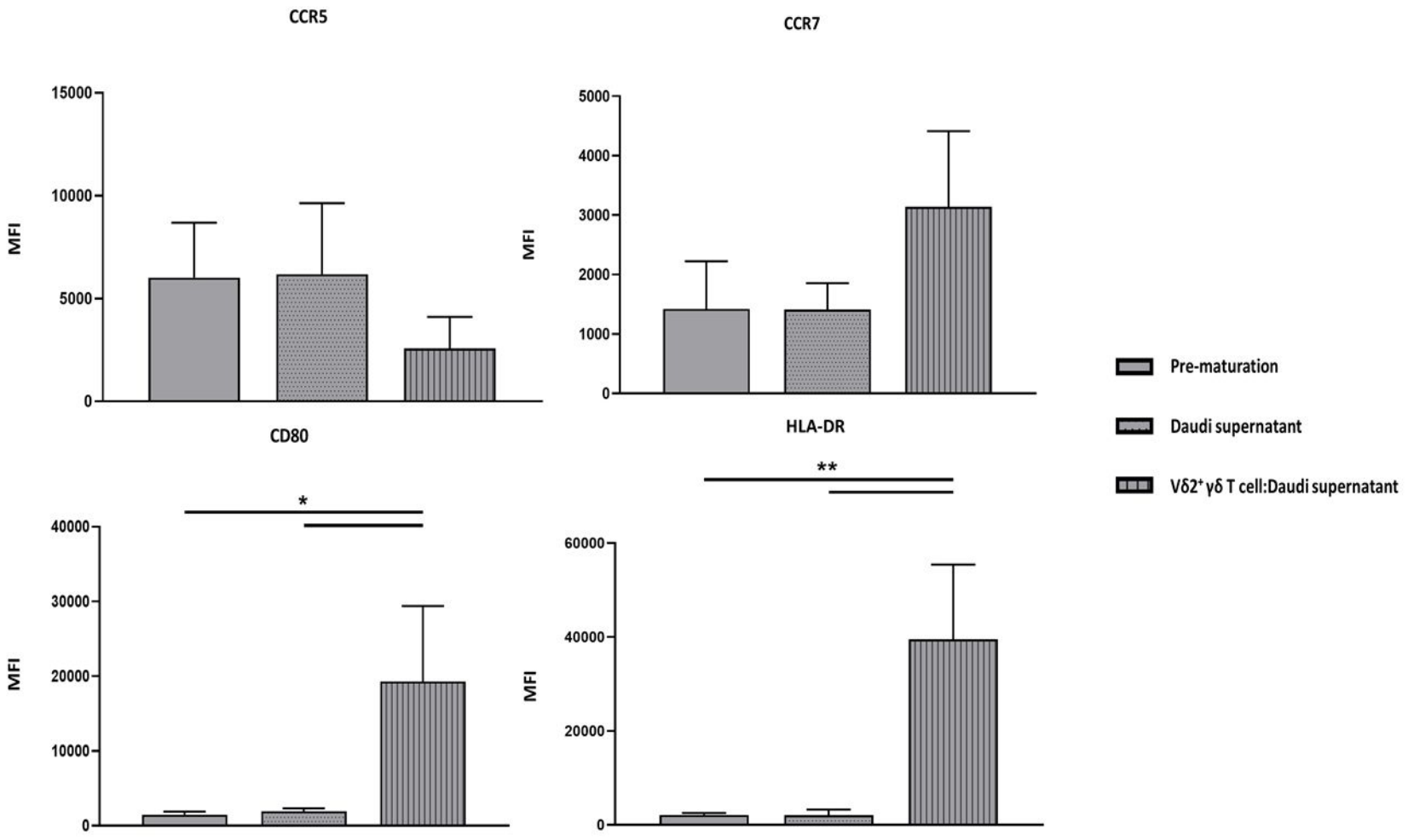

CCR5
MFI
15000
10000
5000
0
CCR7
MFI
5000
4000
3000
2000
1000
0
CD80
*
MFI
40000
30000
20000
10000
0
HLA-DR
**
MFI
60000
40000
20000
0
Pre-maturation
Daudi supernatant
Vδ2+ γδ T cell:Daudi supernatant

Figure S4: Granulysin-containing supernatants can cause maturation of immature DC

*Changes in MFI of cell surface markers CD80, CCR7, CCR5 and HLA-DR on monocyte-derived immature DC following culture with supernatants taken from the 48 hour co-culture of Vδ2$^+$ γδ T cells with Daudi tumour cells (containing 11-52ng/ml granulysin)), as determined by flow cytometry. Treatment of cells with supernatant taken from the culture of Daudi cells alone was included as a negative control. Data shown is the average taken from 3 individual donors. Differences between groups were assessed by two-way ANOVA comparing negative controls (pre-maturation and medium alone) with all other groups. *=p<0.05. **=p<0.01.*

