MATERIALS AND METHODS

Materials

All reagents were from Sigma Aldrich unless otherwise specified. Chemokines were obtained from R&D systems and Peprotech. The CX3CR1 antagonist AZ12201182 (shown as compound 18[a] in Karlström *et al.*) has been described previously[1, 2].

Monocyte isolation

Human blood from anonymous healthy donors was obtained in the form of leukocyte cones from the NHS Blood and Transplant service. Leukocyte cones contain waste leukocytes isolated from individuals donating platelets via apharesis, and consist of a small volume (~10 ml) of packed leukocytes with few red blood cells or platelets. To isolate peripheral blood mononuclear cells (PBMCs), blood was diluted to a final volume of 120 ml with Dulbecco's phosphate buffered saline (D-PBS; Life Technologies) and 30 ml diluted blood overlayed onto 15 ml Ficoll-Paque Plus (GE Healthcare) in 50 ml centrifuge tubes (four tubes per donor). Tubes were centrifuged at 2200 RPM for 30 minutes at room temperature with the brake off. PBMCs were collected to four fresh 50 ml centrifuge tubes and washed three times with D-PBS to remove contaminating platelets. $CD14^+$ monocytes were isolated from PBMCs by labelling with CD14 microbeads (Miltenyi Biotec) and magnetic isolation on LS columns (Miltenyi Biotec) according to manufacturer's instructions. These cells were used in all experiments and comprise almost entirely classical ($CD14^{++}$ $CD16^-$) monocytes with some intermediate subset monocytes ($CD14^{++}$ $CD16^+$) and are simply referred to as 'monocytes' throughout the text. Monocytes were >95% $CD14^+$ as determined by flow cytometry (see below). Monocytes were washed twice in serum-free medium (see apoptosis assay) and used immediately. $CD16^+$ monocytes used in Figures 2B and 4B were isolated using the $CD16^+$ monocyte isolation kit (Miltenyi Biotec) according to manufacturer's instructions. All experiments were performed in 15 ml polypropylene centrifuge tubes (Greiner) to prevent monocyte adherence.

Apoptosis assay

To induce apoptosis, monocytes were incubated at 1×10^6 cells/ml in serum-free medium (RPMI-1640, 10 mM HEPES, 1x Non-essential amino acids; PAA) for 4 hours at 37 °C. Chemokines at the indicated concentrations or fetal-calf serum (10% v/v; PAA) were assayed for their ability to block apoptosis induction. After 4 hours, monocytes were pelleted and resuspended in 100µl annexin-V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2 , pH 7.4) and labelled with 5 µl annexin-V FITC (BD Biosciences) and 0.5 µl 7-AAD (1 mg/ml; Life Technologies) for 15 minutes on ice in the dark. Cells were immediately analysed by flow cytometry using a FACS Calibur instrument (Becton Dickinson). Dead cells were quantified as Annexin-V^+, 7-AAD^+. Data were analysed and compensation performed using FlowJo 7 software. In experiments using the AZ compound, cells were pre-treated for 1 hour with 2 µM AZ12201182 prior to the addition of agonist.

CD14/CD16/CX3CR1 staining

Monocytes (0.5×10^6 per sample) were incubated for 10 mins in blocking buffer (PBS / 5% FCS v/v / 5% human serum v/v; PAA) then stained with either mouse anti-human CD14-PE, mouse anti-human CD16-APC (AbD Serotec), or rat anti-human CX3CR1-APC (Biolegend) antibodies or appropriate isotype controls for 30 mins according to manufacturer's instructions. Cells were washed once then fixed in 1% formalin and analysed by flow cytometry using a FACS Calibur instrument and FlowJo 7 software.

CX3CR1 genotyping

Genomic DNA was prepared from donor PBMCs using the GenElute mammalian genomic DNA miniprep kit (Sigma Aldrich) according to manufacturer's instructions. CX3CR1 was genotyped using a PCR-based allelic discrimination method based on that previously reported[3]. Primers were designed to amplify between the two SNPs G745A (V249I) and

