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Tumour-educated macrophages display a mixed polarisation and enhance pancreatic cancer cell invasion

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Running title: Tumour-educated macrophages in pancreatic cancer

Abstract

At the time of diagnosis, almost 80% of pancreatic cancer patients present with new-onset type 2 diabetes (T2D) or impaired glucose tolerance. T2D and pancreatic cancer are both associated with low-grade inflammation. Tumour-associated macrophages (TAMs) play a key role in cancer-related inflammation, immune escape, matrix remodelling and metastasis. In this study, the interplay between tumour cells and immune cells under the influence of different glucose levels was investigated. Human peripheral blood mononuclear cells were exposed *in vitro* to conditioned medium from BxPC-3 human pancreatic cancer cells, in normal (5 mM) or high (25 mM) glucose levels. Flow cytometry analyses demonstrated that tumour-derived factors stimulated differentiation of macrophages, with a mixed classical (M1-like) and alternatively activated (M2-like) phenotype polarization (CD11c$^+$CD206$^+$). High glucose conditions further enhanced the tumour-driven macrophage enrichment and associated IL-6 and IL-8 cytokine levels. In addition, hyperglycaemia enhanced the responsiveness of tumour-educated macrophages to lipopolysaccharide, with elevated cytokine secretion compared to normal glucose levels. Tumour-educated macrophages were found to promote pancreatic cancer cell invasion *in vitro,* which was significantly enhanced at high glucose. The anti-diabetic drug metformin shifted the macrophage phenotype polarization and reduced the tumour cell invasion at normal, but not high, glucose levels. In conclusion, this study demonstrates that pancreatic cancer cells stimulate differentiation of macrophages with pro-tumour properties that are further enhanced by hyperglycaemia. These findings highlight important crosstalk between tumour cells and TAMs in the local tumour microenvironment that may contribute to disease progression in pancreatic cancer patients with hyperglycaemia and T2D.

Keywords: Pancreatic cancer, TAM, inflammation, type 2 diabetes, hyperglycemia, invasion

Abbreviations

AMPK   Adenosine monophosphate activated protein kinase

LPS   Lipopolysaccharide

PBMC   Peripheral blood mononuclear cell

TAM   Tumour-associated macrophage

TLR   Toll-like receptor

T2D   Type 2 diabetes

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in western societies. The overall 5-year survival rate among pancreatic cancer patients is less than 3% and the median survival is less than 6 months[1-3]. As the cancer-specific symptoms present only at a late stage, the majority of pancreatic cancer patients already have an advanced stage disease at the time of diagnosis. There is an urgent need to identify novel therapeutic approaches and, most importantly, early detection of the disease to improve the survival of patients with pancreatic cancer.

The majority of pancreatic cancer patients present with either new-onset type 2 diabetes or impaired glucose tolerance at the time of diagnosis[4, 5]. Epidemiological studies have reported both obesity and type 2 diabetes to be among the top three modifiable risk factors for pancreatic cancer[6-9]. In addition, among the cases with resectable pancreatic cancer, the majority of patients show improved insulin sensitivity after tumour resection, although a large part of the pancreas is removed[10]. Despite a strong link between the two diseases, the relationship between type 2 diabetes and pancreatic cancer is complex and largely unknown. It remains to be elucidated if the microenvironment in type 2 diabetes is favourable for the development of pancreatic cancer or if cancer cells at an early stage give rise to the diabetes.

Chronic inflammatory conditions increase the risk of different forms of cancer. Patients with chronic pancreatitis have an increased risk of developing pancreatic cancer[11]. Both type 2 diabetes and pancreatic cancer are associated with elevated local and systemic low grade inflammation, which is believed to influence the pathogenesis of both diseases[12-14]. Inflammation plays a central role in the tumour microenvironment and has recently been acknowledged as a novel hallmark of cancer due to its association with the pathogenesis in many types of cancer[14-16]. Macrophages are believed to play a major role in orchestrating the

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cancer-related inflammation and have commonly been divided into two classes; M1 "classically activated, pro-inflammatory" macrophages and M2 "alternatively activated" macrophages[17]. Macrophages originate from blood monocytes being recruited to the tissue where they are activated. Stimulation with LPS and IFNγ leads to M1 activation and the secretion of IL-1β, IL-6 and TNFα along with the expression of HLA-DR[hi] and CD11c, among others. M2 macrophages in contrast, are activated by IL-4 and IL-13, secrete IL-10 and express markers such as HLA-DR[lo] and CD206 (mannose receptor). M1-macrophages have been observed in type 2 diabetes infiltrating adipose tissue and pancreatic islets, and are believed to play a central role in the diabetes-associated inflammation through IL-1 and NFκB activation[12]. Tumour-associated macrophages (TAMs), on the other hand, display a M2-like phenotype and are characterised by CD206^{+} as well as arginase 1 (Arg1) expression, and the secretion of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), IL-6, IL-10, and matrix metalloproteinases (MMPs). TAMs promote carcinogenesis by stimulating angiogenesis, migration, invasion, metastasis and ECM remodelling[14-16, 18-21]. However, recent reviews on TAMs demonstrate that the M1-M2 classification might be over-simplified and no longer sufficient, with intermediate phenotypes emerging due to the plasticity of macrophages, enabling them to adjust to their specific microenvironment[18-21].

Metformin is used as first-line treatment in the management of type 2 diabetes. The key metabolic action of metformin involves the inhibition of hepatic glucose secretion. This mechanism is mediated via activation of the energy-sensing AMP-activated protein kinase (AMPK) in hepatocytes[22]. In addition to its anti-diabetic effects, metformin has recently been shown to possess anti-tumour effects, both in AMPK-dependent and independent manners[23]. Metformin has also been indicated to have anti-inflammatory effects by indirectly inhibit LPS response and/or NFκB-signalling[24, 25].

