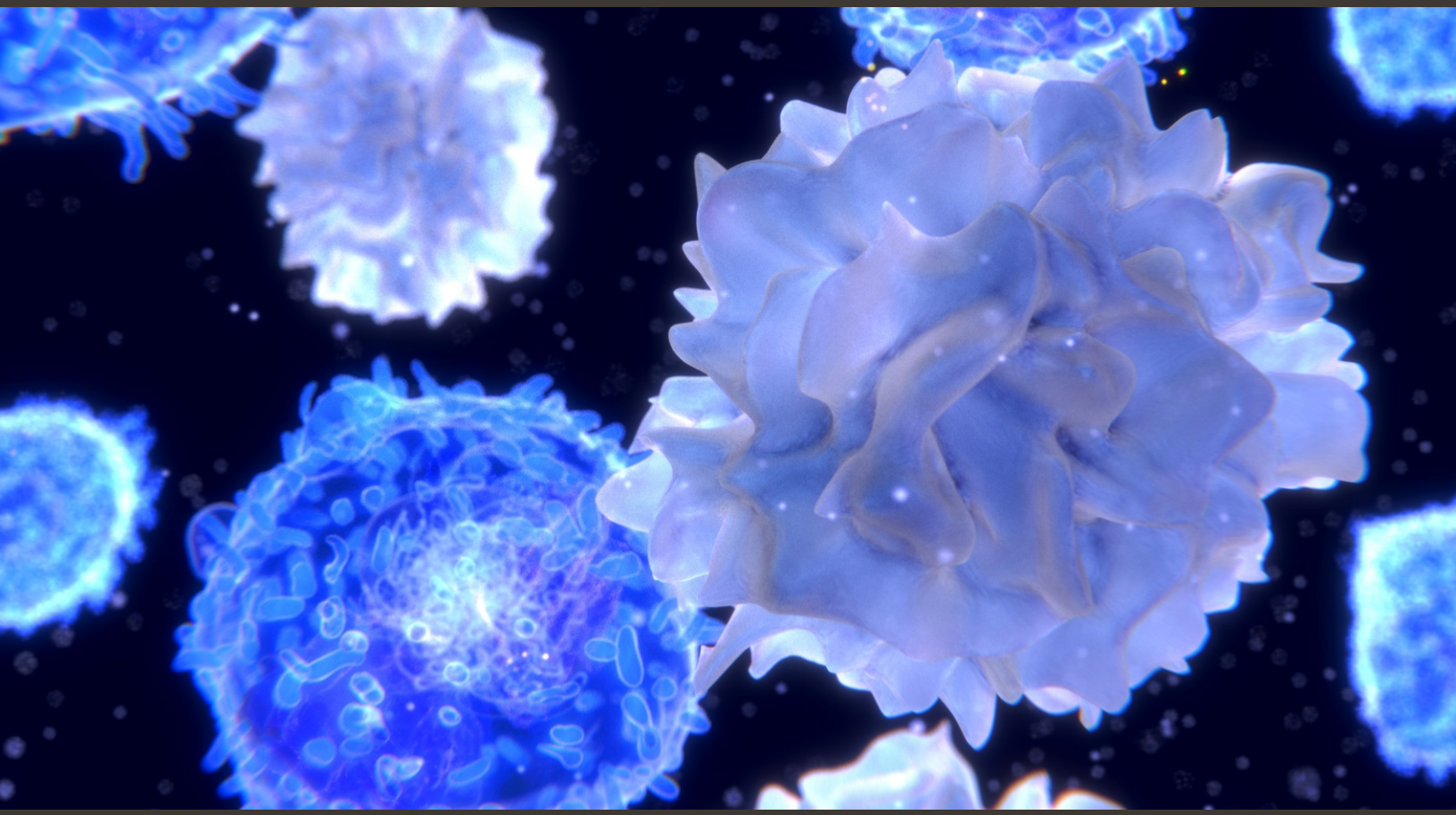
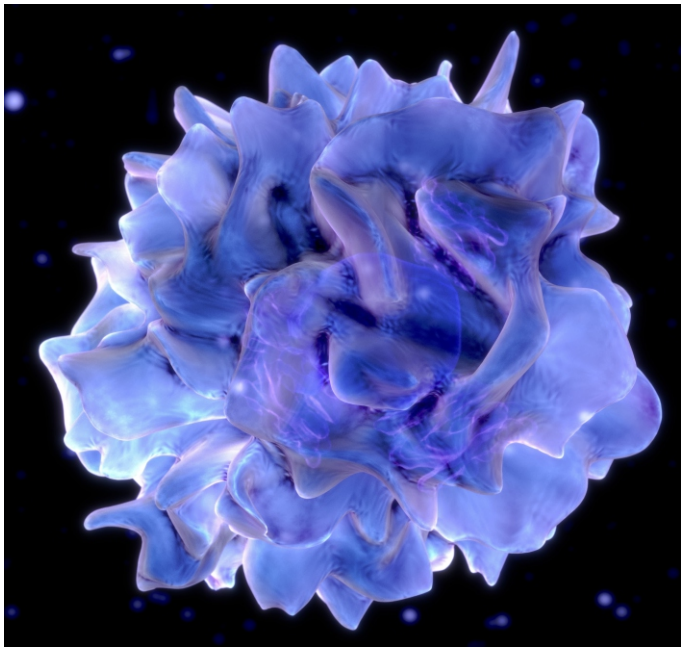
Dendritic Cell (APC) and Autologous T-Cell Response



