

Research paper

# Three-colour flow cytometric method to measure antibody-dependent tumour cell killing by cytotoxicity and phagocytosis

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## Abstract

We have developed a three-colour flow cytometric method to assay the contributions of cytotoxicity and phagocytosis to antibody-dependent cell-mediated tumour cell killing. In this assay, tumour target cells are pre-labelled with CFSE, and mixed with effector cells and a tumour antigen-specific monoclonal antibody. After incubation of the cells with the antibody, effector cells are labelled with PE and dead cells with PI. Using flow cytometry, dead and phagocytosed tumour cells can be quickly and easily counted and the numbers summed to determine the total number of killed cells. One can thereby measure the phagocytic aspect of antibody-dependent cell-mediated tumour cell killing, otherwise only revealed by microscopic examination. The failure to detect phagocytosed, in addition to live and dead target cells, by standard assays may result in an underestimation of tumour cell killing and hence the potential of an antibody for immunotherapy of cancer. We illustrate the new method by analysing human monocyte-mediated cytotoxic and phagocytic cell killing of IGROV1 ovarian tumour cells by the ovarian tumour antigen-specific anti-folate binding protein monoclonal antibody, MOv18 IgE.

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**Keywords:** Antibody-dependent cell mediated cytotoxicity/phagocytosis; MOv18 IgE; NIP IgE; Flow cytometry; U937 cells; Monocytes; Folate binding protein

**Abbreviations:** ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cell-mediated phagocytosis; FBP, folate binding protein; LDH, lactate dehydrogenase; PE, Phycoerythrin; PI, Propidium Iodide; NIP, 4-hydroxy-3-nitro-phenacetyl; CFSE, Carboxy-fluorescein diacetate, succinimidyl ester; FCM, fluorocytometric assay; CM, complete medium; E:T, effector:target; FSC, forward scatter; SSC, side scatter.

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## 1. Introduction

In previous work we demonstrated that IgE might be more effective than IgG for the immunotherapy of solid tumours (Gould et al., 1999; Karagiannis et al., 2003). In the course of this work we sought to understand the mechanisms by which IgE effector cells mediate the killing of tumour cells. We employed a monoclonal antibody (MOv18), directed against an ovarian tumour-specific-antigen, folate binding protein (FBP), monocytes

as effector cells and the ovarian tumour cell line, IGROV1, which expresses FBP, as target cells (Benard et al., 1985; Campbell et al., 1991; Coney et al., 1991; Tomassetti et al., 1993; Toffoli et al., 1997).

IgE has two cell-bound receptors, the high-affinity receptor, FcεRI, and the low-affinity receptor, CD23 (Gould et al., 2003). The former is homologous to IgG receptors, which mediate antibody-dependent cellular cytotoxicity (ADCC) of tumour cells (Ravetch and Kinet, 1991; Clynes et al., 1998, 2000; Dyall et al., 1999; Carter, 2006). The low-affinity IgE receptor, CD23, is a member of the C-type lectin superfamily, and has no counterpart among IgG receptors (Gould et al., 2003). CD23, expressed in monocytes, mediates the phagocytosis of hapten-coated red cells (Yokota et al., 1992). We have shown for the first time, by fluorescence microscopy, that IgE receptors on monocytes can also mediate phagocytosis of tumour cells (Karagiannis et al., 2003). We therefore sought to develop an assay to separately quantify IgE antibody monocyte-mediated tumour cell killing by cytotoxicity (ADCC) and phagocytosis (ADCP). Here, we describe a new three-colour cytometric assay that achieves this purpose.

Standard cytotoxicity (ADCC) assays, such as Chromium-51 ( $^{51}\text{Cr}$ ) release (Brunner et al., 1968) and the lactate dehydrogenase (LDH) release (Korzeniewski and Callewaert, 1983), rely on the loss of membrane integrity, associated with cell death, to release an intracellular substance (in some cases, pre-loaded) into the medium, where it can be measured. In the event of phagocytosis, however, these markers are instead released into phagocytes, where they go undetected. The three-colour flow cytometric assay we describe overcomes this problem. It employs three fluorophores to quantify phagocytosis in addition to cytotoxicity. It is based on the same principle as the fluorocytometric (FCM) assay described by Derby et al. (2001), in which the third fluorophore gives information about the mechanism of cytotoxicity, distinguishing the contributions of lytic granule- and Fas Ligand/TNF-mediated apoptosis.

The assay we describe involves labelling of tumour cells with CFSE (*Carboxy-fluorescein diacetate, succinimidyl ester*), a fluorescent dye normally used to monitor cell proliferation, prior to incubation of antibody with the tumour and effector cells. Following incubation, effector cells are distinguished by Phycoerythrin (PE) labelling of an antigen absent on target cells. Dead cells are labelled with Propidium Iodide (PI). Cell death is then assessed by flow cytometry, whereby five populations can be clearly distinguished: live targets ( $\text{CFSE}^+$ ,  $\text{PE}^-$ ,  $\text{PI}^-$ ), dead targets ( $\text{CFSE}^+$ ,  $\text{PI}^-$ ,  $\text{PI}^+$ ), live effectors

( $\text{CFSE}^-$ ,  $\text{PE}^+$ ,  $\text{PI}^-$ ), dead effectors ( $\text{CFSE}^-$ ,  $\text{PE}^+$ ,  $\text{PI}^+$ ) and phagocytosed target cells ( $\text{CFSE}^+$ ,  $\text{PE}^+$ ,  $\text{PI}^-$ ). The principle of this assay is shown schematically in Fig. 1. We have applied this assay to determine the contribution of cytotoxicity and phagocytosis to MOv18 IgE antibody killing of IGROV1 ovarian tumour target cells by monocytic effector cells, including cells of the U937 cell line and primary peripheral blood monocytes.

## 2. Materials and methods

### 2.1. Antibodies and reagents

Chimeric antibodies MOv18 IgE against the FBP and NIP IgE specific for the hapten 4-hydroxy-3-nitrophenacetyl (NIP) were prepared as before (Gould et al., 1999; Neuberger et al., 1985). We used anti-CD89-PE (BD Biosciences, Oxford, UK) and PE-conjugated mouse IgG isotype-matched control antibody (Dako, Glostrup, Denmark). Propidium Iodide (PI), CFSE dye, tissue culture media, and reagents were from Invitrogen (Paisley, UK). Flow cytometric evaluations of fixed (dead) cells were performed using IntraStain Fixation/Permeabilisation Kit (Dako).