Although inflammation has an established role in carcinogenesis, limited research has been performed in pancreatic cancer and the influence by related type 2 diabetes. The aim of the present study was to investigate the interplay between tumour cells and immune cells under the influence of different glucose levels. Tumour cell-induced macrophage enrichment, phenotype polarisation and associated cytokine secretion was analysed *in vitro*. Furthermore, pro-tumour properties on pancreatic cancer cell invasion by tumour-educated macrophages were investigated, as well as the anti-inflammatory properties of metformin.

Results

Hyperglycaemia enhances pancreatic cancer cell-stimulated macrophage differentiation and cytokine secretion

Given the important pleiotropic roles of macrophages in tumour progression and T2D, we sought to establish the influence by pancreatic cancer cells and glucose levels on monocyte to macrophage differentiation. To mimic a local tumour microenvironment, human PBMCs were exposed to conditioned medium (CM) from BxPC-3 human pancreatic cancer cells cultured in different glucose conditions. CM from BxPC-3 induced a strong enrichment of a population enlarged cells with ruffling membranes, based on the flow cytometry scatter profile (Fig. 1a). This tumour-educated population accounted for 8-18% of the total PBMCs compared to 4% in naïve control cells. Over time, the tumour-educated cell population was better maintained and more numerous in the presence of high (25 mM) compared to normal (5 mM) glucose levels (Fig. 1b). The tumour-educated population was found to be CD68 positive, indicating the cells to be of the monocyte-macrophage lineage (Fig. 1c). In addition, exposure to BxPC-3 CM stimulated a significant increase in IL-6 and IL-8 cytokine levels (Fig. 1d). The cytokine levels were further enhanced in hyperglycaemic conditions, resulting in an almost 3- and 2-fold increase in IL-6 and IL-8 levels, respectively, compared to normal glucose conditions. Taken together, these results indicate that BxPC-3 pancreatic cancer cells stimulate monocyte-to-macrophage differentiation, which is enhanced by hyperglycaemic conditions.

Tumour-educated macrophages display a mixed $CD11c^+CD206^+$ phenotype polarisation

Having demonstrated the enriched population to be $CD68^+$ cells, we further characterized the phenotype polarization and analysed known macrophage surface markers. Figure 2a shows CD14 and HLA-DR expression for the tumour-educated cells after exposure to CM compared to control. The majority of cells in the tumour-educated population were $CD14^+HLA-DR^+$,

which further verified them to be of the monocyte-macrophage lineage. The cell surface levels of the TLR4 co-receptor CD14 were enhanced among tumour-educated macrophages exposed to high (25 mM) relative to normal (5 mM) glucose levels (N.S.; Fig. 2b). The CD14+HLA-DR+ population was further analysed for CD11c and CD206 positivity (Fig. 2c). Initially, the majority of cells were CD11c+CD206-. However, the CD206 levels increased over time and after 72 h differentiation, more than 90% of the macrophages were CD11c+CD206+ (Fig. 2c-d). No significant difference in macrophage polarization was observed between normal (5 mM) and high (25 mM) glucose. The macrophage polarization was further validated with similar findings, when evaluating the CD11c and CD206 positivity among the tumour-educated CD68+HLA-DR+ population (Supplementary figure 1). Similar results were obtained when initial macrophage phenotype polarisation was investigated with CM from two additional pancreatic cancer cell lines, PANC-1 and MIAPaCa-2 (Supplementary figures 2-3). As the tumour-educated cells expressed both M1- and M2- associated surface markers, these results indicate that the pancreatic cancer cells stimulate macrophage differentiation with a mixed phenotype polarisation.

Hyperglycaemia enhances the cytokine secretion in response to LPS

In order to determine the activation status of the BxPC-3 tumour-educated macrophage population in the PBMCs, their functional responsiveness to the TLR4 agonist LPS at different glucose levels was examined. Exposure to LPS significantly increased the IL-1β, IL-6, IL-8 and IL-10 cytokine levels ($P<0.001$; Fig. 3a-d). Hyperglycaemic conditions further enhanced the LPS-induced IL-6 and IL-8 levels, while no obvious influence on IL-1β and IL-10 levels was observed. In contrast, LPS-stimulation strongly promoted TNF-α release by naïve control cells, which was not further altered in the presence of BxPC-3 CM or

hyperglycaemia (Fig. 3e). No effect by LPS on macrophage enrichment or polarization was observed (data not shown).

The macrophage enrichment and associated cytokine secretion is suppressed by metformin

The anti-diabetic drug metformin has been reported to alter inflammatory signatures. Hence, we next investigated the influence by metformin on the tumour-induced macrophage differentiation. Exposure to metformin impaired the responsiveness to BxPC-3 CM differentiating activity and significantly reduced the tumour-educated macrophage population with up to 51% in normal glucose (Fig. 4a-b). Similarly, the tumour-educated macrophage population was reduced after exposure to metformin in hyperglycaemic conditions, although a significantly higher frequency of macrophages remained after the longest exposure time ($P<0.05$; Fig. 4b). In addition, the associated IL-6 and IL-8 levels were significantly suppressed for the tumour-educated cells exposed to metformin, at both normal and high glucose concentrations ($P<0.01$; Fig. 4c). Notably, the cytokine reduction was more pronounced than the reduction in macrophage frequency. This indicates that metformin suppressed both the frequency as well as the activation state of the tumour-educated macrophages.