PBMC-derived DC



The use of peripheral blood mononuclear cell (PBMC) derived dendritic cells (DC) for presenting tumor cell antigens to T-cells has become an important *in vitro* tool in tumor vaccine research and development. Several strategies have been developed for maturing the DC into functional antigen presenting cells (APC) that are loaded with the tumor (or other protein) antigen(s) of interest. One of those methods involves culturing monocytes isolated from human PBMC in the presence of interleukin-4 (IL-4) and granulocyte-monocyte colony stimulating factor (GM-CSF) which then normally mature into DC in 6-7 days. More recently, a novel method that shortens the DC maturation time to 48 hours has been introduced. Human DC are initially incubated with IL-4/GM-CSF for 24 hours followed by activation with prostaglandin E2/ tumor necrosis factor-alpha/interleukin-6/interleukin-1 beta (PGE2/TNF-α/IL-6/IL-1β) for an additional 24 hours, thus yielding mature, functional DC after only two days of culture. Xeno Diagnostics has modified this latter method to generate human DC in three days that are capable of presenting tumor cell (or protein) antigens to T-lymphocytes in a mixed lymphocyte culture setting (MLTuC). Results from ongoing in-house studies that are being conducted to qualify this method are highlighted below.

DC Maturation Time

According to Dauer and coworkers (2003), monocytes that are cultured for 24 hours with GM-CSF and IL-4, followed by stimulation with pro-inflammatory mediators PGE2/TNFα/IL-6/IL-1β for another 24 hours, rapidly undergo all phases of DC differentiation. GM-CSF and IL-4 treated blood monocytes will down-regulate CD14 expression and increase MHC II expression which is consistent with early immature DC development. When proinflammatory cytokines are added in sequence, the cells display a fully mature DC immunophenotype (CD83+CD40+CCR7+CD14-CD80+ CD86*high*MHC II high) (Dauer et al., 2003).

