616 THE NOVEL α-GAL-BASED IMMUNOTHERAPY AGI-134 EVOKES CD8+ T CELL-MEDIATED IMMUNITY BY DRIVING TUMOR CELL DESTRUCTION, PHAGOCYTOSIS AND TUMOR-ASSOCIATED ANTIGEN CROSS-PRESENTATION VIA MULTIPLE ANTIBODY-MEDIATED EFFECTOR FUNCTIONS

Jenny Middleton[1,2], Oliver Schulz[3], Amber Charlemagne[1,2], Sascha A. Kristian[1,2] and Stephen Shaw[1,2]

[1]Agalimmune Limited, Sandwich, UK; [2]BioLineRx Limited, Modi'in; Israel; [3]The Francis Crick Institute, London, UK.

ABSTRACT

AGI-134 is a glycolipid-like, Function-Spacer-Lipid (FSL) molecule being developed as an intratumoral immunotherapy for solid tumors. This synthetic compound is comprised of the α-Gal sugar antigen (Galα1-3Galβ1-4GlcNAc-R), a chemical linker and a lipid tail.

α-Gal is present on the glycolipids and glycoproteins of most mammalian species, and is synthesised by the α1,3-galactosyltransferase (α1,3GT) enzyme. Humans and Old World monkeys carry an inactivated version of the α1,3GT gene and therefore lack the α-Gal antigen[1]. However, constant exposure to α-Gal-bearing commensal gut bacteria results in the development of high-titer natural antibodies against α-Gal, called anti-Gal[2,3].

α-Gal is responsible for the hyperacute rejection of xenogeneic tissue implants in humans, through rapid recruitment of anti-Gal to the transplanted tissue and activation of the complement cascade[4]. Intratumoral injection of a crude extracts of α-Gal glycolipids from rabbit erythrocytes, into primary B16-F10 melanoma lesions on α1,3GT $^{-/-}$ mice, has been shown to induce a tumor-specific T cell response that protected the mice from development of distal lesions[5,6]. We have previously presented data showing the same protection from distal lesion formation by AGI-134 in the B16-F10 melanoma model, as well as synergism of AGI-134 with an anti-PD-1 monoclonal antibody.

We now present *in vitro* data demonstrating the multiple anti-Gal-mediated effector functions, induced by treating α-Gal-negative mammalian cells with AGI-134, that drive the observed *in vivo* anti-tumor immunity.

METHODS

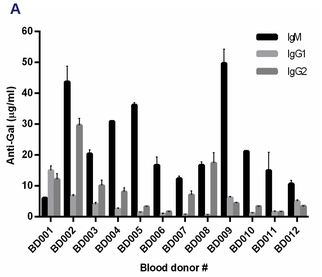
Anti-Gal was quantified by capture onto α-Gal-coated ELISA plates, followed by detection with isotype- and subclass-specific enzyme-conjugated antibodies. Human Ig isotype and subclass standards (eBioscience, Nordic-MUbio) were used to generate standard curves.

For assessment of anti-Gal recruitment and complement opsonization, target cells were treated with AGI-134 by rotating incubation at 37ºC for 1 hour before further incubation with normal human serum (NHS), heat-inactivated NHS (iNHS) or purified anti-Gal IgG, as specified in figure legends. For assessment of downstream effector functions, target cells treated as described above were co-cultured with effector cells as described in figure legends. Analysis was performed by flow cytometry.

RESULTS

Anti-Gal IgM and IgG are present in human serum, with the predominant IgG subclasses being IgG1 and IgG2 in both serum and purified polyclonal anti-Gal IgG

A



B

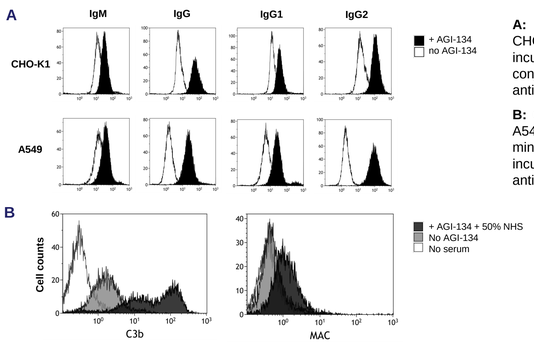
IgG1	IgG2	IgG3	IgG4
187.9	253*	19.7	9.4

Human IgG subclass distribution in purified anti-Gal IgG (µg/ml). *Above limit of quantitation: concentration calculated by subtraction of all other subclasses from total IgG concentration.