Metformin attenuates the tumour-educated macrophage polarisation

Having found that metformin suppressed the tumour-educated macrophage frequency, we further assessed its effect on the phenotype polarization. Exposure to metformin resulted in a pronounced decrease in CD14 positivity, while no apparent change in HLA-DR expression was observed (Fig. 5a). In addition, metformin counteracted the BxPC-3 CM induced CD206 expression. After 24 and 72 h culture, the geometric mean fluorescence intensity (geo-MFI) of

CD206 was 30-40% lower for metformin-treated compared to non-treated cells ($P<0.05$; Fig. 5b-c). The reduced CD206 levels resulted in a shifted phenotype balance with re-polarization towards CD11c$^+$CD206$^-$ cells, which was further emphasized at hyperglycaemic conditions (Fig. 5d).

Metformin suppresses LPS responsiveness and cytokine secretion

To assess the relevance of losing the TLR4 co-receptor CD14 expression and altering the phenotype polarization of the tumour-educated macrophage population, we evaluated the impact of metformin on the functional responsiveness to LPS. Indeed, the sensitivity to the TLR4 agonist LPS was considerably reduced in the presence of metformin, with significantly lower levels of LPS-induced cytokine release ($P<0.01$; Fig. 6). Consistent with the altered phenotype polarization, both IL-6 and IL-8 cytokine levels were strongly suppressed in the presence of high glucose ($P<0.001$; Fig. 6a-b), The metformin-mediated inhibition of IL-1β, IL-10 and TNFα secretion by the tumour-conditioned cells appeared independent of the glucose levels (Fig. 6c-e). In fact, metformin almost completely prevented the LPS-stimulated TNFα secretion to almost undetectable levels at both normal and high glucose levels (Fig. 6e). These results indicate that the sensitivity to tumour-induced macrophage differentiation and functionality was impaired in the presence of metformin.

Tumour-educated macrophages and hyperglycaemia promote invasion of pancreatic cancer cells

Finally, a direct functional effect of the tumour-educated macrophages and hyperglycaemia on pancreatic cancer cell invasion was determined. BxPC-3 cells co-cultured with PBMCs displayed significantly higher invasive capabilities than BxPC-3 cells cultured alone, in accordance with the tumour-promoting role of TAMs ($P<0.001$; Fig. 7a-b). Hyperglycaemia

significantly enhanced the number of invading BxPC-3 cells, in both co-culture ($P<0.01$) and controls ($P<0.001$; Fig. 7B). While metformin was capable of reducing the number of invading pancreatic cancer cells by 60-70% ($P<0.001$) in normal glucose, no reduction in the invasive capabilities was observed at high glucose (Fig. 7c). These results demonstrate that the invasive capability of pancreatic cancer cells was stimulated in the presence of inflammatory cells as well as high glucose levels.

Discussion

There are increasing indications that both type 2 diabetes and pancreatic cancer are associated with an enhanced inflammatory state, and that inflammatory cells may accelerate disease progression[12-14]. However, the complex relationship between type 2 diabetes, inflammation and pancreatic cancer remains unclear. We hypothesise that a symbiotic relationship exists in the tumour microenvironment between inflammatory cells and tumour cells that is fuelled by type 2 diabetes-associated hyperglycaemia. In the present study, we show that pancreatic cancer cells stimulate differentiation of tumour-educated macrophages with a mixed phenotype polarisation and pro-tumour properties. The macrophage frequency, activation status and functional influence on cancer cell motility were further enhanced in high glucose conditions. This study demonstrates important crosstalk between inflammatory cells, cancer cells and glucose levels that may contribute to disease progression.

Previous studies on pancreatic cancer-related inflammation have shown enhanced leukocyte infiltration in the tumour microenvironment and elevated systemic cytokine levels in patients with pancreatic cancer[26-30]. Macrophages, in particular with an alternatively activated M2-associated phenotype, were among the most common leukocyte subsets infiltrating the tumour area[29, 30]. This was further reflected in the systemic cytokine levels, where IL-6, IL-8 and IL-10 were elevated in pancreatic cancer patients[26-28]. In addition, a recent *in vivo* study showed that macrophages infiltrated the pancreatic tumour already at the early pre-invasive stages and remained one of the dominating leukocytes throughout the pathogenesis of the disease[31]. This indicates an important role of tumour-associated macrophages in pancreatic tumourigenesis.

Due to the high plasticity of macrophages, the M1/M2 paradigm is debated and has recently been regarded an oversimplified view and extreme ends of a spectrum of phenotype

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polarisation. The specific TAM phenotype is largely determined by the local microenvironment[18-21]. Herein, we demonstrate that BxPC-3 pancreatic cancer cells stimulate enrichment of CD14$^+$HLA-DR$^+$ macrophages with a mixed CD11c$^+$CD206$^+$ phenotype. In high glucose conditions, the tumour-educated macrophage enrichment as well as IL-6 and IL-8 secretion was enhanced, indicating hyperglycemia to enhance the influence of pancreatic cancer cells on macrophage differentiation. Interestingly, both IL-6 and IL-8 are elevated among type 2 diabetic patients and have been found to accelerate pancreatic cancer progression[32-34]. Similarly to our findings, a previous study performed with various cancer cell lines demonstrated pancreatic cancer cells to induce TAMs with an M2-like phenotype, which was believed to be mediated by M-CSF secreted by the cancer cells[35]. The TAMs were found to secrete both M1 (TNFα) and M2-associated (IL-10, IL-6 and CCL2) cytokines. While promoting macrophage enrichment, pancreatic cancer cells have been shown to inhibit dendritic cells differentiation via secretion of IL-6 and G-CSF[36].

