Journal of Immunology
Research and Therapy
JIRT, 5(1): 212-214
www.scitcentral.com

ISSN: 2472-727X

Review Article: Open Access

Flow Cytometry: A New Vista in Cell Death Methodologies

G Warnes*

*Flow Cytometry Core Facility, The Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary London University, London, UK.

Received October 31, 2019; Accepted December 23, 2019; Published January 28, 2020

INTRODUCTION

Research into Regulated Cell Death (RCD) has been mainly based upon the use of Western Immunoblotting and immunofluorescence microscopy to determine the type of RCD predominating in a cell population [1-5]. Both of these methodologies have limitations in terms of detecting low incident RCD populations in heterogeneous cell populations especially from tissues [1-5]. Flow cytometry has not been as useful in the measurement of cell death processes as perhaps could be envisaged which has been hampered to a degree by the lack of RCD specific reagents [4]. Although the flow cytometric use of the protein annexin V to bind to externalised phospholipids has been useful to detect apoptosis little else has been achieved in extending its use to analyse other forms of RCD [6]. The addition of active fluorescent substrates (*e.g.* active caspases), functional mitochondria and Reactive Oxygen Species (ROS) probes although gives a lot of information does not allow the identification of different forms of RCD [4]. Advances in flow cytometry in recent years including the detection of more than 20 fluorophores simultaneously coupled with the availability of antibodies raised against RCD specific intracellular antigens for use in permeabilised cells as opposed to those raised for immunoblotting has made possible a flow cytometric approach that detects specific forms of RCD [4,5]. The recent development of a flow cytometric necroptosis assay at the Blizard Institute, Queen Mary University London was achieved by the use of a intracellular fluorescenated RIP3 (Receptor-interacting serine/threonine-protein kinase-3) antibody which detects a necroptotic population by the up-regulation of RIP3 within a cell line such as Jurkat T cells [5,7,8]. This assay has also incorporated a means to separate these necroptotic cells from those undergoing caspase dependent apoptosis which was achieved by the intracellular use of an active caspase-3 antibody together with a fixable live-dead fluorescent stain [5,7-10]. The up-regulation of RIP3 was proved to indicate necroptosis by prior incubation of Jurkat cells with necroptosis inhibitor necrostatin-1 which abrogated this up-regulation of RIP3 [5,7,8]. A specific assay for the detection of necroptosis would ideally be an antibody that would detect the phosphorylated form of MLKL (mixed lineage kinase domain-like) the specific biological marker for detecting necroptosis but to my knowledge none are currently available [10]. This approach using flow cytometry has allowed the simultaneous identification of live and dead cells undergoing multiple forms of RCD including necroptosis (phenotype Live-Dead$^{+ve/-ve}$/RIP3^{High}/Caspase-3^{-ve}), early and late apoptosis (phenotype Live-Dead$^{+ve/-ve}$/RIP3^{-ve}/Caspase-3^{+ve}), RIP1-dependent apoptosis (phenotype Live-Dead$^{+ve/-ve}$/RIP3^{+ve}/Caspase-3^{+ve}) and a double negative (DN) cells (phenotype Live-Dead$^{+ve/-ve}$/RIP3^{-ve}/Caspase-3^{-ve}) [5,7-9]. This multiple RCD detection flow cytometry assay became the backbone from which further forms of RCD could then be analysed by the addition of cleaved PARP (poly (ADP-ribose) polymerase 1) and γH2AX antibodies [7,8]. This permitted the identification of multiple sub-populations of cells displaying DNA Damage (γH2AX^{+ve}/PARP^{-ve}), γH2AX hyper-activation of PARP in the presence of capsase-3 or parthanatos in the absence of caspase-3 (γH2AX^{+ve}/PARP^{+ve}), apoptosis (γH2AX^{-ve}/PARP^{+ve}) or DN phenotype (γH2AX^{-ve}/PARP^{-ve} [8,10,11]. Additional analysis for autophagy and ER stress using LC3B (microtubule associated light chain B) and PERK (protein kinase R-like endoplasmic reticulum kinase) respectively were also added to the protocol. The identification of multiple forms of RCD by flow cytometry has also recently been extended to include the measurement of biologically functional parameters such as mitochondrial inner membrane potential and ROS by the use of fixable fluorescent probes (unpublished data) [8,12].

Corresponding author: G Warnes, Flow Cytometry Core Facility, The Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary London University, 4 Newark Street, London E1 2AT, UK, Tel: 44(0)20-7882-2402; E-mail: g.warnes@qmul.ac.uk

Citation: Warnes G. (2020) Flow Cytometry: A New Vista in Cell Death Methodologies. J Immunol Res Ther, 5(1): 212-214.

