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Real-time profiling of NK cell killing of human astrocytes using xCELLigence technology

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ABSTRACT

We have conducted the first profiling of human Natural Killer (NK) cell mediated killing of astrocytes using xCELLigence technology. The sensitivity and applicability of xCELLigence was compared to lactate dehydrogenase (LDH) release and time-lapsed microscopy to validate the killing events. The xCELLigence technology uses electrical impedance measurements from adherent cells and converts into Cell Index (CI). NK cells did not register any Cell Index signal directly, therefore all changes in Cell Index are a direct measure of astrocyte responses. Astrocytes are insensitive to basal NK cells (non-activated NKs). Whereas NK cells activated by IL-2 prior to culture with targets rapidly kill astrocytes. This observation was supported by all methods of analysis. Using the xCELLigence we were able to monitor the longer term killing profile. This demonstrated that at all NK ratios, death was achieved if given long enough. In addition, the development of the killing phenotype was investigated by inducing lymphokine activated killing with IL-2 in the presence of the target astrocytes. In this paradigm of killing, the xCELLigence was the only assay suitable due to the prolonged time-course required for killing, which required 4–5 days to achieve maximal killing (100%). This suggested that the astrocytes can directly suppress the killing activity of the NK cells. These data highlight the sensitivity, applicability and profiling power of the xCELLigence system and support its use for further investigation of NK-killing of healthy and/or tumourogenic astrocytic cells.

1. Introduction

Natural Killer (NK) cells comprise an important part of the innate immune system. They maintain surveillance of tissues and blood, searching for abnormal or unhealthy cells, which they eliminate by cytolytic mechanisms. NK cells are resident in some healthy tissues and actively recruited into diseased tissues. In the brain, the presence of NK cells is associated with states of inflammation (Brehin et al., 2008; Larsson et al., 2000; Mittelbronn et al., 2010), often involving disruption of the blood–brain barrier during encephalitis (Brehin et al., 2008; Hatalski et al., 1998), stroke (Hedtjarn et al., 2004), glioblastoma (Liu et al., 2008; Mittelbronn et al., 2007; Vaquero et al., 1989), or multiple sclerosis (Saikali et al., 2007). In general, the role of NK cells in the diseased human brain is poorly understood, but likely involves their capacity to kill abnormal cells.

The healthy brain has its own resident immune system comprised primarily of microglia and astrocytes. Astrocytes are an integral component of the blood brain barrier (BBB) and comprise one of the most abundant cell types in brain (Prat et al., 2001). Research by others shows that lymphokine-activated NKs have the capacity to kill primary astrocytes (Darlington et al., 2008) and microglia (Lunemann et al., 2008). NKs cells that infiltrate the brain following compromise of the BBB may have the capacity to kill astrocytes, which may impair subsequent inflammatory responses and neuronal support. In contrast, this phenomena could also be beneficial to target astrocytomas and astrogliomas, the most common forms of brain tumour. Therefore, the relationship between human NK cells and astrocytes, in particular the ability of NK cells to kill astrocytes (both healthy and cancerous) in various states of neuroinflammation is particularly important for the advancement of understanding the roles these immune cells have in the aforementioned neurological states.

Here we have investigated the potential of the xCELLigence biosensor technology to determine whether it is capable of reliably measuring killing of astrocytic cells by human NK cells. The xCELLigence technology was chosen, as it has been used previously for quantifying the kinetics of drug cytotoxicity on cells (Ehlers et al., 2010; Hanusova et al., 2010; Smout et al., 2010; Urcan et al., 2010)

Abbreviations: BBB, blood–brain barrier; CI, Cell Index; CNS, central nervous system; E:T, effector to target; LDH, Lactate dehydrogenase; LAK, lymphokine activated killing; NK, Natural Killer; Ntera2/D1, NT2; RTCA, real-time cell analyser.

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and monitoring cell viability in real time (Keogh, 2010; Slanina et al., 2011; Ungefroren et al., 2011). Collectively the data supports the use of the xCELLigence system for measuring the killing of astrocytes by NK cells and we highlight advantages over other classical methods used to measure NK mediated killing. We have established the methods that will allow detailed analysis of the cellular and molecular mechanisms involved in the NK killing of astrocytes and other targets using xCELLigence technology.

2. Materials and methods

2.1. Cell culture

NT2 cell line (Ntera2/D1, CRL-1973) was purchased from ATCC and NT2 precursors were differentiated with retinoic acid using the 10-week differentiation protocol, which yields neurons at 6 weeks (Unsworth et al., 2010) and astrocyte-like cells (NT2As) after 10 weeks (Goodfellow et al., 2011; Unsworth et al., 2011) with functional characteristics of primary astrocytes (Bani-Yaghoub et al., 1999; Lim et al., 2007; Sandhu et al., 2002). Harvested NT2As are routinely stained for GFAP and vimentin. 100% of the NT2As produced by this protocol are vimentin positive, with approximately 30–40% strongly positive for GFAP (Lim et al., 2007; Unsworth et al., 2011). Differentiated NT2As were cryopreserved in 10% DMSO, 40% FBS and 50% DMEM F12 (Invitrogen). NT2As were cultured and maintained in DMEM-F12 media supplemented with 10% FBS. Human primary NK cells were prepared from human blood. Donors gave written informed consent, under protocols approved by the University of Auckland ethics committee. Peripheral blood mononuclear cells (PBMC) were prepared as described previously (Graham et al., 2010) and untouched NK cells were isolated from PBMC using the NK cell negative selection kit (Miltenyi Biotec, #130-092-657). The purity of NK cell preparations was assessed by flow-cytometry and was consistently greater that 95% (data not shown). In all cases, the percentage of $CD3^+$ cells in the NK cell isolates was less than 3%. NK cells were cultured in RPMI-1640 media (Invitrogen) supplemented with 10% FBS (R10 media).