### 2.2. Cells

The human ovarian carcinoma cell line IGROV1 (Benard et al., 1985) and the myelomonocytic cell line U937 (Prof. J.P. Kinet, Harvard University, USA) (Sundström and Nilsson, 1976) were maintained in complete medium (CM), consisting of RPMI 1640, with FCS (10%), glutamine (2 mM), penicillin (5000 U/ml) and streptomycin (100 µg/ml) (Invitrogen), at 37 °C in 5%  $\text{CO}_2$ . Monocytes purified from human venous blood were isolated to >70% purity (Karagiannis et al., 2003) by Rosette-Sep™ human monocyte enrichment cocktail treatment (StemCell Technologies, Vancouver, Canada), followed by Ficoll–Paque gradient centrifugation (Amersham Biosciences, NJ, USA). Monocytes were washed in PBS, 2% FCS, 2 mM EDTA and treated with neuraminidase ( $0.3 \text{ U}/2 \times 10^7$  cells) (Sigma-Aldrich, Dorset, UK) prior to assays (Reischl et al., 1996; Karagiannis et al., 2003). All work was performed with the approval of the Guy's Research Ethics Committee and with the blood volunteers' written informed consent.

### 2.3. Effector cell preparation

Monocytic cells were distinguished from IGROV1 targets by labelling with anti-CD89-PE (BD Biosciences) (Fig. 2). The IgA Fc receptor, CD89, was

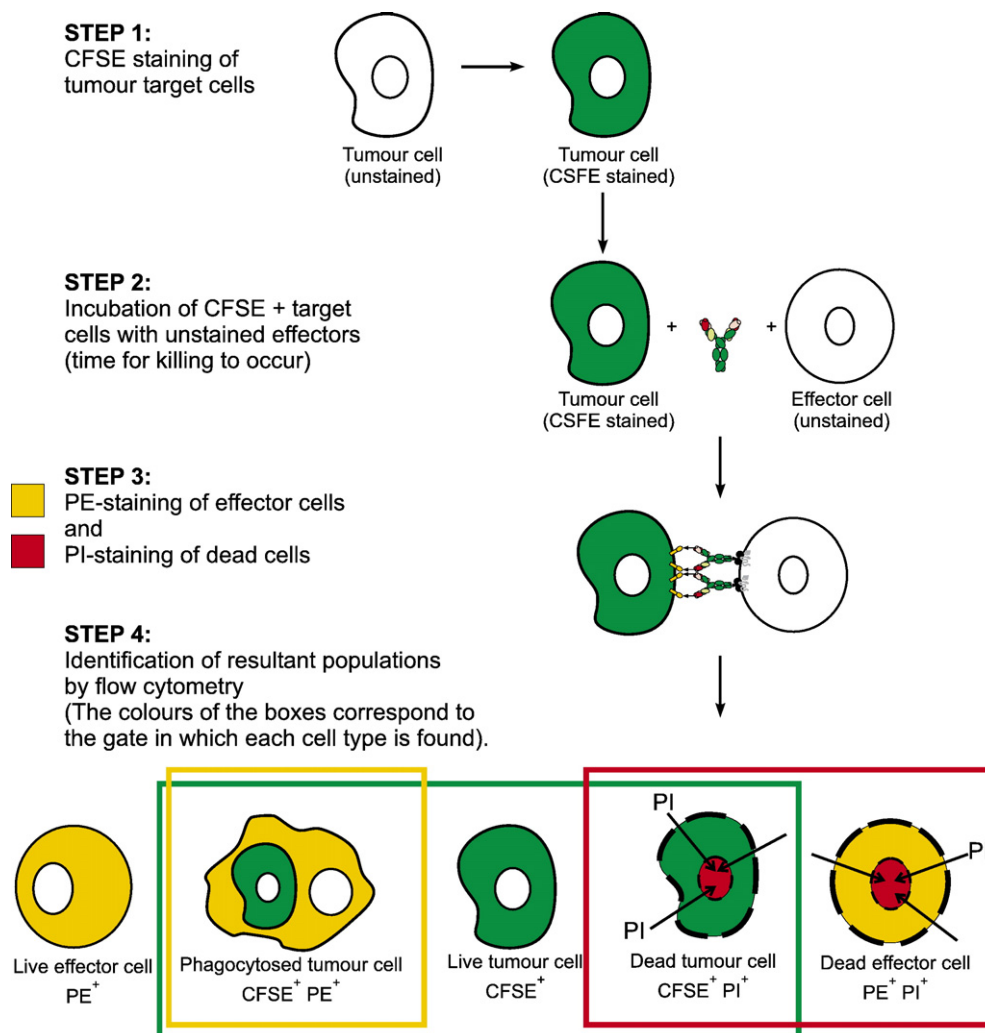


Fig. 1. Schematic representation of the design and set-up of the three-colour flow cytometric assay to simultaneously measure cytotoxicity and phagocytosis. Tumour cells are pre-labelled with CFSE (Step 1), effector and tumour cells are mixed with or without antibodies and incubated at 37 °C (Step 2). After the incubation, cells are stained with PE-conjugated antibody to label effector cells and with PI to label dead cells (Step 3). The resultant populations are identified by flow cytometry using the three fluorophores (Step 4). The coloured boxes correspond to the gates used in Fig. 3C: the green box shows the populations comprising total tumour target cells (Region 1), the orange box depicts phagocytosed tumour cells (Region 2), and the red box shows dead tumour target cells (Region 3).

chosen on the basis of its high expression by U937 (Fig. 2A) and blood monocytes (Fig. 2B) and absence on IGROV1 target cells (Fig. 2C). Effector cells were stained at 4 °C after the killing assay, so as not to interfere with the killing mechanism. We found the optimal concentration of anti-CD89-PE to be 10 µg/ml with  $6.5 \times 10^5$  cells/ml.

#### 2.4. Target cell preparation

The FBP-expressing ovarian cancer cell line, IGROV1, was used as the tumour target (Benard et al.,

1985; Tomassetti et al., 1993). For CFSE staining, IGROV1 cells ( $50 \times 10^6$ /ml) were incubated with 20 µl of 5 mM CFSE in PBS for 10 min at 37 °C (Fig. 2D). Cells were washed twice in CM at 4 °C to stop the reaction and culture conditions resumed for 24 h prior to assays. This was done to allow recovery from staining and minimise background death, but also to allow reduction of the intensity of CFSE staining to a level where any leakage into the FL2 channel could be compensated for by adjustment of settings on the flow cytometer. Where PI<sup>+</sup> dead target cells were required as positive controls, cells were killed by formaldehyde