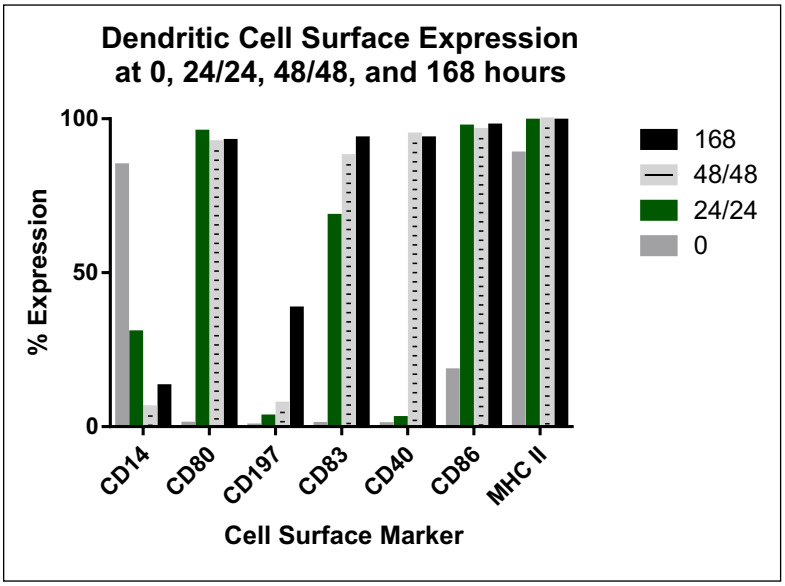


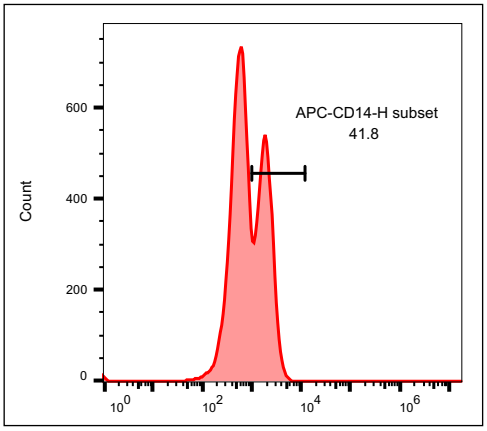
Figure 1

We examined the phenotypic marker profile of human DC that were cultured with IL-4 and GM-CSF (maturation) and then with PGE2/TNF-α/IL-6/IL-1β (activation). The scheme was 24-hour maturation/24-hour activation (24/24), 48-hour maturation/48-hour activation (48/48), and 168-hour incubation with IL-4 and GM-CSF only. It was observed that the CD14 monocyte marker rapidly down regulates between 24 – 48 hours while most markers of the mature DC immunophenotype (CD14-, CD40+, CD83+, CD80+, CD86+, CD197+ (CCR7), and MHC II+) are maximally expressed by 48 hours (Figure 1).

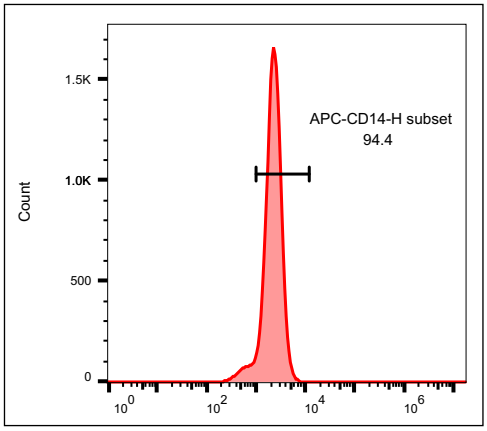
Antigen Loading of DC

When and how to "load" DC with antigen for presentation to T-cells is a subject of debate. Both immature and mature DC have been utilized for antigen loading. Antigen(s) has been loaded as whole cell lysates, tissue or cellular homogenates, or as peptides in the presence of keyhole limpet hemocyanin (KLH).

The current method at Xeno Diagnostics (XD) utilizes human PBMCs isolated by Ficoll gradient density centrifugation of whole blood. Monocytes are in turn isolated from the recovered PBMCs by microbead cell separation (untouched or negative selection) which yields enhanced cell recoveries (Histograms 1 and 2).



Histogram 1: Pre-column CD14 = 41.7%



Histogram 2: Post-column CD14 = 90.1%

The post-column recovered monocytes are cultured with IL-4 and GM-CSF for 48 hours to allow full expression of CD40, followed then by addition of the cytokine activation cocktail of PGE2/TNF-α/IL-6/IL-1β for the final 24 hours of incubation. Antigen is loaded after the first 24 hours of incubation (immature DC stage) and remains until the end of the DC culture period at 72 hours (mature DC stage). The loaded DC are next added to T-cells in media containing soluble CD40 ligand (sCD40L) and incubated for an additional 3 days after which either proliferation and/or cytokines are measured using the BrdU ELISA method or Luminex multiplexing technology (MagPix), respectively.