C839T (T280M) to give a 135bp product. Primer sequences were forward primer 1 (FP1): CTTCTGGACACCCTAACAAC**G**; forward primer 2 (FP2): TCTTCTGGACACCCTACAAC**A**; reverse primer 3 (RP3) AACAATGGCTAAATGCAACC**G**; reverse primer 4 (RP4) CAATGGCTAAATGCAACC**A**. Using a combination of the primers in 4 separate PCR reactions the genotypes could be determined as follows: FP1/RP3 product = VT, FP1/RP4 product = VM, FP2/RP3 product = IT, FP2/RP4 product = IM with the combination of products giving the CX3CR1 haplotype. PCR was carried out using SYBR green real time PCR (Qiagen Quantitect SYBR green) and the following amplification conditions: 95°C 15 mins, followed by 15 cycles of touchdown PCR with annealing temperature reduced by 1°C per cycle (94°C 15 secs, 70-56°C 30 secs, 72°C 30 secs), followed by 30 further cycles (94°C 15 secs, 56°C 30 secs, 72°C 30 secs). The genotype of a subgroup of donors was confirmed by sequencing.

Analysis of ROS with CellROX green

Monocytes were loaded for 30 mins with 5 µM CellROX green reagent (Life Technologies) +/- pegylated superoxide dismutase (PEG-SOD) or AZ12201182 (2 µM) diluted in MACS buffer (D-PBS, 0.5% BSA, 2 mM EDTA). Cells were pelleted and resuspended with agonists (10% v/v FCS or 100 nM chemokine) diluted in SFM (or SFM alone) for a further 30 mins, before cells were pelleted and washed once with ice cold PBS. Cells were fixed with 4% formalin, then analysed by flow cytometry using a FACS Calibur instrument. Dead cells and debris were excluded by gating on forward and side scatter, then geometric mean calculated using FlowJo 7 software. Due to technical variation in geometric mean between experiments, some data have been normalised as fold change compared to either SFM or FCS to allow pooling of data. Since the data are normalised to 1 for the control sample of each individual donor, this does not generate an error bar.

Analysis of ROS with dihydroethidium

Measurement of 2-hydroxyethidium formation by HPLC was used to quantify superoxide production, as previously described[4]. Cells (1 x 10^6 per sample) were incubated for 30 min with 25 µM dihydroethidium (Life Technologies) then agonists (or SFM) added for a further 30 mins. Cells were pelleted, washed twice in ice-cold PBS and pellets frozen at -80 °C. Pellets were thawed, lysed in 200 µl ice-cold methanol then 100 µl hydrochloric acid (0.1 N) was added. Samples were centrifuged at 13000 rpm for 5 mins at 4 °C then the supernatant transferred to amber glass vials prior to loading into the autosampler. All of the samples were stored in darkened tubes and protected from light at all times. Separation of ethidium, 2-hydroxyethidium, and dihydroethidium was performed using a gradient HPLC system (Jasco) with an ODS3 reverse phase column (250 mm, 4.5 mm; Hichrom), and quantified using a fluorescence detector set at 510 nm (excitation) and 595 nm (emission). A linear gradient was applied from Mobile phase A (0.1% TFA (v/v)) to Mobile phase B (0.1% TFA (v/v) in acetonitrile) over 23 min (30% acetonitrile to 50% acetonitrile).

Real time xCelligence chemotaxis assay

Monocyte chemotaxis was performed using an xCelligence DP instrument (ACEA biosciences) and 16 well CIM plates essentially as described previously[5]. Agonists were made to desired concentrations (final volume of 160 µl) in chemotaxis buffer (RPMI 1640, 25 mM HEPES, 0.5% (w/v) BSA) and loaded in the lower wells of the CIM-16 plate. Following upper chamber attachment, the upper wells were filled with 50 µl pre-warmed chemotaxis buffer and the plate left for 30 mins at RT to pre-equilibrate. Monocytes were resuspended to 8×10^6 cells/ml in chemotaxis buffer and 50 µl cell suspension (4×10^5 cells) was placed into the top wells. The assembled plate was transferred to the RTCA-DP machine and data was collected every 5 s over the course of 3 hours. As cells pass through the 8 µm pores towards a selected chemoattractant they adhere to the underside of the filter on which is embedded a gold micro-electrode. This produces a signal of electrical impedance which is reflected in the cell index. Area under the curve was analysed using RTCA Software 1.2.1. and provides a combined measure for both migration and spreading.

Data analysis

All data were analysed using GraphPad Prism software version 6.02. All data were analysed using one-way ANOVA and Dunnett's or Tukey's posthoc test as described in the figure legend.

References

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