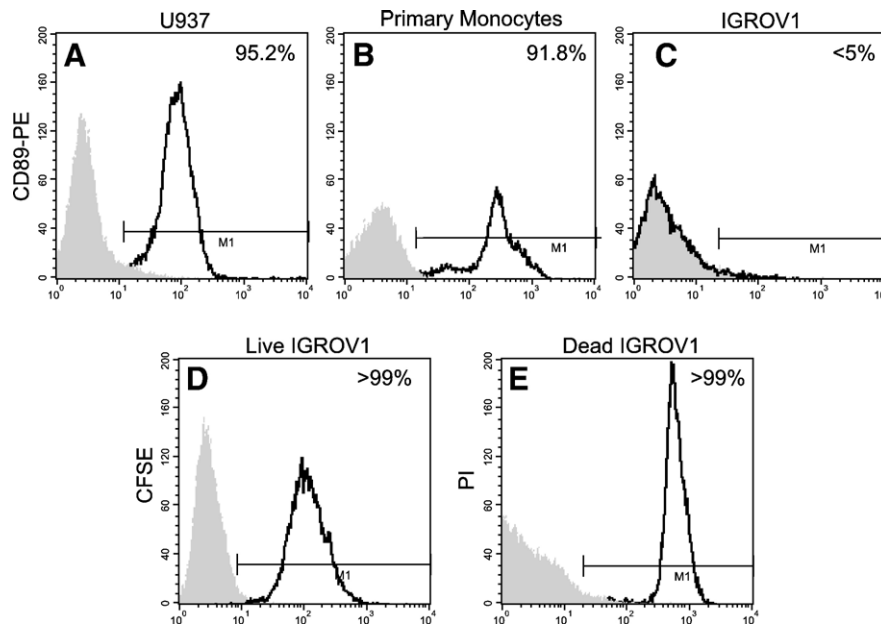


Fig. 2. Immunofluorescence profiles of the labelled effector and target cell populations used to set up the three-colour flow cytometric assay. An anti-CD89-PE mAb was employed to label effector cells: U937 monocytic cells (A) and primary peripheral blood monocytes (B) express CD89, while the ovarian carcinoma cell line IGROV1 does not (C). Cells stained with anti-CD89-PE (solid line white histograms) and background fluorescence of the PE-conjugated mouse IgG1 isotype control (grey histograms). Live IGROV1 tumour target cells were labelled with CFSE (white solid line histogram) and unlabelled live IGROV1 cells (grey histogram) (D). Dead CFSE<sup>+</sup> tumour cells were stained with PI (solid line white histogram) and dead CFSE<sup>+</sup> tumour cells not given PI (grey histograms) (E).

fixation and membranes permeabilised using the IntraStain kit (Dako) according to the manufacturer's instructions (Fig. 2E).

### 2.5. Cell incubations and labelling for the cytotoxicity/phagocytosis assay

Labelled IGROV1 tumour target cells (CFSE<sup>+</sup>) were incubated in triplicate in 12×75 mm FACS tubes (Falcon, BD Biosciences) with unstained effector cells and MOv18 IgE, control NIP IgE or without antibody at 37 °C, 5% CO<sub>2</sub>. The total number of cells in each tube was  $2.6 \times 10^5$  in a 400 µl volume (effector and target cell numbers were adjusted according to the effector:target ratio required). MOv18 IgE was titrated and shown to give maximal cell killing at 5 µg/ml. Following an incubation at 37 °C (optimised for maximum killing at 2.5 h), cells were washed in FACS buffer (PBS, 5% normal goat serum) at 4 °C and effector cells were labelled with anti-CD89-PE (10 µg/ml) for 25 min at 4 °C. Following a further wash, cells were labelled for 15 min at 4 °C with PI (optimised at 0.25 µg/ml) to identify dead cells. After a final wash, cells were mixed thoroughly to interrupt cell–cell contact and re-sus-

pended at 250 µl/tube in FACS buffer at 4 °C for immediate analysis.

### 2.6. Flow cytometry

Cells were analysed on a dual laser FACSCalibur™ (BD Biosciences). A threshold was set on a Forward Scatter vs. Side Scatter (FSC/SSC) dot plot to exclude the minimal amount of cell debris. To ensure that measurements of single cell events were collected, acquisition and measurement of single cell events were monitored by FSC/SSC dot plots and compared to control single- and mixed-population samples. Cells were excited by an Argon ion laser emitting at 488 nm. CFSE-labelled cells were detected in the FL1 channel (530/30 nm band pass filter). PE-labelled cells were detected in the FL2 channel (585/42 nm band pass filter). PI<sup>+</sup> dead cells were detected in the FL3 channel (670 nm LP band pass filter). Photomultiplier voltages were set to place the CFSE<sup>+</sup> cells predominantly in the third log decade of the FL1 channel and the first log decade of the FL2 channel. Voltage and compensation adjustments were made using a series of control tubes, such that PE<sup>+</sup> cells were placed predominantly in the second log decade of the FL2

channel and the first log decade of both FL1 and FL3 channels, and that PI<sup>+</sup> cells were placed in the fourth log decade of the FL3 channel and first log decade of both FL1 and FL2 channels. All settings were kept constant for all ADCC/ADCP assay experiments.

### 2.7. Immunofluorescence imaging and measurements of cell–cell interactions

Cell–cell contact and phagocytosis were visualised by two-colour immunofluorescent imaging. CFSE-labelled IGROV1 tumour cells were incubated in glass chamber slides (Lab-Tek II, SLS, Nottingham, UK) with unstained effector cells and either no antibody, control NIP IgE or MOv18 IgE (5 µg/ml) for 2.5 h at 37 °C, 5% CO<sub>2</sub>, and washed twice with FACS buffer at 4 °C. U937 effector cells were then stained with anti-CD89-PE (20 µg/ml) for 25 min at 4 °C. Slides were washed twice, fixed in 1% paraformaldehyde and mounted in fluorescence preserver (Dako). Slides were observed at an original magnification of 40× under an Axioscop 20 upright Carl Zeiss microscope using a Zeiss A-Plan 40×/0.65 Ph2 lens, an AxiCam camera (Carl Zeiss Ltd., Hertfordshire, UK) and AxioVision Version 3.0.2 imaging system (Imaging Associates Ltd., Oxfordshire, UK). Tumour:effector cell interactions were measured on 10 microscopic fields per sample condition, by counting the total number of IGROV1 tumour cells and U937 cells in contact with tumour cells per field. U937 cells that made contact with tumour cells and also contained green-fluorescent material, depicting phagocytosis, were also measured. For each condition, effector and target cell interactions were expressed as % Mean±SD.

### 2.8. Data handling and statistical analysis

Flow cytometric assays were performed in triplicate and data are shown as mean ADCC±SD and ADCP±SD of 4 independent experiments (*n*=4). Statistical analyses of ADCC/ADCP assays and quantitative observations of cell interactions by microscopy were performed by means of the unpaired two-tailed Student's *t* test, and significance was accepted at *p*<0.05.

## 3. Results

### 3.1. Flow cytometric ADCC and ADCP assay set up

Voltage and compensation adjustments were made using a series of control tubes performed for each effector or target cell population (Fig. 3). These included live

unstained effectors, live PE<sup>+</sup> effectors, live CD89-PE-, and PI-stained effectors (Fig. 3A). IGROV1 target cell population controls included live CFSE<sup>+</sup> and live CFSE<sup>+</sup> with and without CD89-PE, live CFSE<sup>+</sup> and PI-stained, or killed (fixed and permeabilised) CFSE<sup>+</sup> and PI-stained targets (Fig. 3B).

