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Establishment of a heterotypic 3D culture system to evaluate the interaction of T_{REG} lymphocytes and NK cells with breast cancer

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ABSTRACT

Three-dimensional (3D) culture approaches to investigate breast tumour progression are yielding information more reminiscent of the in vivo microenvironment. We have established a 3D Matrigel system to determine the interactions of luminal phenotype MCF-7 cells and basal phenotype MDA-MB-231 cells with regulatory T lymphocytes and Natural Killer cells. Immune cells were isolated from peripheral blood using magnetic cell sorting and their phenotype validated using flow cytometry both before and after activation with IL-2 and phytohaemagglutinin. Following the establishment of the heterotypic culture system, tumour cells displayed morphologies and cell–cell associations distinct to that observed in 2D monolayer cultures, and associated with tissue remodelling and invasion processes. We found that the level of CCL4 secretion was influenced by breast cancer phenotype and immune stimulation. We further established that for RNA extraction, the use of proteinase K in conjunction with the Qiagen RNeasy Mini Kit and only off-column DNA digestion gave the best RNA yield, purity and integrity. We also investigated the efficacy of the culture system for immunolocalisation of the biomarkers oestrogen receptor-α and the glycoprotein mucin 1 in luminal phenotype breast cancer cells; and epidermal growth factor receptor in basal phenotype breast cancer cells, in formalin-fixed, paraffin-wax embedded cultures. The expression of these markers was shown to vary under immune mediation. We thus demonstrate the feasibility of using this co-culture system for downstream applications including cytokine analysis, immunolocalisation of tumour biomarkers on serial sections and RNA extraction in accordance with MIQE guidelines.

1. Introduction

The breast tumour microenvironment is an intricate network involving multiple cell types, the interactions of which play pivotal roles in cancer progression. Ex vivo experimentation has attempted to construct tumour microenvironments to better investigate cellular interactions; however, the reductionist approach of two-dimensional (2D) culture systems fails to recreate the complexity of the in vivo microenvironment (Pinto et al., 2011). While three-dimensional (3D) culture systems are more successful in this regard, studies of breast tumourigenesis have focused on metastatic processes primarily using homotypic cultures to investigate breast cancer cell invasion (Poincloux et al., 2011; Chandrasekaran et al., 2012; Yu and Machesky, 2012) or heterotypic cultures investigating breast tumour cell and fibroblast interaction (Olsen et al., 2010). While few studies have assessed the invasion capacity of lymphocytes in 3D cultures (Albertsson et al., 2007; Edsparr et al., 2011), to date, no studies have investigated the heterotypic interactions between immune cells and breast cancer cells in such a system.

Immunity is a fundamental determinant of tumour progression and response to therapy. The vast majority of studies have noted regulatory T (T_{REG}) lymphocyte accumulation in the tumour infiltrating lymphocyte (TIL) population and peripheral blood of breast cancer patients (Bates et al., 2008; Pooi et al., 2006; Bohling and Allison, 2008; Decker et al., 2012). T_{REG} lymphocytes, functionally described as the principal T cell subset responsible for maintenance of self-tolerance; are however, also associated with the induction of tumour tolerance and the suppression of anti-tumour responses (Sakaguchi et al., 1995; Beyer and Schultze, 2006; Mougiakakos et al., 2010). Elevated T_{REG} lymphocyte numbers are associated with poor prognosis in oestrogen receptor $(ER)^+$ invasive ductal carcinoma and human epidermal growth factor receptor 2 $(HER2/neu)^+$ carcinomas (Pooi et al., 2006; Bates et al., 2008). In triple negative breast cancers, T_{REG} lymphocytes have

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; BPCM, basal-phenotype culture model; ER, oestrogen receptor; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; GFRM, growth factor-reduced Matrigel; HER2/neu, human epidermal growth factor receptor 2; IL, interleukin; IFN-γ, interferon-γ; LPCM, luminal-phenotype culture model; MUC1, mucin 1; NK cells, Natural Killer cells; PBMCs, peripheral blood mononuclear cells; PR, progesterone receptor; TIL, tumour infiltrating lymphocyte; TNF, tumour necrosis factor; TGF-β, transforming growth factor-β; T_{REG} lymphocyte, regulatory T lymphocyte.

