The development of anti-Human Calcitonin Receptor antibodies to track receptor expression in blood diseases and activity in apoptosis using flow cytometry

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Background

- CT Receptor is the most ancient member of Family B G protein-coupled receptors (GPCRs) with orthologues found in primitive metazoan chordates (*Ciona intestinalis*, lineage split from vertebrates over 520mya), more complex invertebrates and vertebrates. In mammals CT Receptor can function with or without receptor activity modifying proteins (RAMPs).
- The expression of CT Receptor is widespread in vertebrates during foetal (1) and postnatal development, in wound healing (1), pre-apoptotic cell stress (2), autophagy (3) and in several diseases including leukaemia, brain and prostate cancers, and cardiovascular disease.
- Anti-human CT Receptor antibodies to an intracellular (mAb1H10, epitope 1) and an extracellular (mAb1C11 & mAb2C4, epitope 4) epitope have been developed to facilitate FACS analysis and studies of CT Receptor-positive cells.
- An anti-CT Receptor antibody:fluorophore conjugate (mAb2C4:AF568, CalRexin^{TM}:568) was developed, is accumulated into live cells and used to investigate a role for CT Receptor in cell stress induced with cytotoxins and subsequent apoptosis (2) or spontaneous apoptosis in cultured retinal organoids.
- CalRexin^{TM}:647 identifies additional dying populations not identified by Annexin V.

Aim

Anti-human calcitonin receptor (CT Receptor) antibodies have been developed with refinements that allow increasing facility for measurement by flow cytometry. Here we report the detection of CT Receptor in blood disease and in apoptotic cells.

Methods

- Mouse monoclonal anti-human CT Receptor antibodies (mAb1C11 & mAb2C4) were developed that bind epitope 4 within the extracellular domain. Further, mAb2C4 was conjugated with AZDye^{TM} 568 (mAb2C4:AF568, CalRexin^{TM}:568). The high degree of labelling (4.4 AZDye^{TM} 568/mAb2C4) results in a high fluorescent signal when accumulated into dying cells. CalRexin^{TM}:647 is a brighter reagent offering greater scope for differentiating subpopulations (CalRexin^{TM}:fluorophores, www.apopbiosciences.com).
- FACS analysis of Jurkat cells treated +/- 1µM staurosporine was performed in a Beckman Coulter Astrios flow cytometer following live cell staining with 1µg/mL of CalRexin^{TM}:568 (buffer 1% BSA/PBS) or annexin V:biotin/ streptavidin:488. Sytox dyes & Zombie Aqua were used to identify dead cell populations.
- Retinal organoids are cultured at the Childrens Medical Research Institute (NSW) and grown for different periods over several weeks.

References

1. Wookey PJ, Zulli A, Lo C, Hare DL, Schwarer AP, Darby IA, Leung A. Calcitonin receptor (CTR) expression in embryonic, foetal and adult tissues: developmental and pathophysiological implications. In: "The calcitonin gene-related peptide family; form, function and future perspectives", Eds Debbie L Hay & Ian M Dickerson. Netherlands: Springer. 2010, pp199-233 [ISBN: 978-90-481-2908-9].
2. Furness SGB, Hare DL, Kourakis A, Turnley AM, Wookey PJ. A novel ligand of calcitonin receptor reveals a potential new sensor that modulates programmed cell death. Cell Death Discovery 2,16062, 2016.
3. Wookey PJ, Gupta P, Bittencourt L, Cheung S, Hare DL, Furness SGB. Methods to measure calcitonin receptor activity, up-regulated in cell stress, apoptosis and autophagy. F1000Research 10:1019, 2021.

Results

Figure 1: **Cartoon of the human CT Receptor** indicating the binding sites for mouse monoclonal antibodies: **epitope 1:** mAb1H10 (IgG2A); **epitope 2:** mAb10G6; **epitope 4:** mAb1C11 (IgM), mAb2C4 CalRexin^{TM}(IgG1) & mAb9B4 (IgG2A).

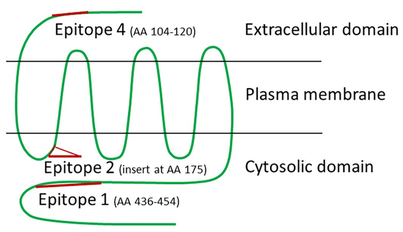
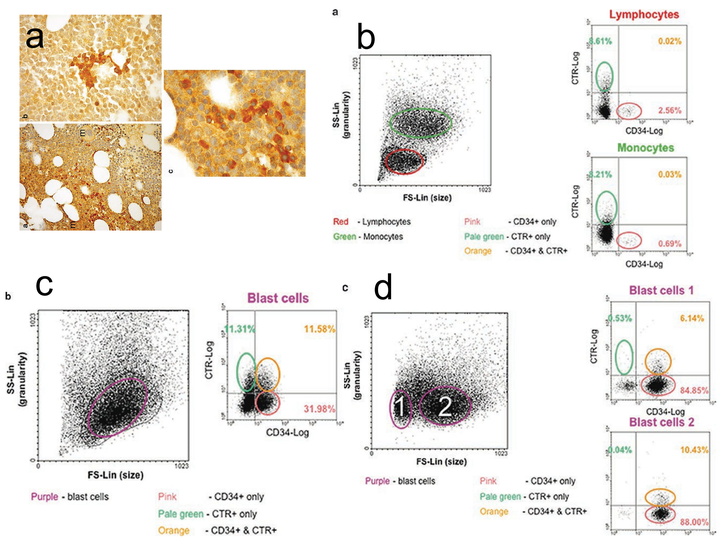