A: Anti-Gal isotype and subclass distribution in human serum. Not shown: anti-Gal IgG3 and IgG4 levels for all samples were either below or just above the limit of quantitation.

B: Anti-Gal IgG subclass distribution in purified anti-Gal IgG. Anti-Gal IgG was purified from human IVIG by affinity chromatography (Rockland Immunochemicals). The IgG subclass distribution was determined using Invitrogen's Human IgG Subclass Profile kit.

Anti-Gal in human serum is recruited to the surface of AGI-134-treated mammalian cells and drives complement deposition



A: Anti-Gal recruitment. AGI-134-treated CHO-K1 and A549 cells were sequentially incubated with 50% iNHS and fluorochrome-conjugated isotype- or subclass-specific antibodies.

B: Complement deposition. AGI-134-treated A549 cells were incubated with 50% NHS for 45 mins at RT, then stained by sequential incubation with mouse anti-C3b/anti-MAC and anti-mouse IgG FITC.

Complement dependent cytotoxicity (CDC) of AGI-134-treated mammalian cells is driven by anti-Gal IgM

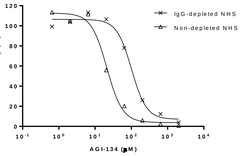
A: Anti-Gal and CH50 levels in IgG-depleted and non-depleted NHS. IgG was depleted from NHS using a protein G column (GEHealthcare). Total and anti-Gal IgG/M was quantified by ELISA. CH50 values were determined using a CH50 ELISA kit (Quidel).

B: CDC assay. CHO-K1 cells were treated with a dose-titration of AGI-134 then washed and incubated for 1 hour with IgG-depleted or non-depleted NHS. Cell viability was determined using ATPLite (PerkinElmer).

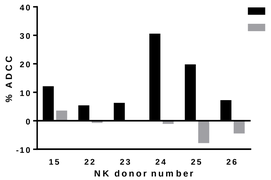
A

	IgG-depleted NHS	Non-depleted NHS
IgG (µg/ml)		
Total	<1.56	5383
Anti-Gal	<0.003	23.8
IgM (µg/ml)		
Total	5683	4082
Anti-Gal	7.98	8.75
CH50 (U Eq/ml)	61.3	112.5

B



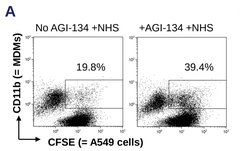
Anti-Gal IgG drives antibody dependent cell-mediated cytotoxicity (ADCC) of AGI-134-treated mammalian cells



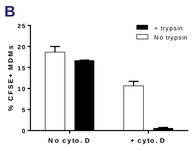
NK (CD3- CD56+) cells were enriched from freshly isolated PBMCs using magnetic beads (eBioscience), and cultured overnight in the presence of IL-2. CFSE-stained CHO-K1 target cells were incubated with or without 1 mg/ml AGI-134 and 30 µg/ml anti-Gal IgG before being co-cultured with NK cells for 4 hours (8:1 E:T ratio). A viability dye was added prior to analysis by flow cytometry. The % of dead target cells was determined as the % of 7AAD+ cells within the CFSE+ gate. % ADCC = (% dead target cells in presence of anti-Gal) – (% dead target cells in absence of anti-Gal).