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traditionally been linked with poor overall, and relapse-free survival (Bohling and Allison, 2008; Cimino-Mathews et al., 2013). However, recent data indicates that a T_{REG} lymphocyte infiltrate conversely correlates with good prognosis in triple-negative cancers (West et al., 2013).

The immunosuppressive action of T_{REG} lymphocytes against effector T lymphocytes and Natural Killer (NK) cells has been documented in both in vitro and in vivo studies (Ghiringhelli et al., 2005; Ralainirina et al., 2007; Salagianni et al., 2011); however, interleukin (IL)-activated NK cells have been shown to be resistant to these inhibitory effects (Chikileva et al., 2010). The primary mechanisms of NK cell killing, facilitated by direct contact with stressed, infected or malignant cells, include the activation of death receptors on target cells and the secretion of degradative granules including perforin, a membrane-disrupting protein and granzymes, a family of proteases (Smyth et al., 2005). NK cells further produce a range of cytokines and chemokines implicated in controlling tumour progression (Wilk et al., 2008). These include: interferon-γ (IFN-γ), IL-2, IL-8, IL-15, tumour necrosis factor (TNF), transforming growth factor-β (TGF-β) and CCL3, amongst others; which allow for interaction with other cell types including malignant cells and T_{REG} cells of the adaptive immune system (Chambers, 2010).

In patients presenting with breast cancers, NK cells are scarce in TIL populations (Georgiannos et al., 2003; Macchetti et al., 2006), despite an elevated presence in peripheral blood (Mozaffari et al., 2007). This may indicate inadequate NK cell homing mechanisms to tumour sites (Albertsson et al., 2003) and possibly the dominance of adaptive immune responses. Alterations in NK cell phenotype and functionality are dependent on tumour stage and presentation, with NK cells exhibiting poor cytotoxic capacity dominating NK populations in advanced breast cancer immune infiltrates (Dewan et al., 2009; Mamessier et al., 2011, 2013). The prognostic significance of NK cells in breast cancer has yet to be established (Roberti et al., 2012), however, gene expression studies have indicated that signatures associated with NK cells are predicative of relapse free-survival in primary breast cancer patients (Ascierto et al., 2013). The scarcity of NK cells and the dominance of T_{REG} lymphocytes in advanced TIL populations may be linked to breast cancer immune evasion and tumour tolerance strategies, where the induction of a local inflammatory response is employed for tumour progression.

The reciprocal interaction between cancer cells, immune cells and the extracellular matrix (ECM) is mediated by a variety of growth factors, cytokines and chemokines. These secreted factors allow, either directly or indirectly, for the hallmarks of tumour progression to occur, including invasion and remodelling of the ECM, cellular proliferation, neovascularisation, immune evasion and extravasation into the vascular or lymphatic system for the establishment of distant secondary sites (de Visser and Coussens, 2005). There is an increasing body of evidence indicating that tumour cells are not only able to induce tumour tolerance and suppress or evade cytotoxic lymphocyte function, but also to induce TILs to become active participants in tumour progression (Whiteside et al., 1992; Drescher and Lynch, 2005; Prestwich et al., 2008; Emens et al., 2012; Cimino-Mathews et al., 2013; West et al., 2013). This stresses the need to investigate the interaction between T_{REG} cells and NK cells in breast cancer in order to shed more light on tumour escape from immunological control, which may or may not be dependent on breast cancer phenotype.

In order to compare the response of luminal and basal phenotype breast cancer cells to immune infiltration, the luminal phenotype ER^+ progesterone receptor $(PR)^+$ MCF-7 cell line and the basal phenotype phenotype (ER^-PR^-HER2/neu^-) MDA-MB-231 cell line (Lostumbo et al., 2006; Neve et al., 2006; Prat and Perou, 2010) were thus selected for this study. To recreate a geometric space more reminiscent of the tumour microenvironment, the commercially available laminin-rich extract established from Engelbreth–Holm–Swarm tumour cell-secreted basement membrane (Matrigel, BD Biosciences) (Kleinman and Martin, 2005; Nyga et al., 2011) was used as a scaffold within which to create 3D heterotypic cultures. Matrigel is regarded as a more biologically relevant scaffold for both normal mammary and cancerous mammary epithelial cell cultures compared to synthetic scaffolds (Ampuja et al., 2013), with morphology therein directly linked with phenotype (Kenny et al., 2007).