### 3.2. Calculation of ADCC and ADCP

Twenty thousand events were acquired from each tube and dot plots were created to calculate the proportion of tumour cells killed by ADCC or ADCP (Fig. 3C); gates were set with the control tubes used to optimise instrument settings (Fig. 3A and B). Region 1 (R1, green gate, Fig. 3C) included all CFSE<sup>+</sup> events acquired, regardless of their PI and PE status. When effectors and targets were mixed at an original ratio of 1:1, R1 was expected to contain approximately 10,000 events, representing half of the 20,000 events acquired in total. Due to both background death, perhaps by apoptosis during the assay, and additional antibody-mediated death, the number of acquired events in R1 was consistently lower than 10,000, reflecting the increase in ratio of live effectors to target cells.

To quantify the 'background' R1 population, and thus determine the number of IGROV1 cells acquired, the number of events in R1 of each of three no-antibody control tubes (containing only effector and target cells) was averaged, and referred to as the "R1 spontaneous loss (SL) control". For each sample (MOv18 IgE, NIP IgE or no Ab control) the difference between its R1 value and the R1 SL control was determined. The amount by which the number of events in R1 is reduced represents the proportion of tumour cells that had been killed and fragmented to the extent that they disappeared below the FSC threshold and were therefore unrepresented on the FACS plot. For the purposes of MOv18 IgE/U937-mediated tumour cell death, the assumption was made that the death of this population of killed IGROV1 cells did not involve phagocytosis. The justification for this is based on the assumption that the time frame of the assay (2.5 h) was insufficient for tumour cell phagocytosis, death, complete disintegration of the tumour cells and total quenching of the CFSE dye inside effector cells.

Region 2 (R2, orange box, Fig. 3C) was set to include all CFSE<sup>+</sup>/PE<sup>+</sup> events, regardless of their PI status. This region reflects the number of tumour cells that have been phagocytosed by effector cells, with each event representing a CFSE<sup>+</sup> tumour target (or portion of) inside a PE-positive effector cell. To ascertain that the CFSE<sup>+</sup>/PE<sup>+</sup> events detected represented tumour cell phagocytosis and

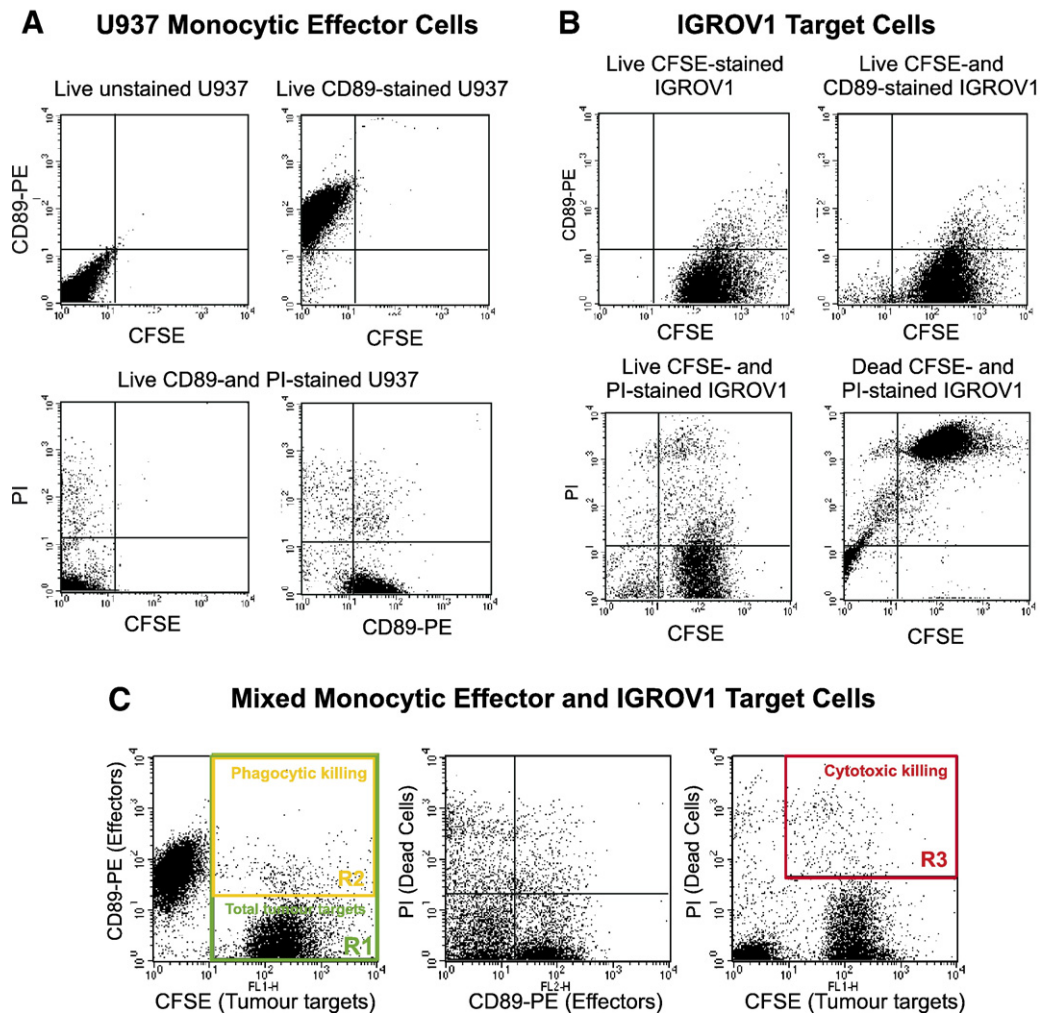


Fig. 3. Example of the three-colour flow cytometric assay set-up. Control cell populations of U937 monocytic cells (A) and ovarian cancer IGROV1 cells (B) used for instrument settings and determination of where Regions should be set. (C) Dot plots of mixed U937 monocytic effector and tumour target cells from which calculations were made: Region 1 (R1, green) includes events representing total CFSE<sup>+</sup> tumour cell targets. The CFSE<sup>+</sup> tumour cell targets may be present within PE-stained effector cells (where they have been phagocytosed); these events are found in Region 2 (R2, orange). Region 3 (R3, red) contains tumour targets killed externally by effector cells (cytotoxicity) and are thus CFSE<sup>+</sup>/PI<sup>+</sup>. Schematic representations of cells found within these gates are depicted in Fig. 1.

not cell–cell contact, all samples were thoroughly mixed at the end of incubations to disrupt cell interactions and FSC/SSC dot plots were used to monitor acquisition of single-cell events. Where the mean fluorescence intensity of the CFSE<sup>+</sup>/PE<sup>+</sup> population in the FL1 channel remained equal to that of the corresponding CFSE<sup>+</sup>/PI<sup>−</sup> population, it may represent phagocytosis of one target cell by one effector cell.

