iLite® ADCC mTNF-alpha
ASSAY READY CELLS

iLite ADCC anti- mTNF-alpha Assay Ready Cells allow you to measure the ADCC activity of your anti- mTNF-alpha antibody in a simple, rapid and sensitive manner.

Monoclonal anti-TNF-alpha inhibitors are one of the most commons types of antibody drugs on the market. The mechanism by which these drugs act is most often blocking of soluble TNF-alpha or the TNF receptor. Since TNF-alpha is produced through cleavage of a membrane bound form of the protein, there is a risk that anti-TNF-alpha antibodies bind the membrane bound TNF-alpha and trigger effector functions, such as ADCC. By using the *iLite* ADCC mTNF-alpha Activity Assay, the risk of an anti-TNF-alpha antibody drug inducing ADCC can be quantified.

iLite ADCC Effector (V) Assay Ready Cells are human cells which express high levels of FcγRIIIa (CD16) and signals to a Firefly Luciferase (FL) reporter gene. The cells are engineered to have a high tolerance for serum, and include a second reporter gene, Renilla Luciferas (RL), which allows for normalization of cell counts, serum matrix effects or lysis of the effector cells by the target cells.

The *iLite* ADCC Target mTNF-alpha (+) Assay Ready Cells are human cells engineered to overexpress mTNF-alpha and optimized to give high sensitivity and specificity when used together with ***iLite*** ADCC Effector (V) Assay Ready Cells. Since unspecific activation of ADCC can be a confounding factor when performing ADCC assays, we have also developed ***iLite*** ADCC Target mTNF-alpha (-) Assay Ready Cells which are depleted of mTNF-alpha expression, to be used as an internal control. The control cells can also be used for adjusting background levels when analyzing serum samples, thereby effectively removing any issues with varying background between samples.

This cell-based assay is unique in its setup – it does not require any culturing and can be run within one workday.

The iLite anti- mTNF-alpha ADCC Activity Set can be used for the quantification of ADCC activity of anti- mTNF-alpha antibodies

- Unparalleled sensitivity
- Normalization readout available
- High precision, due to Assay Ready Target cells with a constant high expression of mTNF-alpha
- Assay Ready Target negative cells for use as internal control and background adjustment

***iLite*® ADCC mTNF-alpha Assay Ready Cells**	
Product code	BM5001 *iLite*® ADCC Effector (V) Assay Ready Cells BM5013 *iLite*® ADCC Target mTNF-alphaα(+) Assay Ready Cells BM5014 *iLite*® ADCC Target mTNF-alphaα(-) Assay Ready Cells BM4095 *iLite*® anti-mTNF-alphaαADCC Activity Set
Host Cell	For BM5001: Human T lymphocyte cell line, Jurkat (ATCC #TIB-152) For BM5013, BM5014: Human embryonic kidney cell line, HEK293 (ATCC# CRL-1573)
Format	Assay Ready Cells
Application	The *iLite*® ADCC Effector (V) Assay Ready Cells can be used together with matched *iLite*® ADCC Target mTNF-alphaα(+) and *iLite*® ADCC Target mTNF-alpha (-) Assay Ready Cells for the quantification ADCC activity. • Quantification of anti-mTNFα ADCC activity (E-281-GB)
Assay time	4 hours (incubation)
Detection system	Luminescence
Availability	Research Use Only (RUO)*

**These products are intended for professional research use only. The data and results originating from using the products, should not be used either in diagnostic procedures or in human therapeutic applications.*

In accepting delivery of iLite® Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third party, and recipient is only to use them directly in assays. The iLite® cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered iLite® Assay Ready Cells would constitute an infringement.

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Quantification ADCC Activity of Therapeutics Anti-TNFα in a Cohort of Patients with RA and Crohn's Disease

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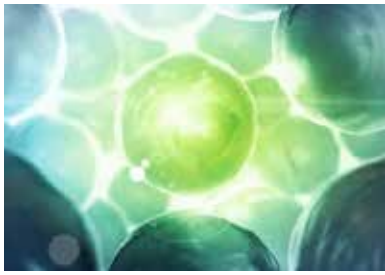

Introduction

Patients treated with therapeutic antibodies often respond in a different manner to therapy and some patients also develop anti-drug antibodies (ADA) that can negatively impact their ability to respond to therapy. Thus, there is a need to monitor individual patients over time for both circulating levels of the therapeutic antibody and for anti-drug antibodies. Furthermore, since the activity of a number of therapeutic antibodies is mediated in part by antibody-dependent cell-mediated cytotoxicity (ADCC) there is also a need to monitor patients for the ADCC activity of the therapeutic antibody. This is rendered difficult, however, due to the high concentration of IgG present in human serum that can bind to the surface of the target cells resulting in a nonspecific increase in the activity of the effector cells that can confound the results obtained. The development of recombinant target cells expressing noncleavable membrane bound TNFα and homologous control cells that do not express membrane bound TNFα provides a means of quantifying ADCC activity in samples of serum from patients with autoimmune diseases treated with different TNFα antagonists.