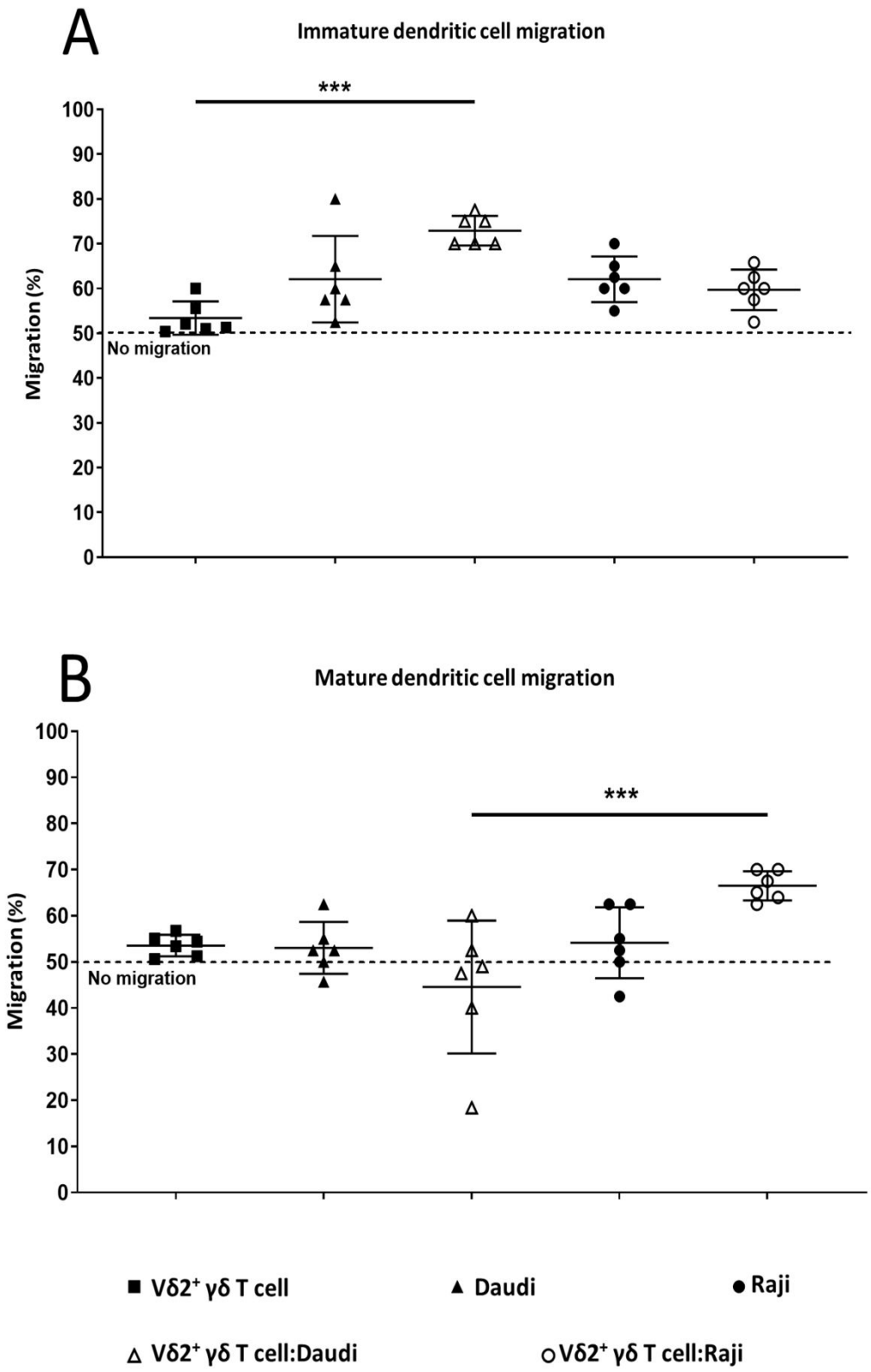

A
Immature dendritic cell migration
***
Migration (%)
No migration
B
Mature dendritic cell migration
***
Migration (%)
No migration
Vδ2⁺ γδ T cell
Daudi
Raji
Vδ2⁺ γδ T cell:Daudi
Vδ2⁺ γδ T cell:Raji

Figure S5: Granulysin-containing supernatants cause differential migration of immature and mature DC

*A) The migration of immature DC in response to supernatants taken from cultures of Daudi or Raji tumour cell lines alone, supernatants taken from cultures of Vδ2$^+$ γδ T cells alone, or supernatants taken from the 48 hour co-cultures of Vδ2$^+$ γδ T cells with Daudi or Raji tumour cell lines, as determined by Ibidi μ-migration assays. B) The migration of mature DC in response to supernatants taken from cultures of Daudi or Raji tumour cell lines alone, supernatants taken from cultures of Vδ2$^+$ γδ T cells alone, or supernatants taken from the 48 hour co-cultures of Vδ2+ γδ T cells with Daudi or Raji tumour cell lines, as determined by Ibidi μ-migration assays. Data shown is from 6 independent experiments using immature and LPS-matured DC differentiated from the monocytes of 6 individual donors, with error bars (SD). Differences between groups were assessed using one-way ANOVA. ***=$p<0.001$*