We thus present the feasibility of using a 3D heterotypic culture system to investigate the reciprocal interactions of T_{REG} lymphocytes and NK cells with luminal and basal phenotype breast cancers. The effectiveness of downstream applications including RNA extraction for RT-PCR, immunocytochemistry for selected biomarkers and chemokine analysis is demonstrated.

2. Materials and methodology

2.1. Isolation of T_{REG} lymphocyte and NK cell populations

Approximately 30 ml blood from seemingly healthy female volunteers (n = 13) between ages 18–35 (exclusion criteria included pregnancy, autoimmune diseases, immunodeficiency, cancer and a previous history of cancer) was collected via venipuncture in ethylenediaminetetraacetic acid (EDTA)-coated Vacutainers (BD Biosciences, Woodmead, South Africa). Following 1:1 dilution with phosphate buffered saline (PBS), peripheral blood mononuclear cells (PBMCs) were obtained via density gradient centrifugation using Ficoll-Hypaque (1.077 g/cm^3) (GE Healthcare Biosciences AB, Sweden, 17-1440-03). PBMCs were washed in PBS and resuspended in 80 μl PBS (pH 7.2) supplemented with 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, A9418) and 2 mM EDTA, in preparation for magnetic cell sorting. All reagents used in magnetic cell sorting were maintained at a temperature of 8 °C as per the manufacturer's instruction.

The $CD4^+$ Multisort Microbead Kit (Miltenyi Biotec, 130-055-101) was employed for positive selection of the $CD4^+$ lymphocyte compartment followed by removal of residual magnetically labelled cells and enrichment of the $CD4^+$ population using MACS Multisort Release agent (Miltenyi Biotec, 130-055-101) and MACS Multisort Stop Reagent (Miltenyi Biotec, 130-055-101). $CD4^+$ lymphocytes were thereafter labelled with CD25 Microbeads (Miltenyi Biotec, 130-055-101) followed by positive selection and magnetic isolation of the prototypic T_{REG} lymphocyte population. From the unlabelled PBMC fraction, NK cells were labelled with APC-NKp46 (Miltenyi Biotec, 130-092-609) and magnetically isolated using anti-APC microbeads (Miltenyi Biotec, 130-090-855). The median yield of live cells and viability were assessed using the trypan blue exclusion assay and the Bio-Rad Automated Cell Counter TC-20 (Bio-Rad, Parkwood, South Africa).

For activation of lymphocyte populations, T_{REG} cells and NK cells were resuspended at a minimum of 1×10^5 cells/100 μl in RPMI medium 1640 (Lonza, Bloemfontein, South Africa) supplemented with 0.1% penicillin/streptomycin (P/S), 10% foetal bovine serum (FBS), 1 μg/ml phytohaemagglutinin (PHA) (Sigma-Aldrich, L1668) and 0.8 ng/ml IL-2 (Miltenyi Biotec, 130-093-901) for 18 h. Subsequently, cell suspensions were harvested, centrifuged at 400 ×g for 5 min and resuspended in RPMI 1640 supplemented with 0.1% P/S, 10% FBS and 0.8 ng/ml IL-2 for a further 30 h after which the establishment of co-cultures was undertaken (Domaica et al., 2009).

2.1.1. Flow cytometry analysis of lymphocyte subpopulations

Flow cytometry (LSRFortessa, BD Biosciences, South Africa) was used to analyse the samples of isolated T_{REG} and NK cell populations to ascertain the efficacy of the magnetic cell sorting technique and the activation procedure. Cells were resuspended at a maximum of 1×10^6 cells/100 μl PBS. Magnetically isolated T_{REG} cells were incubated with 10 μl CD4-APC (BD Biosciences, Woodmead, South Africa, 555349) and 10 μl CD25-PE (BD Biosciences, 555432). Magnetically isolated NK cells were incubated with 10 μl CD56-PE-Cy7 (BD Biosciences, 557747) and 10 μl NKp46-APC (BD Biosciences, 558051). Both cell populations were incubated with their respective antibodies for

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