Methods

I: Establishment of an Engineered Target Cell Line Expressing High Constant Levels of mTNFα at the Cell Surface

Quantification of the ADCC activity of TNFα antagonists requires a target cell expressing membrane bound TNFα. Although TNFα is initially membrane TNFα RII -/- HEK293 bound it is subsequently cleaved by the ADAM17 (TACE) protease. Thus, in order to establish a cell line that expresses membrane-bound non-cleavable TNFα site-directed mutagenesis was used to mutate the protease cleavage suite in order to change amino acids AV to LL at position 76-77 of the gene encoding human TNFα (Figure 1A). Noncleavable TNFα expressed on the surface of a cell will bind, however, to the TNFα RII receptor present on the surface of neighboring cells resulting in cell death and rendering the establishment of a permanent cell line difficult. Thus, in order to obviate such difficulties the TNFα RII receptor was invalidated using genome editing.

The gene encoding the TNFα RII receptor was invalidated in HEK293 cells (ATCC® CRL-1573) using CRISPR-Cas9 genome editing. Briefly two guide RNA sequences were designed, synthetized and cloned into the nuclease vector GeneArt CRISPR in order to guide the Cas9 double stranded DNA endonuclease to a specific site within exon 2 of the TNFα RII gene located on chromosome 12, and exon 2 of the TNFα RII gene located on chromosome 1, in order to isolate TNFα RII -/- HEK293 cells. The TNFα RII -/- HEK293 cells were then transfected with a mTNFα expression vector and stable clones were isolated and characterized for mTNFα expression (Figure 1B).

10	20	30	40	50
MSTESMIRDV	ELAEEALPKK	TGGPQGSRRC	LFLSLFSFLI	VAGATTLFCL
60	70	80	90	100
LHFGVIGPQR	EEFPRDLSLI	SPLAQLLSS	SRTPSDKPVA	HVVANPQAEG
110	120	130	140	150
QLQWLNRRAN	ALLANGVELR	DNQLVVPSEG	LYLIYSQVLF	KGQGCPSTHV
160	170	180	190	200
LLTHTISRIA	VSVQTKVNLL	SAIKSPCQRE	TPEGAEAKPW	YEPIYLGGVF
210	220	230		
QLEKGDRLSA	EINRPDYLDF	AESGQVYFGI IAL		

Figure 1A. Site Directed Mutagenesis of the Protease Cleavage Site of Human TNFα cells.

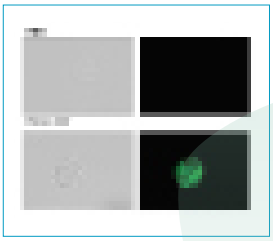

Figure 1B. Cell surface expression of mTNFα +/+ was visualized using an inverted fluorescent microscope and Infliximab and a FITC labelled anti hIgG1 monoclonal antibody.

Results

I. Quantification of the ADCC Activity of TNFα antagonists

Stable clones were isolated and characterized for ADCC activity in the presence the J5.35 effector cells and Remicade (infliximab) and then sub-cloned. A suitable sub-clone was isolated, characterized, and propagated giving rise to the mTNFα +/+ target cell line. The J5.35 effector cells and the mTNFα +/+ target cells were then used to quantify the ADCC activity of Remicade (infliximab), Adalimumab, and Etanercept as shown for cells in culture in Figure 2. Vials of J5.35 effector cells and vials of mTNFα +/+ target cells were frozen separately using standard techniques. Upon thawing, effector cells and target cells were mixed at an E:T ratio of 3:1 and incubated for 6 hours in the presence of increasing concentrations of Remicade. FL and NL activity was then determined using the Nano-Glo dual luciferase substrate and results were expressed as relative luciferase units (RLU).

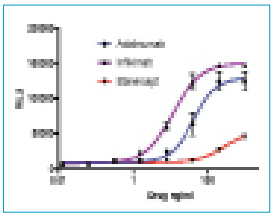

Figure 2. Quantification of the ADCC Activity of TNFα Antagonists using J5.35 Effector & mTNFa +/+ Target Cells

II. Quantification of the ADCC Activity of TNFα antagonists in Patients with Autoimmune disease

The use of homologous target negative cells in which the specific drug target has been invalidated by genome editing provides a means of estimating the level of non-specific activation in the presence of a given concentration of human serum. This value can then be subtracted from that observed with the homologous cells expressing the specific drug target in the presence of the same concentration of human serum. This technique has been use to quantity the ADCC activity of the TNFα antagonist infliximab in samples of serum from patients with Crohn's disease (Table 1). A reading for Target ++ /Target -/- cells of 5.0 or greater was considered to be positive from the results obtained from the control samples with and without a 1/20 final dilution of normal human serum (Table 1). Thus, samples 1, 3, and 9 were found to exhibit ADCC activity using this technique (Table 1). Samples 1, 3, and 9 were also found to contain detectable levels of infliximab using either an ELISA (Matriks Bioteck, TR-QS-INF) or bioassay (Lallemand et al., J Immunol. Methods 2011 373 (229-39) as shown in Table 1.