Region 3 (R3, red box, Fig. 3C) was set to include CFSE<sup>+</sup>/PI<sup>+</sup> events. This reflects the population of IGROV1 tumour target cells (CFSE<sup>+</sup>) killed by cytotoxicity, such that their membranes have lost integrity

sufficient for PI to enter and fluoresce, but not fragmented to the extent that they have become undetectable as single events (and formed part of the population responsible for deviations in the R1 value from the 10,000 events expected to be acquired with a 1:1 E:T ratio). The final calculation for determination of cells killed by cytotoxicity and phagocytosis, was therefore as follows:

Percentage of IGROV1 cells killed by cytotoxicity (ADCC):

$$R1 \text{ SL control} - R1 = X.$$

$$[(X + R3)/R1 \text{ SL Control}] \times 100$$

Percentage of IGROV1 cells phagocytosed (ADCP):  
[R2/R1 SL Control]  $\times 100$

### 3.3. Identification of target cell death by ADCC and ADCP

FSC/SSC dot plots of single population samples (Fig. 4A) were compared to those with mixed U937 and IGROV1 populations to monitor acquisition of single cell events (Fig. 4B and C, top panels). In ADCC/ADCP flow cytometric killing assays, at a 1:1 effector:target ratio, all FSC/SSC dot plots of mixed effector and target cells (Fig. 4B, top panel) had similar size and granularity to those acquired in single effector and target cell populations seen in Fig. 4A. This suggested that single cell events were acquired. Target cells phagocytosed by U937 effector cells were identified as a population of CFSE<sup>+</sup>/PE<sup>+</sup> cells within Region 2, (Fig. 4B, middle panel, R2 orange boxes), and measured using the equation described in Section 3.2. These showed that in the presence of MOv18 IgE, low levels of tumour cells phagocytosed by U937 effector cells could be detected compared with controls.

Dead target cells were distinguished as CFSE<sup>+</sup>/PI<sup>+</sup> cells in Region 3 (Fig. 4B, R3 bottom row, red boxes) and quantified using the equation described in Section 3.2. In the presence of MOv18 IgE, low levels of PI-positive tumour cells were detected (Fig. 4B bottom right, red box), compared to those seen with the control NIP IgE (bottom middle) or in the absence of antibody (bottom left).

### 3.4. Enhanced phagocytosis of IGROV1 cells

When the ratio of U937 effectors to IGROV1 tumour target cells was increased to 2:1, all FSC/SSC dot plots of mixed effector and target cells (Fig. 4C, top panel) revealed cells of similar size and granularity to those acquired in single cell populations (Fig. 4A), confirming acquisition of single cell events. U937 cells displayed enhanced MOv18 IgE-dependent phagocytosis of IGROV1 tumour cells, compared to controls (Fig. 4C, middle panel, R2 orange boxes), which was maximal after 2.5 h. This was in agreement with previous published work describing macrophage-mediated phagocytosis (Jadus et al., 1998). The identity of the FL1 signal intensity for both the CFSE<sup>+</sup>/PE<sup>+</sup> and CFSE<sup>+</sup>/PI<sup>+</sup> populations indicates that one target cell is being phagocytosed by one effector cell. As in assays using effector:target ratio of 1:1, U937 cells used at a ratio of 2:1 mediated MOv18 IgE-dependent cytotoxicity of tumour cells, manifested by an increased CFSE<sup>+</sup>/PI<sup>+</sup> tumour cell population (R3, red box, bottom right plot), compared to controls (bottom middle and left).

### 3.5. Quantification of tumour cell death

Fig. 4D and E show total tumour target cell killing by cytotoxicity (black bars) and phagocytosis (white bars) in the presence of either no antibody, control NIP IgE or MOv18 IgE ( $n=4$ ). At an effector:target ratio of 1:1, the proportion of tumour cells killed by ADCC was  $33.3 \pm 6.1\%$  with MOv18 IgE compared to  $8.4 \pm 1.8\%$  with NIP IgE and  $6.5 \pm 1.2\%$  in the absence of antibody (Fig. 4D). At the same effector:target ratio of 1:1, U937 monocytes phagocytosed a small proportion,  $10 \pm 1.9\%$ , of IGROV1 cells, compared to  $4.6 \pm 1.9\%$  with NIP IgE and  $4.7 \pm 1.3\%$  without antibody. At an effector:target ratio of 2:1, U937 monocytes mediated MOv18 IgE-dependent ADCC of tumour cells, measured at  $31.2 \pm 6.4\%$ , compared to  $7.7 \pm 2.2\%$  with NIP IgE and  $7.2 \pm 1.8\%$  for the no antibody controls (Fig. 4E). With MOv18 IgE, the proportion of phagocytosed cells was  $16.8 \pm 2.9\%$ , significantly greater than  $5.8 \pm 1.4\%$  with NIP IgE, or  $4.6 \pm 0.8\%$  with the no antibody control. U937 monocytes assayed with MOv18 IgE and tumour cells at the effector:target ratio of 2:1 exerted significantly greater ADCP of IGROV1 cells than at the 1:1 ratio ( $p=0.0078$ ). In contrast, MOv18 IgE-dependent ADCC of tumour cells was not affected by this difference in effector:target ratio ( $p=0.65$ ).

### 3.6. Observation and quantification of phagocytosis by immunofluorescent imaging

To confirm that CFSE<sup>+</sup>/PE<sup>+</sup> cells detected by flow cytometry were a true representation of phagocytosed IGROV1 tumour cells by U937 monocytes, CFSE<sup>+</sup> IGROV1 cells were co-cultured with U937 cells in the presence of NIP IgE or MOv18 IgE. After 2.5 h, effector cells were stained with anti-CD89-PE, contact between cells were visualised (Fig. 5) and effector:target cell interactions were measured (Table 1). In accordance with results obtained using the flow cytometric assay (Fig. 4), when the effector:target ratio was 1:1, contact of effector (red) and target (green) cells was evident in the presence of MOv18 IgE (Fig. 5A), but not with NIP IgE (Fig. 5B). We measured 39.7% of IGROV1 tumour cells in contact with U937 cells with MOv18 IgE compared to 8.4% contact with NIP IgE (Table 1). Only 8.0% of tumour cells were in contact with effector cells that engaged in tumour cell phagocytosis compared to even fewer seen with NIP IgE (4.1% of tumour cells) confirming low or undetectable phagocytosis of IGROV1 cells (Fig. 5A).