Recruitment of anti-Gal and complement enhances phagocytosis of AGI-134-treated human cancer cells by monocyte-derived human macrophages (MDMs)

A



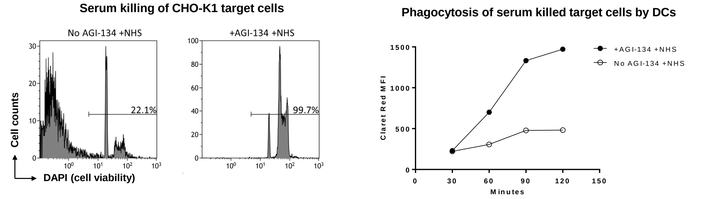
B



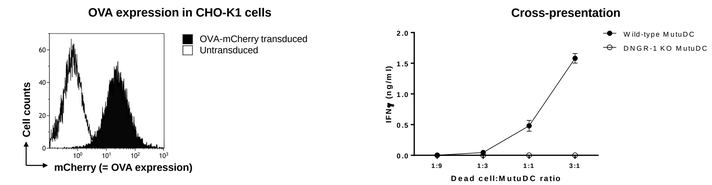
CFSE-stained A549 cells were treated with AGI-134 and NHS under conditions sufficient to opsonize cells with anti-Gal and complement but not resulting in cell lysis. These viable target cells were co-cultured with MDMs, generated by 7-day culture of PBMCs in the presence of M-CSF, for 4 hours. MDMs were identified by staining with anti-CD11b-APC **(A)** or CellTrace Far Red **(B)**. To demonstrate engulfment, cytochalasin D was added to co-cultures, and bound cells were separated by trypsin treatment prior to analysis **(B)**.

Complement-killed AGI-134-treated mammalian cells are preferentially taken up by murine CD8α dendritic cells and cell-associated antigens from dead cells are cross presented to CD8+ T cells

A



B



A: Uptake assay. CHO-K1 target cells were killed by treatment with AGI-134 and NHS, but remained viable following treatment with AGI-134 alone. Treated cells were stained with Claret Red and co-cultured with MutuDCs for up to 2 hours. Uptake of target cells by MutuDCs was determined by incorporation of Claret Red into the DC population.

B: Cross-presentation assay. OVA-expressing CHO-K1 target cells were killed by treatment with AGI-134 and NHS then co-cultured with WT and DNGR-1-KO MutuDCs. After 4 hours, pre-activated OT-I T cells (which recognise the SIINFEKL-OVA peptide in combination with the MHC-I molecule H-2K^b) were added for overnight culture. Supernatants were collected and levels of IFNγ quantified by ELISA.

CONCLUSIONS

- Anti-Gal in human serum is predominantly IgM, IgG1 and IgG2.
- AGI-134-treatment recruits the predominant anti-Gal isotypes and subclasses to the cell surface.
- Anti-Gal IgM effectively drives the deposition of, and activation of, complement, resulting in CDC of AGI-134-treated cells; whilst anti-Gal IgG drives ADCC.
- Opsonization of AGI-134-treated target cells with anti-Gal and complement enhances their uptake by both human MDMs and CD8α murine DCs.
- Cell-associated antigens from target cells killed by treatment with AGI-134 and human serum are cross presented by CD8α murine DCs to CD8+ T cells, thereby enabling generation of anti-tumor immunity.
- AGI-134 holds promise as a new immunotherapy for solid tumors, alone and in combination with checkpoint inhibitors. AGI-134 will be entering clinical trials shortly.

REFERENCES & ACKNOWLEDGEMENTS

1. Galili, U. *et al, The Journal of Biological Chemistry.* 1988; 263(33): 17755-62.
2. Galili, U. *et al. The Journal of Experimental Medicine.* 1984; 160(5): 1519-21.
3. Galili, U. *et al. Infection and Immunity.* 1988; 56(7): 1730-7.
4. Parker, W. *et al. Journal of Immunology.* 1994; 153: 3791-3803.
5. Galili, U. *et al. Journal of Immunology.* 2007; 178: 4676-4687.
6. Abdel-Motal, U. *et al. Cancer Immunology, Immunotherapy: CTII.* 2009; 58(10): 1545-56.

The authors wish to thank Prof. Caetano Reis e Sousa (The Francis Crick Institute, UK) for helpful discussion and advice. We thank Prof. Hans Acha-Orbea (University of Lausanne, Switzerland) for permitting experiments with MutuDC cells.