No ADCC activity was detected in samples 7, 8, 14 & 20 that were found to contain anti-infliximab antibodies determined using a commercially available ELISA (Matriks Bioteck reference TR-ATIv4). Furthermore, the anti-infliximab antibodies detected by ELISA were also shown to be neutralizing using a bioassay (Lallemand et al., J Immunol. Methods 2011 373 (229-39).

The ADCC activity of adalimumab and etanercept was also quantified in samples of serum from patients with rheumatoid arthritis (Table 2). Thus, samples 7 to 11 and sample 17 from patients treated with etanercept were found to contain readily detectable ADCC activity (Table 2). Similarly, samples 4, 5, 8, 12, 13, 14, 17, & 19 from patients treated with adalimumab were also found to contain readily detectable ADCC activity (Table 2).

Infliximab	ADCC Reporter Gene Assay				Residual Drug		ADA	
Sample	Target Negative Cells (RLU)	Target Positive Cells (RLU)	Target+/Target-	Serum Sample/Control	Bioassay (% of Drug Activity)	ELISA (µg/ml)	Bioassay (% of Drug activity)	ELISA (µg/ml)
No Hum. Serum	1122	4264	3,80	0,33	0%	0,026	0%	0
Hum. Serum	2607	12830	4,92	1,00	0%	0,026	14%	0
No Hum. Serum+Infliximab	1382	39370	28,49	3,07	100%	3	80%	0,7
Hum. Serum+Infliximab	1902	39075	20,55	3,05	100%	3	79%	0,7
Patient Sample 1	1975	36750	18,61	2,86	100%	2,58	90%	0
Patient Sample 2	3490	17315	4,96	1,35	33%	0,033	80%	0
Patient Sample 3	2032	11740	5,78	0,92	40%	0,032	83%	0
Patient Sample 4	1607	7422	4,62	0,58	0%	0,025	89%	0
Patient Sample 5	3619	15380	4,25	1,20	0%	0,027	81%	0
Patient Sample 6	1456	6900	4,74	0,54	17%	0,026	79%	0
Patient Sample 7	1826	7228	3,96	0,56	17%	0,025	0%	0,554
Patient Sample 8	1189	4756	4,00	0,37	0%	0,025	0%	0,554
Patient Sample 9	2494	51750	20,75	4,03	100%	0,97	79%	0
Patient Sample 10	1605	4882	3,04	0,38	17%	0,025	84%	0
Patient Sample 11	1721	7670	4,46	0,60	33%	0,027	81%	0
Patient Sample 12	1312	4724	3,60	0,37	17%	0,025	83%	0
Patient Sample 13	2260	8855	3,92	0,69	0%	0,032	80%	0
Patient Sample 14	2736	9099	3,33	0,71	33%	0,024	21%	0,503
Patient Sample 15	3063	12785	4,17	1,00	0%	0,026	91%	0
Patient Sample 16	1984	6331	3,19	0,49	0%	0,025	90%	0
Patient Sample 17	1452	5628	3,88	0,44	0%	0,025	93%	0
Patient Sample 18	1379	4492	3,26	0,35	0%	0,024	90%	0
Patient Sample 19	1428	5981	4,19	0,47	0%	0,029	87%	0
Patient Sample 20	1250	4300	3,44	0,34	0%	0,025	51%	0,509

Table 1. Quantification of the ADCC Activity of Infliximab in Patients with Crohn's Disease

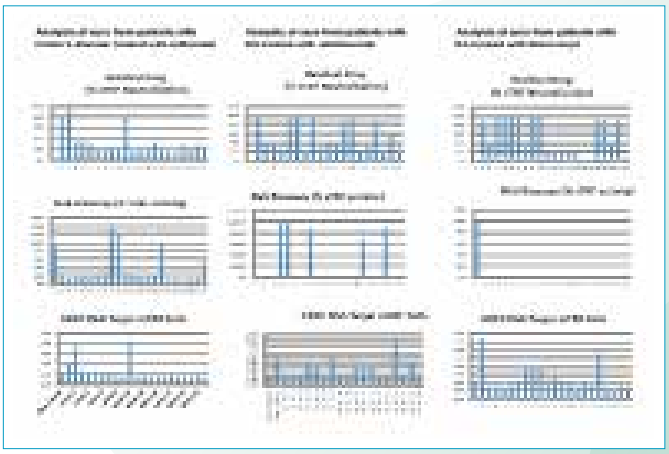

Table 2. Quantification of the ADCC Activity of Infliximab in Patients with Crohn's Disease and R.A.

Conclusion

All the samples of serum containing residual infliximab or adalimumab activity were found to be positive for ADCC activity. In contrast, none of the samples containing neutralizing anti-drug antibodies exhibited ADCC activity. None of the samples from patients treated with Etarnecept contained neutralizing anti-drug antibodies determined using a reporter gene assay. Several samples that were shown to contain residual drug activity did not exhibit detectable ADCC activity suggesting that some binding anti-drug antibodies may be directed towards the Fc moiety of the drug.