When the effector:target ratio was 2:1, ample contact (44.4%) of target cells with effector cells was evident in the presence of MOv18 IgE (Fig. 5C, long arrow),

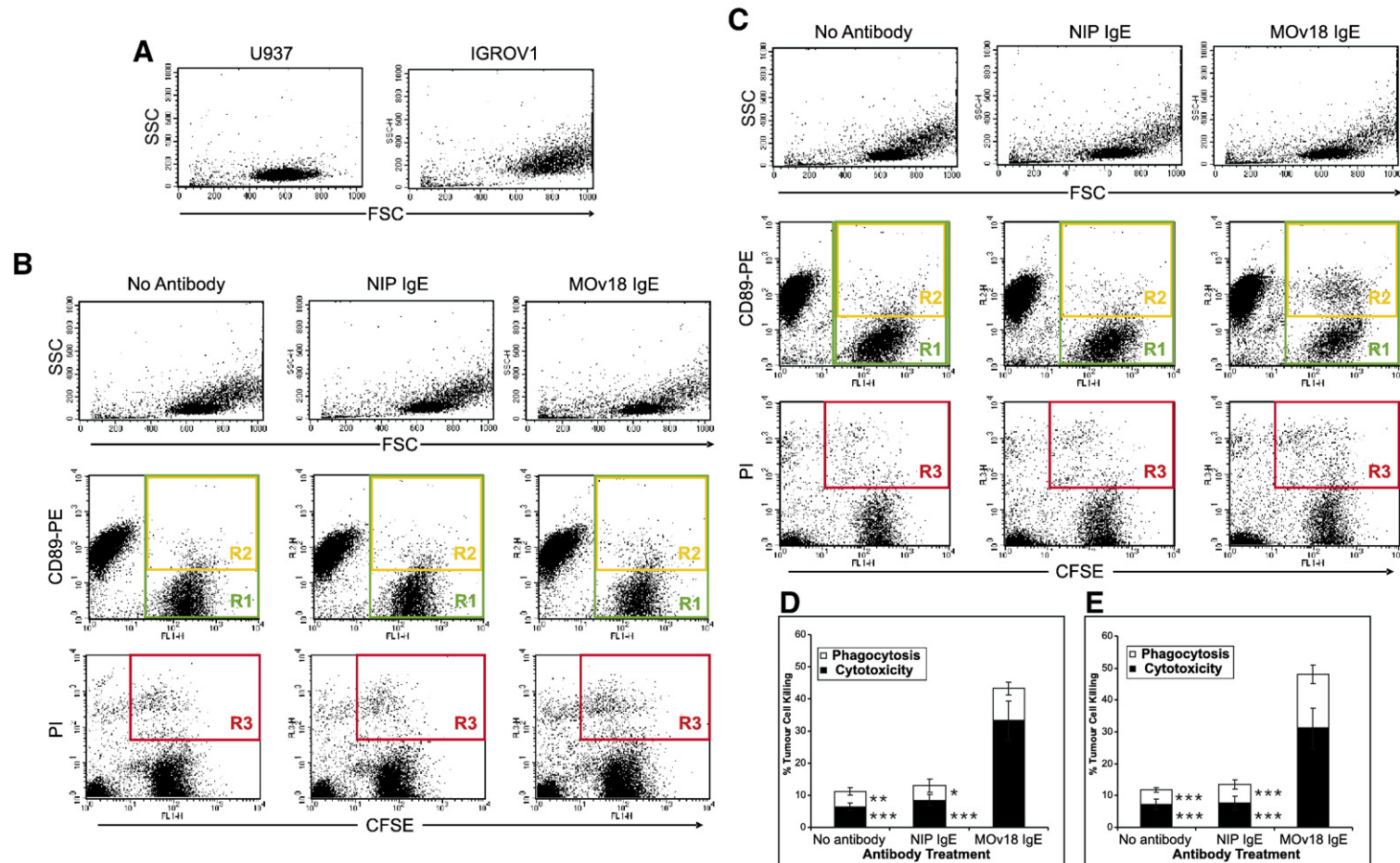


Fig. 4. Use of the three-colour flow cytometric assay to measure cytotoxicity and phagocytosis of IGROV1 ovarian tumour cells by U937 monocytic effector cells. Forward Scatter vs. Side Scatter (FSC/SSC) dot plots of single U937 (left) and IGROV1 (right) populations (A). U937 and IGROV1 cells at effector:target ratios of 1:1 (B) and 2:1 (C). FSC/SSC dot plots of mixed U937 and IGROV1 cell populations (top) and two-colour fluorimetric dot plots showing phagocytosis (middle) and cytotoxicity (bottom). Coloured boxes represent the gates set in Fig. 3C: R1 (green) total tumour target cells, R2 (orange) phagocytosed tumour cells, and R3 (red) dead tumour target cells, following incubations with no antibody (left), NIP IgE (middle) or MOv18 IgE (right). Quantitation of IGROV1 tumour cell killing by U937 cells and MOv18 IgE at effector:target ratios of 1:1 (D) and 2:1 (E). Cytotoxicity: black bars; phagocytosis: white bars. Results are means  $\pm$  SD of four independent experiments. Significance is shown for comparisons to samples given MOv18 IgE by the Student's *t*-test: n/s  $p > 0.05$ , \*  $p < 0.05$ , \*\*  $p < 0.005$ , \*\*\*  $p < 0.0005$ .