A primary function of macrophages is to rapidly increase cytokine and chemokine biosynthesis and release upon activation by different TLRs[17]. Interestingly, the TLR4 co-receptor CD14 was upregulated on the tumour-educated macrophages under hyperglycaemic conditions that may influence the TAM activation and sensitivity to LPS stimulation. A study investigating the LPS/NFκB-pathways in TAMs demonstrated that TAMs accumulate inhibitory p50 homodimers of NFκB in the nucleus, which alters the TAMs classic response to LPS and leads to a reduced TNFα secretion[37]. In the present study, elevated levels of IL-1β, IL-6, IL-8 and IL-10 were seen in response to LPS. In contrast, no increase in TNFα was observed by TAMs compared to basal levels of naïve cells. This is in line with the M2-like phenotype previously described for TAMs, and the indications of an altered LPS response.

The glucose-lowering drug metformin is used as first-line treatment in the management of type 2 diabetes. It has recently been shown to have anti-tumour properties, besides its anti-diabetic effects[23]. In addition, metformin has been indicated to have anti-inflammatory effects by indirect inhibition of inflammatory mechanisms[24, 25]. In the present study, metformin effectively suppressed the macrophage enrichment, phenotype polarisation and cytokine secretion regardless of the glucose levels. Metformin also strongly reduced cytokine secretion induced by LPS, which further supports its implied anti-inflammatory effects. Metformin might therefore have a wider range of action than the initial anti-diabetic, and later anti-tumour, effects seen.

TAMs have several roles in the tumour microenvironment where they stimulate migration, invasion and metastatic abilities[18-20]. We found that BxPC-3 pancreatic cancer cells displayed an enhanced motility after co-culture with tumour-educated macrophages. In addition, we made the novel observation that these invasive capabilities were further emphasized in the presence of hyperglycaemia. In previous studies with colon cancer[38] and pancreatic cancer cells[39], co-culture with monocytes or macrophages increased the cancer cell migration rate via paracrine mechanisms involving SDF-1α, VEGF and TNFα. Consistent with our findings, hyperglycaemia alone, without the influence of TAMs, increased the pancreatic cancer cell proliferation and migration[40]. However, our study has uniquely shown the combined influence of leukocytes and glucose on pancreatic cancer cell motility. While metformin influenced the macrophage phenotype under both glucose conditions, the inhibitory effect by metformin on the pancreatic cancer cell invasiveness was strongly reduced or abolished in the presence of high glucose. This may relate to an altered communication between the macrophages and cancer cells, while more likely to an altered responsiveness by the pancreatic cancer cells to metformin under hyperglycaemic conditions, as previously reported[23].

In conclusion, this study shows that pancreatic cancer cells induce tumour-educated macrophages with intermediate mixed phenotype polarisation, which in turn stimulated pancreatic cancer cell invasion. These effects were further triggered by hyperglycaemia. Treatment with the anti-diabetic drug metformin suppressed the induction of tumour-educated macrophages, which indicates a potential role of metabolic pathways in the phenotype polarisation and associated cytokine secretion. This study highlights an important crosstalk between pancreatic cancer cells and macrophages that may contribute to disease progression in pancreatic cancer patients with hyperglycaemia and T2D. However, further studies are required to determine the effect by metformin and glucose on the communication between TAMs and pancreatic cancer cells with influence on tumour invasiveness. With increased understanding of the role of associated inflammation and T2D in pancreatic cancer, new potential targets and treatment strategies can be investigated to hopefully improve the outcome of this fatal disease.

Methods

Materials

All chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA) unless stated otherwise. Cell culture media, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen (Paisley, UK). Fluorochrome-labelled antibodies specific for human CD11c (clone B-ly6), CD14 (clone M5E2), CD68 (clone Y1/82A), CD206 (clone 19.2) and HLA-DR (clone G46-6) were purchased from BD Biosciences (San Diego, CA, USA).

Cell culture

The human pancreatic adenocarcinoma cell lines BxPC-3, PANC-1, MIAPaCa-2 were purchased from ATCC-LGC Standards (Manassas, VA, USA). The cells were maintained in RPMI1640 or DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) in a humified 5% CO_2 atmosphere at 37^oC. All experiments were performed in glucose-free RPMI1640 or DMEM supplemented with 5 mM (normal) or 25 mM (high) D-glucose, 2 mM L-glutamine and antibiotics as above (serum free media; SFM). Conditioned medium (CM) was obtained by culturing BxPC-3 cells to 70-80% confluence, washing twice and changing to SFM. CM was collected after 48 h incubation, residual cells removed by centrifugation, and aliquots stored at -80^oC until further use.

***In vitro* macrophage differentiation**

This study was approved by the Regional Ethical Review Board in Lund, Sweden. Peripheral blood mononuclear cells (PBMCs) were obtained by separation with Ficoll-Paque (GE Healthcare, Freiburg, Germany) from blood samples given by healthy volunteers. The cells were *in vitro* differentiated (5 x 10^5 cells/well) in 6-well plates with SFM or CM, with or

without 20 mM metformin, for 24-72 h. Supernatants were collected, aliquoted and stored for cytokine analysis.