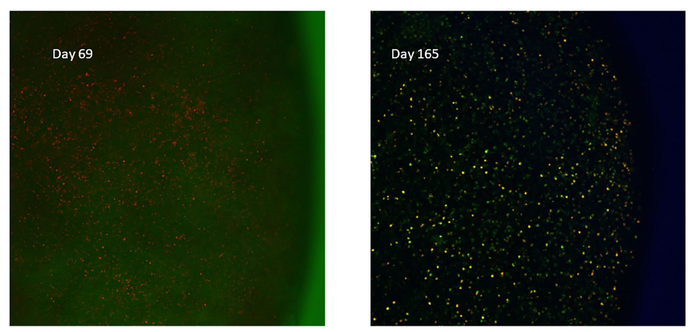
Figure 2: FACS analysis using **anti-human CTR antibody** (MAb 1C11) and anti-CD34 antibody (1).

(**a**) AML bone marrow stained for CT Receptor.
(**b**) normal PBSCs, lymphocytes (CTR+/CD34+, 0.02%; CTR+/CD34-, 8.6%) and monocytes (**CTR+/CD34+, 0.03%**; CTR+/CD34−, 8.2%).
(**c**) AML bone marrow (**CTR+/CD34+, 11.3%;** CTR+/CD34−, 11.6%).
(**d**) ALL bone marrow, population 1 (**CTR+/CD34+, 6.1%;** CTR+/CD34−, 0.5%), population 2 (**CTR+/CD34+, 10.4%;** CTR+/CD34−, 0.4%).



Type of Leukaemia	Sub-category	Number of samples	% CT Receptor+ve/CD34+ve[a]		% of disease category	
			0.5-2.5%	>2.5-15%	0.5-2.5%	>2.5%
ALL	Not available	12	4	5	33%	42%
AML	-	8	1	1	31%	12.5%
	MLD	2	1	1		
	M1	1	1	0		
	M2	2	1	0		
	M3	3	1	0		
Normal BM		6	2	0	33%	0%

Figure 3: Merged images of retinal organoid IPE2 cells at days 69 & 165 stained with CalRexin^{TM}:568, sytox (green) and colocalised (orange).



Retinal organoid cells were FAC sorted at days 77 & 177 in which a high background with annexin V was evident.

Day	P3 CalRexin^{TM}:568	P4 Sytox	P5 Annexin V
77	6%	2%	11%
177	10%	9%	21%

Figure 4: Flow cytometry of live Jurkat cells treated with 0.5µM staurosporine and stained with CalRexin^{TM}:647 and Zombie Aqua identified live, dying (apoptotic), transition and dead cell populations (central panel). Annexin V was unable to differentiate intermediate subpopulations (left panel). Cells untreated with staurosporine and stained with CalRexin^{TM}:647 were negative. The isotype IgG1:AF647control with treated cells was negative.

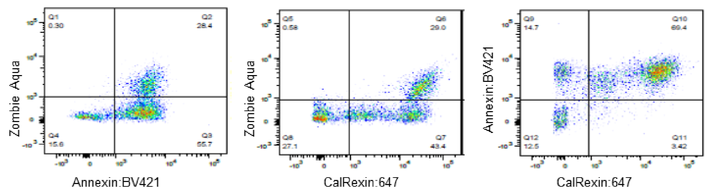
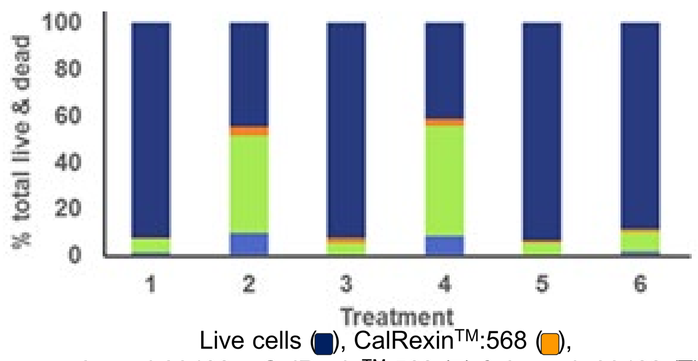


Figure 5: Live, dying and dead cell populations of Jurkat cells treated with different cytotoxins and inhibitors, detected with CalRexin^{TM}:568 . Treated (24 hours) with (**1**) 10µM rapamycin (induces autophagy), and induction of apoptosis with (**2**) 50µM etoposide, (**3**) 50µM etoposide + caspase inhibitor z-VAD-fmk, (**4**) 15ng/mL TRAIL, (**5**) 15ng/mL TRAIL+ z-VAD-fmk, (**6**) untreated.



Live cells ( ), CalRexin^{TM}:568 ( ),
AnnexinV:488 + CalRexin^{TM}:568 ( ) & Annexin V:488 ( )

Conclusions

- CT Receptor is found activated in several blood diseases including B-CLL, multiple myeloma and blast cells of AML and ALL patient samples all of which express high levels of Receptor.
- FACS analysis with CalRexin^{TM}:647 and Zombie Aqua was useful to define four subpopulations not identified with annexin V, including live, apoptotic, transition and dead cell populations of Jurkat cells treated with staurosporine.
- CalRexin^{TM}:568 identified dying and dead cells in live staining of retinal organoids in which there was a high background with annexin V.
- CalRexin^{TM}:568 detects dying and dead cells in cell lines induced with a variety of cytotoxins (2).