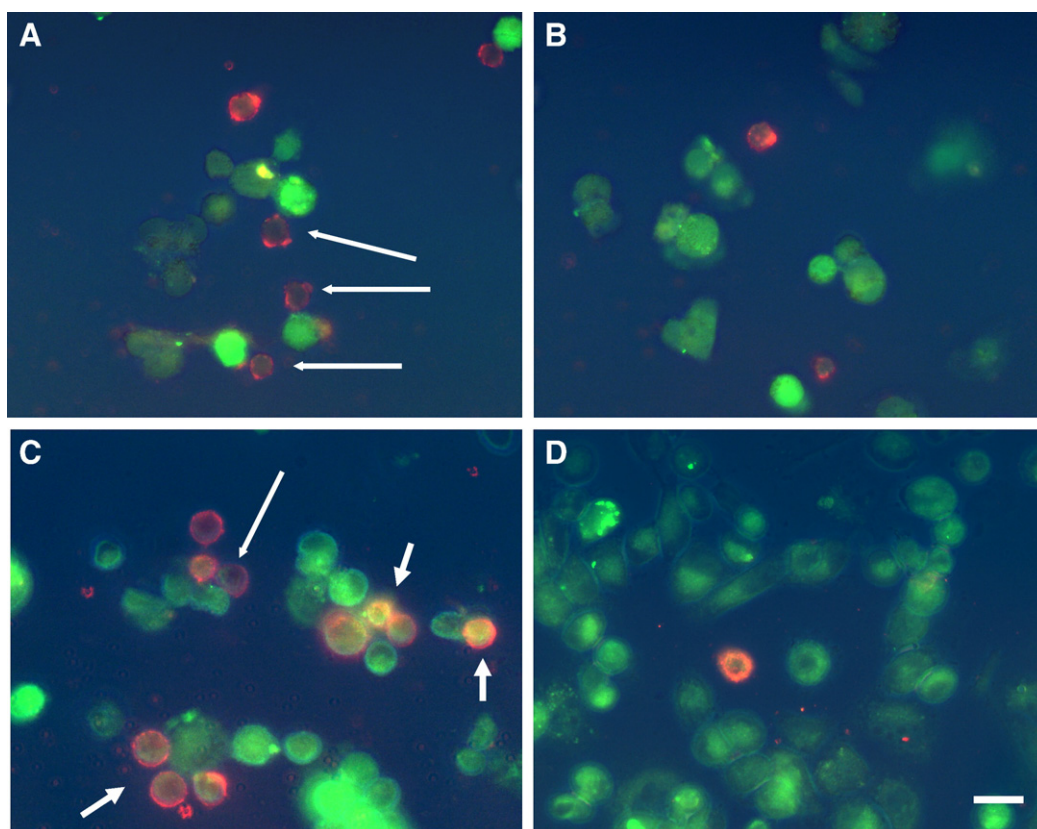


Fig. 5. Superimposed bright-field and fluorescent images of IGROV1-U937 interactions. CFSE-stained IGROV1 tumour cells (green) and CD89-PE-labelled U937 monocytic cells (red) were incubated for 2.5 h in culture. When U937 and IGROV1 cells at effector:target ratio of 1:1 (A) were incubated with MOv18 IgE, U937 (red) were in contact with IGROV1 cells (green, long white arrow) but not when incubated with NIP IgE (B). When U937 and IGROV1 cells at effector:target ratio of 2:1 (C) were incubated with MOv18 IgE, U937 cells (red) showed contact with IGROV1 (green, long white arrow) and enhanced phagocytosis of IGROV1 cells (green CFSE inside U937, yellow-orange, short white arrows). Reduced effector:target contact was observed with NIP IgE (D).

compared to 11.5% of IGROV1 cells with NIP IgE (Fig. 5D and Table 1). A proportion of these tumour cells (17.5%) were associated with effector cells that appeared orange/green in colour suggesting the presence of CFSE<sup>+</sup> target cell material within the PE-positive effector cells (Fig. 5C, short arrows). These CFSE<sup>+</sup>/PE<sup>+</sup> cells were not frequently observed with NIP IgE (5.3%) (Fig. 5D).

### 3.7. Analysis of MOv18 IgE antibody-dependent ADCC and ADCP of ovarian tumour cells by primary blood monocytes

Further to using U937 monocytic cells as effector cells, we studied IgE antibody-dependent IGROV1 tumour cell killing by human peripheral blood monocytes. These cells also express the CD89 antigen (Fig. 2C) and can therefore

Table 1  
Microscopic measurements of IGROV1:U937 cell interactions

| Treatment | E:T ratio 1:1                        |                                           | E:T ratio 2:1                        |                                           |
|-----------|--------------------------------------|-------------------------------------------|--------------------------------------|-------------------------------------------|
|           | % E:T contact (mean±SD) <sup>a</sup> | % E:T phagocytosis (mean±SD) <sup>a</sup> | % E:T contact (mean±SD) <sup>a</sup> | % E:T phagocytosis (mean±SD) <sup>a</sup> |
| MOv18 IgE | 39.7±13.9***                         | 8.0±4.2*                                  | 44.4 ± 15.0***                       | 17.5±6.9***                               |
| NIP IgE   | 8.4±5.6                              | 4.1±3.9                                   | 11.5±4.9                             | 5.3±3.2                                   |

<sup>a</sup> Mean±SD values were calculated from 10 microscopic fields for each sample condition. The Student's *t*-test was used to generate significance for MOv18 IgE compared to NIP IgE. <sup>n/s</sup>*p*>0.05, \**p*<0.05, \*\**p*<0.005, \*\*\**p*<0.0005.

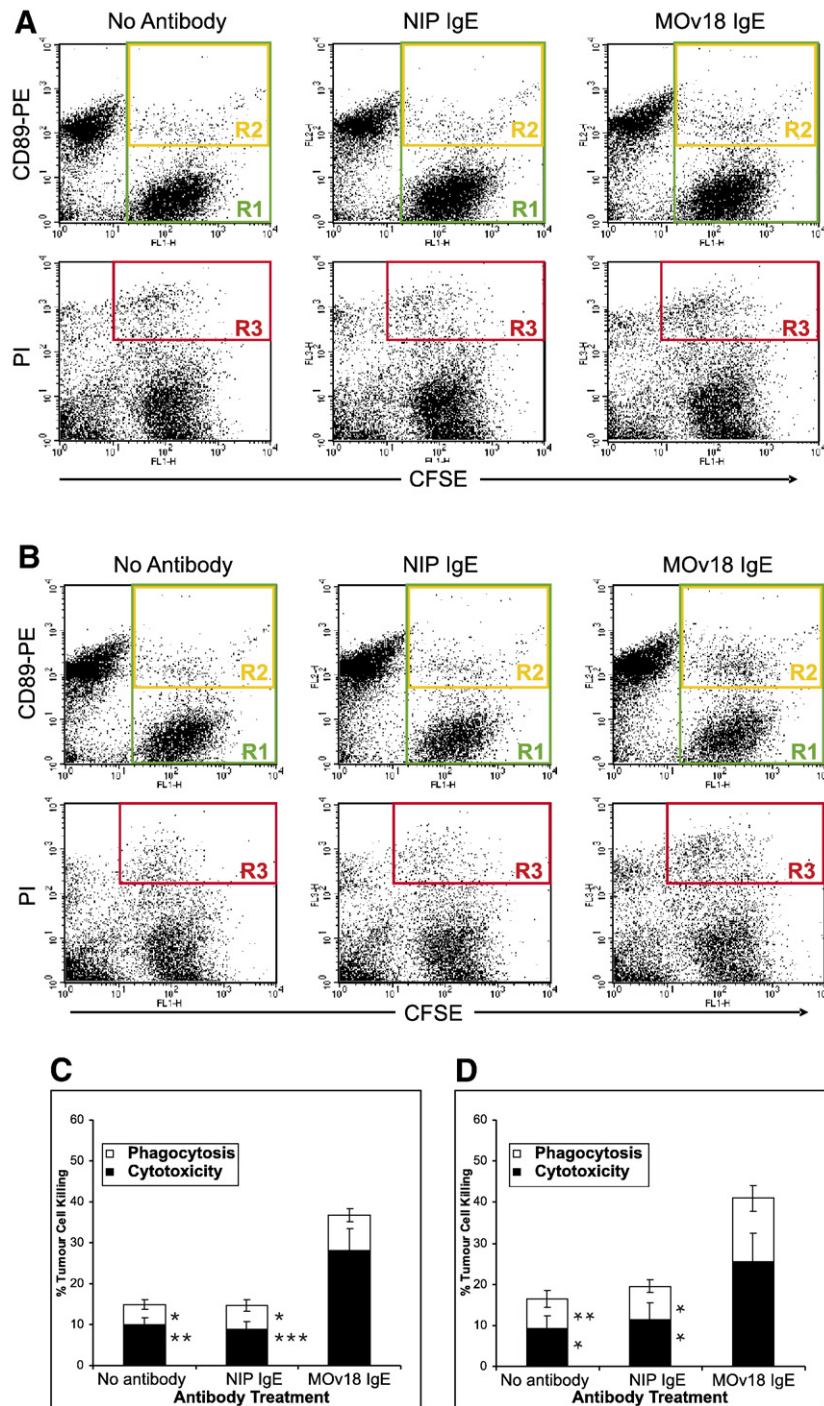


Fig. 6. The three-colour flow cytometric assay is used to measure cytotoxicity and phagocytosis of IGROV1 ovarian tumour cells by primary monocytes. Dot plots of purified monocytes incubated with IGROV1 cells at effector:target ratios of 1:1 (A) and 2:1 (B) with no antibody (left), NIP IgE (middle) or MOv18 IgE (right). The coloured boxes represent the gates set in Fig. 3C: R1 (green) shows total tumour target cells, R2 (orange) phagocytosed tumour cells, and R3 (red) dead tumour target cells. Quantification of IGROV1 tumour cell killing by peripheral blood monocytes and MOv18 IgE at effector:target ratios of 1:1 (C) and 2:1 (D). Cytotoxicity: black bars; phagocytosis: white bars. Results are means  $\pm$  SD of four independent experiments. Significance is shown for comparisons to samples given MOv18 IgE by the Student's *t*-test:  $n/s$   $p > 0.05$ ,  $*p < 0.05$ ,  $**p < 0.005$ ,  $***p < 0.0005$ .