Flow cytometry

Cells were incubated for 5 min on ice with human Fc Receptor Binding Inhibitor (eBioscience, Hatfield, UK) in PBS + 1% BSA. Cells were then incubated with manufacturer's suggested dilutions of fluorochrome-conjugated primary antibodies in four-color panels (CD14/HLA-DR/CD11c/CD206 or CD68/HLA-DR/CD11c/CD206) for 30 min at 4°C. Before intracellular CD68 staining, cells were fixed and permeabilised with Cytofix-Cytoperm solution (BD Biosciences) according to the manufacturer's instructions. Approximately 5×10^4 events (cells) were collected for each sample using a FACSCalibur (BD Biosciences) dual laser, flow cytometer with CellQuest Pro Software (BD Biosciences), and analysed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

LPS stimulation of *in vitro* differentiated macrophages

PBMCs were seeded (5×10^5 cells/well) in 6-well plates with SFM or CM, with or without 20 mM metformin, and incubated for 72 h. LPS (100 ng/ml) was spiked into the cell cultures during the last 24 h of incubation. Cells were analysed by flow cytometry as above and supernatants collected and stored for ELISA.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels of cell supernatants were assessed using DuoSet ELISA according to the manufacturer's instructions (R&D systems, Abingdon, UK). Samples were measured in duplicate on a Labsystems Multiskan Plus plate reader (test wavelength 450 nm, reference wavelength 540 nm) using the DeltaSoft JV software (BioMetallics Inc., Princeton, NJ, USA).

Assay sensitivities for the analytes were: IL-1β (3.91 pg/ml), IL-6 (9.38 pg/ml), IL-8 (31.25 pg/ml), IL-10 (31.25 pg/ml), and TNFα (15.63 pg/ml).

Invasion studies

PBMCs (5 x 10^5 cells/insert) were seeded into Transwell inserts (3 μm pores; BD Biosciences) and co-cultured with BxPC-3 cells (2.5 x 10^5 cells/well) in the lower chamber without cell contact. The BxPC-3 cells were harvested after 48 h incubation with or without co-culture and 1.5 x 10^5 cells were re-suspended in SFM and added to inserts (8 μm pores; BD Biosciences). SFM supplemented with 10% FBS was added to the lower chamber. After 8 h, cells on the upper side of the membrane were removed using cotton swabs and invaded cells were fixed in 1% glutaraldehyde and stained with 0.5% crystal violet. Cells were photographed and the number of invaded cells counted in nine microscope fields per insert (magnification x10).

Statistics

A minimum of three independent experiments were done using at least three different PBMC donors. Statistical analyses were performed using two-tailed Student's *t*-test or mixed models with Excel 2010 or SPSS version 20 softwares, respectively. A *P*-value of <0.05 was considered statistically significant.

Acknowledgements

Authors' contributions

AHR and EK designed the study. EK and AHR made significant contributions to the experimental design, acquisition and interpretation of data, manuscript preparation and editing. RA and AHR provided funding for the study and manuscript editing. All authors read and approved the final manuscript.

Supplementary information is available at Immunology and Cell Biology's website.

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Legends to figures

Figure 1. Effects of pancreatic cancer cells and hyperglycaemia on macrophage differentiation and cytokine secretion. (**a**) Flow cytometry analysis showing live scatters of human PBMCs with gated macrophage populations after 24 h *in vitro* exposure to CM from BxPC-3 pancreatic cancer cells or SFM control, in normal (5 mM) or high (25 mM) glucose. (**b**) Graph shows the percentage of gated tumour-educated macrophages for three individual donors over time, compared to SFM control (mean ± SE values for three donors). (**c**) Histogram showing CD68 expression for general PBMCs and gated macrophages after 48 h. (**d**) IL-6 and IL-8 levels for four donors, measured by ELISA on supernatants after 24-72 h incubation with CM. Basal cytokine levels by naïve unstimulated cells are subtracted. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's *t*-test.

Figure 2. Tumour-educated macrophages display a mixed CD11c$^+$CD206$^+$ phenotype polarisation. Flow cytometry analyses demonstrating (**a**) CD14 and HLA-DR expression for the gated "macrophage" population and (**c**) CD11c and CD206 expression for CD14$^+$HLA-DR$^+$ cells after 24 h (control) and 24-72 h incubation (CM BxPC). (**b**) Graph displaying changes in CD14 geo-MFI for four donors between normal (5 mM) and high (25 mM) glucose conditions after 72h. (**d**) Graph showing tumour-educated macrophage polarization with percentage CD11c$^+$CD206$^+$ cells in the CD14$^+$HLA-DR$^+$ population, as mean of three individual repeats.

Figure 3. Enhanced cytokine secretion in response to LPS. Cytokine levels as measured by ELISA in supernatants for (**a**) IL-6, (**b**) IL-8, (**c**) IL-1β, (**d**) IL-10 and (**e**) TNFα for four donors, after 72 h incubation of which the last 24 h in the presence or absence of 100 ng/ml LPS. Basal cytokine levels by naïve unstimulated cells are subtracted (A-D). Statistical

significance represents the effect by LPS for both glucose levels. *P<0.05, **P<0.01, ***P<0.001, mixed models (random factor: donor, fixed factors: LPS, glucose).

Figure 4. Metformin suppresses the tumour-educated macrophage enrichment and cytokine secretion. (**a**) Flow cytometric analysis showing live scatters of human PBMCs after 72 h *in vitro* exposure to CM from BxPC-3 pancreatic cancer cells, with or without 20 mM metformin, in normal (5 mM) or high (25 mM) glucose. (**b**) Graph shows the percentage of gated tumour-educated macrophages after metformin exposure, compared to CM control. (**c**) IL-6 and IL-8 cytokine levels measured by ELISA in supernatants after 24-72 h incubation with or without 20 mM metformin. Basal cytokine levels by unstimulated cells are subtracted. Bars represent mean ± SE of four determinations. Statistical significance represents the effect by metformin for all time points at 5 mM or 25 mM glucose. *P<0.05, **P<0.01, ***P<0.001, (**b**) Student's t-test, (**c**) mixed models (random factor: donor, fixed factors: glucose, time, metformin).