The assay has been used on the Jurkat T-cell line in published work to date and unexpected findings amongst a plethora of data are that dead cells have a similar phenotype (Live-Dead^{+ve}/RIP3$^{+ve/-ve}$/Caspase-3$^{+ve/-ve}$) no matter what form of RCD was induced [5,7-9]. So it is worth reflecting upon the relative incidence of live and dead cells in studies employing immunoblotting techniques. So it is now possible with flow cytometry more than any other technique to identify a range of RCDs including necroptosis, apoptosis, autophagy, ER stress, DNA Damage, hyperactivated PARP, cleaved PARP and parthanatos all within the same sample after induction of cell death by a single drug for example. This methodology strikingly has made it possible to show that autophagic cells have less DNA Damage than non-autophagic cells thus highlighting a mechanism by which autophagy can preserve cell health [8]. Recent drug research at the Blizard Institute highlights the fact that a single cytotoxic drug can induce multiple forms of RCD with induction of autophagy, ER stress and necroptosis in keratinocyte cell lines (personal observation). Current research at Queen Mary University employing heterogeneous cell populations is also underway to investigate how γδ T-cells die in response to fungal infection and the associated RCD and ACD DAMPS (Damage Associated Molecular Patterns) signals from the gut tissue in a diseased state [1,13-15]. The role of leucocyte cell death in trauma patient's survival is similarly under study [1,13-15]. It can be envisaged that this type of flow cytometric approach to the simultaneous measurement of multiple forms of RCD can be broadened to investigate their role in heterogeneous preparation of cells present in other disease states including infection (e.g. HIV), autoimmunity, cardiovascular, nervous and respiratory diseases.

The use of MitoTracker probes has been to date misused by the lack of use of cell viability probes when analysing the effects of cytotoxic drugs [16,17]. Dead cells do not have mitochondrial function and so should be excluded; this has led to misleading conclusions in some respects about the nature of the role of mitochondria in RCDs [16,17]. However recent work in this laboratory making use of cell viability dyes and an active caspase fluorescent substrate along with MitoTracker and ROS probes has shown that live cells after induction of necroptosis (*e.g.* by shikonin) have hyper-polarized mitochondrial membrane potential with increased ROS levels (manuscript in preparation). I have also previously reported these types of findings with other drugs such as staurosporine, rapamycin and UV-irradiation [18,19]. The results of such an approach to the detection and measurement of RCDs induced by etoposide, shikonin and thapsigargin is beginning to show indeed the complexity of such RCD responses to the action of cytotoxic drugs [8,20]. Recent publications have shown that multiple forms of necroptotic, apoptotic, oncotic and undefined (DN) cells can be defined by their level of γH2AX, PARP, parthanatos, ROS and mitochondrial function which can be modulated by drugs and their inhibitors for example [8,20].

Shortcomings of the assay besides locating permeability resistant MitoTracker and ROS probes would also include the additional use of a RIP1 antibody. This would then allow the specific detection of RIP1-dependent apoptosis rather than by the current indirect approach by using RIP3 which assumes that this molecule is always associated with RIP1. Previous studies employing shikonin or TNFα to induce necroptosis with blockade by necrostatin-1 showed that necroptosis was indeed inhibited but the incidence of RIP1-dependent apoptosis was increased rather than reduced as would be expected [5,7,8]. Work employing a non-phosphorylated MLKL antibody (rather than the specific phosphorylated form, which is not available) has shown no up-regulation of this specific biological marker of necroptosis (personal observation). So the development of these missing antibodies would hopefully improve the current assay as would the inclusion of the biological marker for gasdermin for the detection of pyroptosis rather than activated caspase-1 [1].

Future work would include the addition of a mechanism for the detection of ferroptosis, lysosomal-dependent, immunogenic, MPT driven cell and endtotic deaths. While NETosis appears to be mainly cell type specific for which a flow cytometric methodology has already been developed [1,21]. The phenotyping of the biological markers for each form of RCD so far developed has also recently been applied to Accidental Cell Death (ACD) [9]. In which it has been shown that dead oncotic (or necrotic) cells can be phenotyped to be either RIP3$^{+ve/-ve}$ whilst being Caspase-3^{-ve} and were shown to be a DNA Damage phenotype (γH2AX^{+ve}/PARP^{-ve}), parthanatos (γH2AX^{+ve}/PARP^{+ve}), cleaved PARP (γH2AX^{-ve}/PARP^{+ve}) or a DN phenotype (γH2AX^{-ve}/PARP^{-ve}), respectively [9]. This has allowed the identification of eight forms of oncosis whose incidence interestingly could be modulated by inhibitors of necroptosis and apoptosis which perhaps indicates the mechanistic signalling routes involved in ACD [9]. This approach to the simultaneous measurement of multiple forms of RCD and ACD expressed in heterogeneous cell populations could also be transferred to tissues by the use of heavy metal antibody labels rather than fluorescence. Thus enabling the use of Hyperion Imaging CyTOF technology to broaden the concept of this type of approach to the measurement of RCD still further. This would allow the detection and analysis of multiple forms of RCD from relatively low numbers of *ex vivo* cells isolated from tissue and within *in situ* sections of tissue in the numerous diseases states were a role for a form of cell death is known. This would include for example RCDs occurring in carcinomas, inflammation of gut tissue [13], neurodegenerative diseases [22], Gaucher's disease [23], ischemia-reperfusion injury [14], liver disease [23] and the targeting of drugs for the treatment of these diseases [24]. This type of approach to gastroenterological diseases

and trauma is being undertaken at Queen Mary University and is hoped to reveal cellular mechanisms involved with a clarity not observed before that could aid the treatment of such diseases and may lead to the development of new approaches to their treatment in the near future.