be directly tested as effector cells in the assay system described above. At a 1:1 effector:target ratio, two colour fluorimetric dot plots show low levels of tumour cells phagocytosed by primary monocytes (Fig. 6A, top panel, R2 orange boxes). In the presence of MOv18 IgE, increased proportion of dead CFSE<sup>+</sup>/PI<sup>+</sup> IGROV1 cells was detected (Fig. 6A, bottom right, R3 red box), compared to controls, illustrating MOv18 IgE ADCC. When the ratio was increased to 2:1, similarly to U937 cells, monocytes displayed enhanced MOv18 IgE-dependent ADCP (Fig. 6B, top panel, R2 orange box) and ADCC (bottom panel, R3 red box) of IGROV1 tumour cells, compared to controls.

Bar graphs show the calculated tumour cell killing (Fig. 6C and D;  $n=4$ ). At an effector:target ratio of 1:1, MOv18 IgE-mediated ADCC was elevated,  $28.1 \pm 5.3\%$ , compared to  $8.9 \pm 1.9\%$  with NIP IgE and  $10 \pm 1.6\%$  without antibody (Fig. 6). ADCP measured at  $8.6 \pm 1.6\%$  with MOv18 IgE, compared with  $5.7 \pm 1.4\%$  for NIP IgE and  $4.9 \pm 1.2\%$  with no antibody. When the effector:target ratio was 2:1, monocytes mediated  $25.4 \pm 7.2\%$  MOv18 IgE-dependent ADCC of tumour cells, compared with  $11.3 \pm 4.2\%$  for NIP IgE and  $9.2 \pm 3.0\%$  with the no antibody control (Fig. 6D). With MOv18 IgE,  $15.6 \pm 3.2\%$  of tumour cells were phagocytosed, compared with  $8.2 \pm 1.6\%$  for NIP IgE and  $7.3 \pm 2.1\%$  with the no antibody control. Similarly to U937 cells, primary monocytes co-cultured with MOv18 IgE and tumour cells at an effector:target ratio of 2:1 exerted greater ADCP of IGROV1 tumour cell targets than at the 1:1 ratio ( $p=0.008$ ). MOv18 IgE-dependent ADCC of tumour cells was not affected by this difference in effector:target ratio ( $p=0.57$ ).

#### 4. Discussion

We describe here a new flow cytometric method for mechanistic studies of antibody-dependent cell-mediated tumour cell killing, applied to a realistic system. This assay allows the measurement of ADCP, ADCC and total cell killing. IGROV1 tumour target cells are pre-labelled with the green fluorescent dye, CFSE. The CFSE<sup>+</sup> target cells are incubated with human U937 or primary monocytic effector cells and anti-FBP MOv18 IgE for tumour cell killing. The monocytes are labelled with PE and dead cells with PI. Using flow cytometry, killed tumour cells (CFSE<sup>+</sup>/PI<sup>+</sup>) and phagocytosed tumour cells (CFSE<sup>+</sup>/PE<sup>+</sup>) are then clearly distinguished and quantified.

To confirm that in this assay we truly observed phagocytosis of tumour cells by monocytic cells, we complemented our ADCC/ADCP measurements with FSC/SSC dot plots, as well as quantitative and qualitative assessments of cell–cell interactions by fluores-

cence microscopy. To achieve consistency and to validate tumour cell killing calculations, flow cytometric voltage settings, fluorescence compensation adjustments and gates were kept constant for all studies. Flow cytometric FSC/SSC dot plots clearly showed that samples used to measure different levels of phagocytosis displayed identical light scatter properties, thus excluding the possibility that variations of FSC/SSC dot plots may interfere with phagocytotic assay measurements (Nuutila and Lilius, 2005). These plots also confirmed that single cell events were acquired and measured, excluding the possibility that acquired CFSE<sup>+</sup>/PE<sup>+</sup> events represented effector cells still in contact with IGROV1 tumour target cells. In agreement with flow cytometric assay measurements, contact of effector with tumour target cells was evident and measurable independently of phagocytosis, suggesting that cytotoxic killing of tumour cells was taking place. In the same samples, monocytic cells engaged in contact and phagocytosis of tumour cells were also observed and quantified, further confirming this mode of killing in this assay system. These observations are in agreement with previous reports by us and others documenting continuous contact, tumour cell killing and phagocytosis by monocytes/macrophages. These associations lead to lysis, structural disintegration of tumour cells and continued phagocytosis of fragments of killed tumour cell material, leading to phagocytosis of whole tumour cells by monocytes taking place over a period of 3 h *in vitro* (Karagiannis et al., 2003; Lu et al., 2005).

An advantage of this three-colour cytometric assay over standard assays is the inclusion in the total tumour cell death count, of those tumour cells that have been killed by phagocytosis. Furthermore, as for all FCM cytotoxicity assays, this ADCC/ADCP assay has the advantage of allowing analysis to be conducted on a single cell basis (Munn and Cheung, 1989; Radosevic et al., 1990; Derby et al., 2001; Lee-MacAry et al., 2001), providing a higher level of sensitivity and greater discrimination than the standard <sup>51</sup>Cr- and LDH-release assays. The latter fail to determine whether the marker of cell death has been released in its entirety from a small proportion of the target cells, or partially released from all the cells. Other advantages include the ease and uniformity of target cell labelling with CFSE, cost-effectiveness, and simplicity and speed of analysis.

This assay can be adapted to use with different tumour target cell lines that can be optimised to be pre-labelled with the CFSE dye. We demonstrate that the assay can be used with the U937 human monocyte cell line and primary human monocytes as effector cells. We have also applied the assay to eosinophils as IgE effector cells against IGROV1 cells (results not shown). We have

tested the assay effectively with higher effector:target ratios (up to 50:1; results not shown), which may be useful with effector cells and antibodies (e.g. IgG) that are less efficient mediators of tumour cell killing than IgE. Thus, this assay could be applied to other combinations of tumour antigen-specific antibody, regardless of the antibody class, with different effector and target cells, and may help to uncover mechanisms that could be exploited for immunotherapy of cancer.

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