Figure 5. Effect of metformin on macrophage phenotype polarisation. (**a**) CD14 and HLA-DR expression for the gated macrophage population after 24 and 72 h incubation with or without 20 mM metformin. (**b**) CD11c and CD206 expression for CD14$^+$HLA-DR$^+$ cells after 24 h and 72 h. (**c**) Graph represents CD206 geo-MFI after 24 h and 72 h incubation with or without 20 mM metformin as mean ± SE of three individual repeats. (**d**) Graph shows CD11cCD206 expression after 72 h in the CD14$^+$HLA-DR$^+$ population, as the mean of three separate repeats. *P<0.05, **P<0.01, ***P<0.001, Student's *t*-test.

Figure 6. Reduced LPS-responsiveness and cytokine levels in the presence of metformin. Cytokine levels as measured by ELISA in supernatants for (**a**) IL-6, (**b**) IL-8, (**c**) IL-1β, (**d**)

IL-10 and (**e**) TNFα, after 72 h incubation with or without 20 mM metformin, of which the last 24 h in the presence or absence of 100 ng/ml LPS. Basal cytokine levels by naïve unstimulated cells are subtracted (A-D). Bars represent mean ± SE of four determinations. Statistical significance represents the effect by metformin for both glucose levels. *$P<0.05$, **$P<0.01$, ***$P<0.001$, mixed models (random factor: donor, fixed factors: LPS, glucose, metformin).

Figure 7. Influence of tumour-educated macrophages, high glucose and metformin on pancreatic cancer cell migration. BxPC-3 pancreatic cancer cell migration after 48 h co-culture with or without PBMCs, followed by 8 h transwell migration with 10% FBS added to the lower chamber. (**a**) Representative images (x20 magnification) from one of three independent experiments are shown. (**b**) Graph represents number of invaded cell as mean ± SE in nine fields for each insert (x10 magnification). (**c**) Representative images (x10 magnification) from one of three independent experiments of 48 h co-culture, with or without 10 mM metformin, followed by 8 h transwell migration. (**d**) Graph represents fold change in tumour cell invasion after metformin treatment as mean ± SE of four independent repeats. Dashed line indicates the reference level for cells without metformin treatment. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Student's *t*-test.

Supplementary figure 1. Macrophage phenotype polarisation and characterisation with CD68. (**a**) Flow cytometry analysis showing live scatters of human PBMCs with gated macrophage populations after 48 h *in vitro* exposure to CM from BxPC-3 pancreatic cancer cells or SFM control, in normal (5 mM) or high (25 mM) glucose. (**b**) CD68 and HLA-DR expression for the gated "macrophage" population after 48 h. (**c**) CD11c and CD206 expression for CD68^{+}HLA-DR^{+} cells after 48 h.

Supplementary figure 2. Effect of PANC-1 pancreatic cancer cells on macrophage differentiation and phenotype polarisation. (**a**) Flow cytometry analysis showing live scatters of human PBMCs with gated macrophage populations after 24 h (control) and 24-72 h *in vitro* exposure to CM from PANC-1 pancreatic cancer cells, in normal (5 mM) or high (25 mM) glucose. Flow cytometry analyses demonstrating (**b**) CD14 and HLA-DR expression for the gated "macrophage" population and (**c**) CD11c and CD206 expression for CD14$^+$HLA-DR$^+$ cells after 24 h (control) and 24-72 h incubation (CM PANC-1).