2.2. Target seeding determined by the xCELLigence DP system

The DP version of the xCELLigence system was used throughout these studies. The DP version comprises a measurement unit housed within a standard tissue culture incubator with 3 stations that each take E16 plates (each E16 plate has 16 wells). The 3 stations can be controlled independently (max 16 wells each) or combined into a larger experiment (max 48 wells). The format of the DP version provides flexibility, however this is at the cost of being able to conduct high-throughput applications, as the maximum number of wells in any single experiment is 48. For these studies this impacts the number of NK conditions that can be compared simultaneously (e.g. different donors X ET ratios X activation). The SP or MP version of xCELLigence are better suited to high-throughput applications. The DP unit is wired to an external laptop which runs the xCELLigence software (version 1.2.1).

2.3. Target seeding determined by the xCELLigence DP system

The growth characteristics following seeding were monitored to determine the ideal seeding density and time to beginning killing. The xCELLigence DP system measures the electrical impedance across the high-density electrode array coating the bottom of the well and converts the impedance values to a Cell Index (Ke et al., 2011; Keogh, 2010; Rahim and Uuml Ren, 2011; Smout et al., 2010). Cell Index measurements directly correspond to the strength of cell adhesion and cell number. As cells detach and die (i.e. during cytotoxic events) Cell Index values decrease. An optimal seeding density of 6250 target NT2As per well of the xCELLigence DP E-16 plates (area of E-16 well is equivalent to standard 96-well plate) was selected; this seeding density was used for all experiments.

2.4. Basal killing experiments

To determine the sensitivity of NT2As to human NK cells, different effector to target ratios (E:T ratios) were tested ranging from 1:1 to 10:1 using freshly isolated NK cells. In these experiments, NK cells were not exposed to inflammatory lymphokines and were used immediately following isolation. The xCELLigence DP system killing assays were monitored in real-time with measurement sampling typically every 10–30 min and in some experiments tracked for 3–5 days. Data were processed and plotted using the xCELLigence RTCA software package (version 1.2.1).

2.5. Generation of LAK activity

For the generation of LAK-activity (lymphokine activated killing), NK cells were cultured in the presence of human recombinant IL-2 (Peprotech) 50 ng/ml for 20 h. After this activation period the NK cells were used in each type of killing assay. In additional experiments (Fig. 4 only), the influence of the target NT2As on the generation LAK-activity was investigated by adding IL-2 (50 ng/ml) to NK-astrocyte co-cultures. The IL-2 was added to the co-cultures 5 min after the NK cells were seeded onto the target NT2As.

2.6. LDH assays

The LDH Cytotoxicity Detection Kit (#11 644 793 001) was purchased from Roche and used following the manufacturer's recommendations. In brief, NT2As and NK cells were prepared in an identical manner to those used for the xCELLigence experiments except they were seeded into standard 96-well plates rather than the E-16 xCELLigence plates. At the end of the killing assay the plate was centrifuged at $250 \times g$ for 5 min and 100 μl of the clarified media from each sample was transferred to a new clear-96 well plate well. To each well, 100 μl of LDH reaction mixture was added and incubated for 30 min at room temperature in the dark. The absorbance was measured at 492 nm using a FLUOstar Optima plate reader. Minima (MIN) LDH values were obtained from media only and maxima (MAX) LDH release values were obtained from target cells lysed with 1% triton X-100. In all LDH assays, each treatment was replicated at least three times. The percentage cytotoxicity was calculated as $[=(\text{SAMPLE} - \text{MIN})/(\text{MAX} - \text{MIN}) \times 100\%]$. Following each LDH assay, the remaining cells were fixed with 4% PFA, permeabilised with triton and the NT2As were stained for vimentin (#AB15248, Abcam, Sapphire Biosciences) using standard immunocytochemistry methods (Graham et al., 2006) to assess number of surviving NT2As. Vimentin is not an astrocyte specific marker but used here to stain the entire extremities of the NT2As cytoplasm. The vimentin antibody was used at 1:1000 dilution and detected with anti-rabbit IgG (1:500 dilution) conjugated with biotin (Sigma). The biotinylated secondary was detected with Extravidin peroxidise (1:250 dilution). DAB (Sigma) was incubated for 10 min with cells to achieve the required level of immunoprecipitate. The primary and secondary were incubated overnight at 4 °C with the tertiary incubated for 2 h at room temperature.

2.7. Live cell imaging using Nikon BioStation

Real-time killing events were observed using a Nikon BioStation live-cell imaging platform. In brief, the same experimental design used for the xCELLigence experiments were replicated on glass-slides with NT2As seeded into silicon chambers (Ibidi, GmbH).