REFERENCES

1. Galluzzi L, Vitale I, Aaronson SA, Abrams JM, Adam D, et al. (2018) Molecular mechanisms of cell death: Recommendations of the Nomenclature committee on cell death differ. Cell Death Differ 25: 486-541.
2. Galluzzi L, Bravo-San Pedro JM, Vitale I, Aaronson SA, Abrams JM, et al. (2015) Essential versus accessory aspects of cell death: Recommendations of the NCCD. Cell Death Differ 22: 58-73.
3. Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke EH, et al. (2012) Molecular definitions of cell death subroutines: Recommendations of the nomenclature committee on cell death. Cell Death Differ 19: 107-120.
4. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, et al. (2009) Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ 16: 1093-107.
5. Lee HL, Pike R, Chong MHA, Vossenkamper A, Warnes G (2018) Simultaneous flow cytometric immunophenotyping of necroptosis, apoptosis and RIP1-dependent apoptosis. Methods 134-135: 56-66.
6. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C (1995) A novel assay for apoptosis flow cytometric detection of phosphatidyl serine early apoptotic cells using fluorescein labeled expression on Annexin V. J Immunol Methods 184: 39-51.
7. Vossenkamper A, Warnes G (2018) A flow cytometric immunophenotyping approach to the detection of regulated cell death processes. J Immunol Sci 25: 6-12.
8. Bergamaschi D, Vossenkamper A, Lee WYJ, Wang P, Bochukova E, et al. (2019) Simultaneous polychromatic flow cytometric detection of multiple forms of regulated cell death. Apoptosis 24: 453-464.
9. Vossenkamper A, Warnes G (2019) Flow cytometry reveals the nature of oncotic cells. Int J Mol Sci 20: 1-13.
10. Grootjans S, Vanden Berghe T, Vandenabeele P (2017) Initiation and execution mechanisms of necroptosis: An overview. Cell Death Differ 24: 1184-1195.
11. Andrabi SA, Dawson TM, Dawson VL (2008) Mitochondrial and nuclear cross talk in cell death: Parthanatos. Ann N Y Acad Sci 1147: 233-241.
12. Popat A, Patel A, Warnes G (2019) A flow cytometric study of ER stress and autophagy. Cytometry A 95: 672-682.
13. McCarthy NE, Eberl M (2018) Human gamma delta T-cell control of mucosal immunity and inflammation. Front Immunol 9: 985.
14. Manson J, Cole E, De'Ath HD, Vulliamy P, Meier U, et al. (2016) Early changes within the lymphocyte population are associated with the development of multiple organ dysfunction syndrome in trauma patients. Crit Care 20:176.
15. Brown GD (2011) Innate antifungal immunity: The key role of phagocytes. Annu Rev Immunol 29: 1-21.
16. Sun W, Wu X, Gao H, Yu J, Zhao W, et al. (2017) Cytosolic calcium mediates RIP1/RIP3 complex-dependent necroptosis through JNK activation and mitochondrial ROS production in human colon cancer cells. Free Radic Biol Med 108: 433-444.
17. Chen S, Lv X, Hu B, Zhao L, Li S, et al. (2018) Critical contribution of RIPK1 mediated mitochondrial dysfunction and oxidative stress to compression-induced rat nucleus pulposus cells necroptosis and apoptosis. Apoptosis 23: 299-313.
18. Warnes G (2014) Measurement of autophagy By Flow Cytometry. Curr Protoc Cytom 68: 1-10.
19. Warnes G (2016) The multiplexing of assays for the measurement of early stages of apoptosis by polychromatic flow cytometry. In: Schmid I, editor. Flow Cytometry Select Topics. On-line: InTech Open, pp: 85-99.
20. Vossenkamper A, Warnes G (2019) Thapsigargin induced apoptosis occurs with a delayed DNA damage response. Br J Cancer Res 2: 314-319.
21. Masuda S, Shimizu S, Matsuo J, Nishibata Y, Kusunoki Y, et al. (2017) Measurement of NET formation in vitro and in vivo by flow cytometry. Cytometry A 91: 822-829.
22. Rosello A, Warnes G, Meier U (2012) Cell death pathways and autophagy in the central nervous system and its involvement in neurodegeneration, immunity and CNS infection: To die or not to die - that is the question. Clin Exp Immun 168: 52-57.
23. Zhou W, Yuan J (2014) Necroptosis in health and diseases. Semin Cell Dev Biol 35: 14
24. Suvarna V, Singh V, Murahari M (2019) Current overview on the clinical update of Bcl-2 anti-apoptotic inhibitors for cancer therapy. Eur J Pharmacol 862: 172655.