Supplementary figure 3. Effect of MIAPaCa-2 pancreatic cancer cells on macrophage differentiation and phenotype polarisation. (**a**) Flow cytometry analysis showing live scatters of human PBMCs with gated macrophage populations after 24 h (control) and 24-72 h *in vitro* exposure to CM from MIAPaCa-2 pancreatic cancer cells, in normal (5 mM) or high (25 mM) glucose. Flow cytometry analyses demonstrating (**b**) CD14 and HLA-DR expression for the gated "macrophage" population and (**c**) CD11c and CD206 expression for CD14$^+$HLA-DR$^+$ cells after 24 h (control) and 24-72 h incubation (CM MIAPaCa-2).
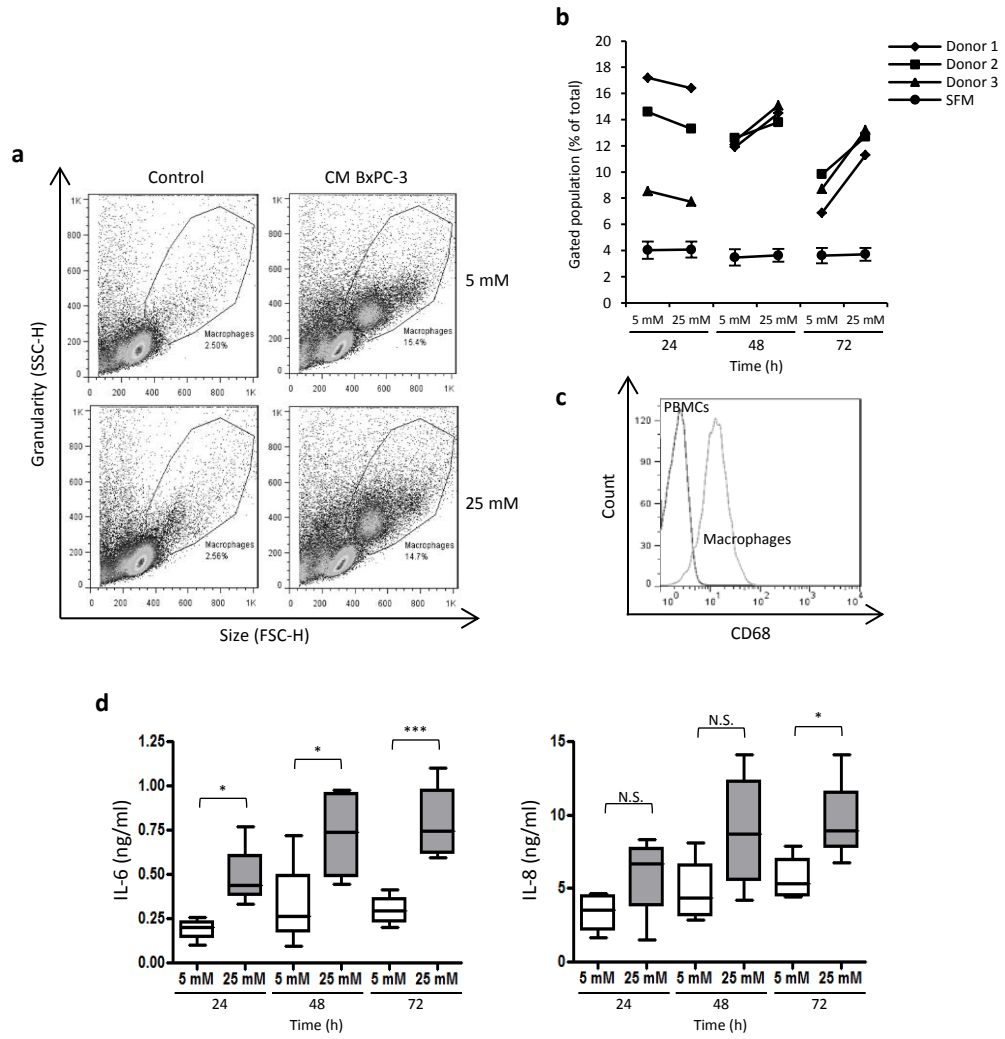
Figure 1

Figure 2

a

CM BxPC-3

Control	24h	48h	72h	

CD14 / HLA-DR

5 mM: Control 6.58% / 31.2% / 37.2% / 25.0%; 24h 5.91% / 85.3% / 4.25% / 4.50%; 48h 30.5% / 58.9% / 5.99% / 4.66%; 72h 11.6% / 74.7% / 9.51% / 4.15%

25 mM: Control 4.11% / 31.0% / 38.9% / 26.0%; 24h 2.26% / 88.2% / 5.23% / 4.27%; 48h 51.2% / 41.3% / 4.24% / 3.25%; 72h 21.2% / 68.0% / 7.24% / 3.50%

b

CD14 (GeoMFI) vs Glucose (mM)

◇ Donor 1
○ Donor 2
□ Donor 3
△ Donor 4

c

CM BxPC-3

Control	24h	48h	72h	

CD11c / CD206

5 mM: Control 63.5% / 33.2% / 1.18% / 2.12%; 24h 56.9% / 42.6% / 0.275% / 0.165%; 48h 20.4% / 78.7% / 0.302% / 0.589%; 72h 4.56% / 94.8% / 0.184% / 0.430%

25 mM: Control 60.8% / 36.4% / 0.637% / 2.12%; 24h 45.1% / 54.6% / 0.103% / 0.191%; 48h 14.3% / 84.7% / 0.256% / 0.753%; 72h 2.45% / 96.9% / 0.200% / 0.411%

d

% of CD14+HLA-DR+ cells

Time (h): 0 24 48 72 | 0 24 48 72
5 mM | 25 mM

□ CD11c+CD206-
■ CD11c-CD206+
□ CD11c-CD206-
■ CD11c+CD206+

Figure 3

Figure 4

Figure 5

a

| | SFM | 24h | | 72h | |
| | | − metformin | + metformin | − metformin | + metformin |

CD14 / HLA-DR

5 mM row:
SFM: 2.30%, 27.5%, 21.5%, 48.7%
−metformin: 2.46%, 78.9%, 4.66%, 14.0%
+metformin: 3.80%, 68.1%, 9.49%, 18.6%
−metformin 72h: 6.88%, 81.8%, 5.70%, 5.64%
+metformin 72h: 0.477%, 34.3%, 6.83%, 58.4%

25 mM row:
SFM: 1.44%, 29.1%, 19.4%, 50.1%
−metformin: 2.47%, 76.8%, 5.69%, 15.0%
+metformin: 4.00%, 62.5%, 11.0%, 22.5%
−metformin 72h: 17.1%, 71.6%, 5.31%, 5.97%
+metformin 72h: 0.620%, 55.6%, 4.05%, 39.8%

b

| | SFM | 24h | | 72h | |
| | | − metformin | + metformin | − metformin | + metformin |

CD11c / CD206

5 mM row:
SFM: 66.0%, 25.5%, 6.83%, 1.67%
−metformin: 21.5%, 70.2%, 2.94%, 5.43%
+metformin: 55.1%, 35.7%, 6.55%, 2.58%
−metformin 72h: 2.64%, 95.6%, 0.224%, 1.52%
+metformin 72h: 26.7%, 68.9%, 1.60%, 2.78%

25 mM row:
SFM: 58.6%, 36.1%, 2.56%, 2.73%
−metformin: 26.3%, 65.6%, 2.77%, 5.30%
+metformin: 49.9%, 39.1%, 7.01%, 4.03%
−metformin 72h: 6.86%, 91.7%, 0.176%, 1.23%
+metformin 72h: 41.6%, 56.9%, 0.728%, 0.777%

c

Geo-MFI (CD206); □ − metformin, ■ + metformin

24 h: 5 mM ***, 25 mM *
72 h: 5 mM **, 25 mM

d

% of CD14+HLA-DR+ cells

Legend:
□ CD11c+CD206−
▨ CD11c−CD206+
□ CD11c−CD206−
■ CD11c+CD206+

Metformin: − + − +
5 mM   25 mM

Figure 6

Figure 7

a

	5 mM	25 mM
CTRL		
After co-culture		

b

Number of invaded tumour cells

□ 5 mM
■ 25 mM

CTRL   After co-culture

c

	- metformin	+ metformin	- metformin	+ metformin
CTRL				
After co-culture				

5 mM glucose   25 mM glucose

d

Fold difference in invading tumour cells after metformin treatment

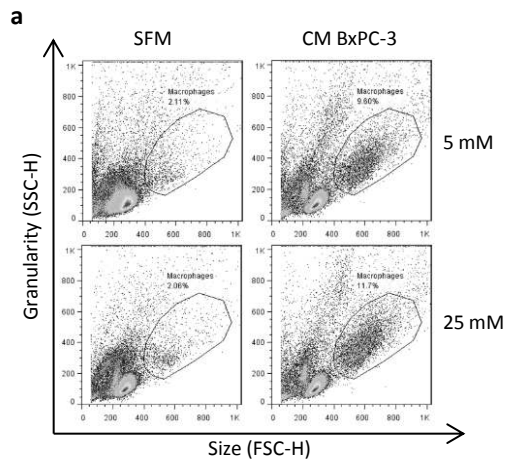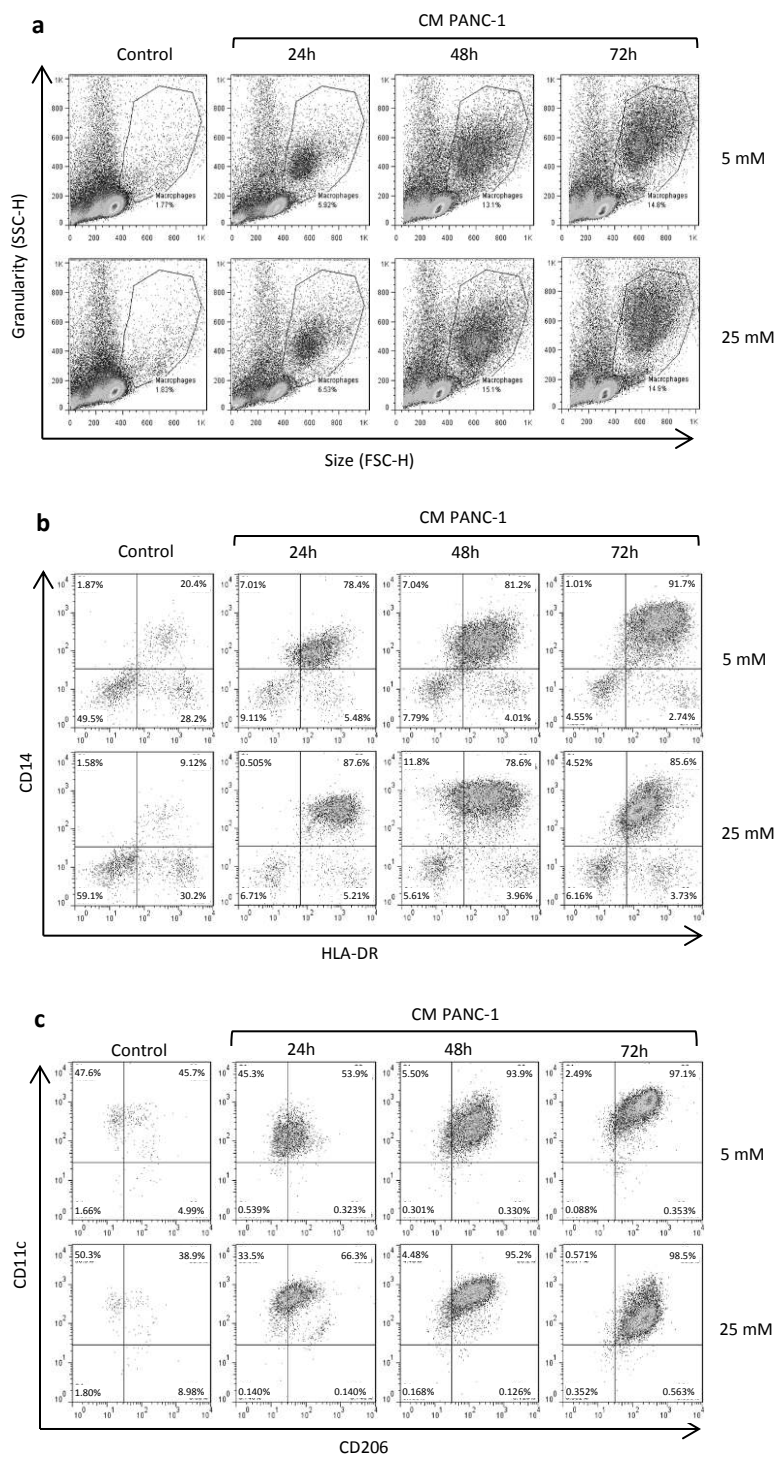□ CTRL
■ After co-culture

5 mM   25 mM

Supplementary figure 1

Supplementary figure 2

Supplementary figure 3