Co-culture: T-Cells and DC Loaded with Jurkat or BDCM Cell Homogenate

An initial autologous T-cell proliferation study was performed with DC loaded with antigen derived from either Jurkat (Creative Bioarray Clone E6-1; CSC-C9455L) or BDCM (ATCC® CRL-2740™) cell homogenates. Homogenates from Jurkat and BDCM cells were prepared using a gentleMacs™ Disassociator and the protein concentration determined by a bicinchoninic acid assay (BCA). Suspensions of BDCM homogenate protein in PBS were prepared at concentrations of 500, 100, 50, 10, and 5 µg/mL and Jurkat homogenate protein concentrations of 250, 100, 50, 10, and 5 µg/mL (final concentration). The homogenates were added to maturing DC (GM-CSF and IL-4) in culture at 24 hours. After an additional 24 hours, a DC activation cocktail (PGE2/TNF-α/IL-6/IL-1β) was added and the cultures maintained for another 24 hours (72 hours total). All media was then removed and replaced with previously isolated T-cells in AIMV media containing sCD40L. To assess cell proliferation, bromo-deoxyuridine (BrdU) was added on Day 5 and total BrdU incorporation was measured by ELISA on Day 6 (Fig 2).

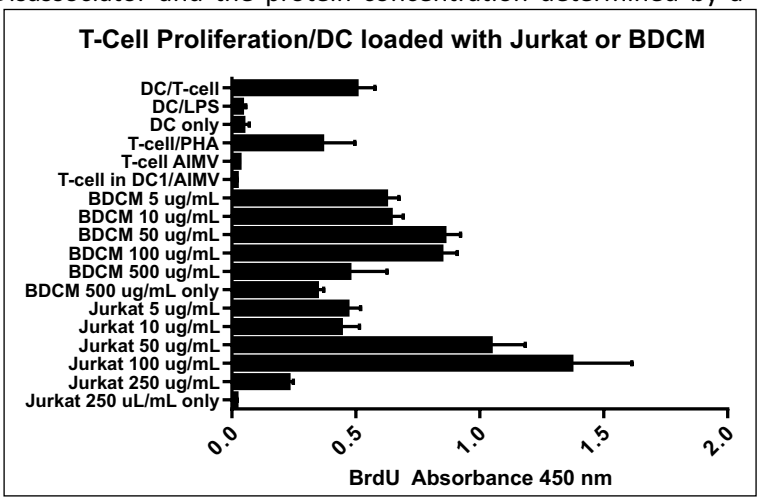


Figure 2: T-Cell Proliferation with Dendritic Cell Laded with BDCM or Jurkat Antigen

T-Cells Co-cultured with DC Loaded with Tetanus Toxoid

We examined the effects on T cell proliferation and cytokine production after co-culturing different ratios of DC [(loaded with tetanus toxoid (TT)] with purified T lymphocytes (1:10, 1:20, 1:40, 1:80, and 1:160). Pan-T cells were isolated by magnetic bead separation from a portion of the recovered PBMCs and were placed in AIMV media. Human monocytes were isolated from PBMC and matured for 24 hours with IL-4 and GM-CSF (10% FBS/RPMI). Tetanus toxoid (5 µg/mL) was mixed with the maturing DC after 24 hours. At 48 hours, activation media (PGE2/TNFα/IL-6/IL-1β) was added. After 72 hours media was removed and replaced with previously isolated T-cells in AIMV media containing sCD40L. Media was collected for cytokine analysis utilizing a Luminex MagPix™ and for proliferation by BrdU incorporation and ELISA on Day 6 (6 days total) (Figure 3 and Table 1).

Optimal stimulation by the TT-loaded dendritic cells was observed at DC/T cell ratios of 1:10 and 1:20 (Figure 3). Similarly, co-cultures with these cell ratios (1:10 and 1:20) had increases in each of the 7 cytokines analyzed (IFN-γ, IL-4, IL-6, MIP-1α, TNF-α, IL-10 and IL-12). At higher DC/T cell ratios there was a general tendency for a reduction in cytokine release, especially for IL-6 and IL-12 (Table 1). The relative order of magnitude of cytokine secretion was IL-10> MIP-1α>TNF-α>IFN-γ>IL-4>IL-6.

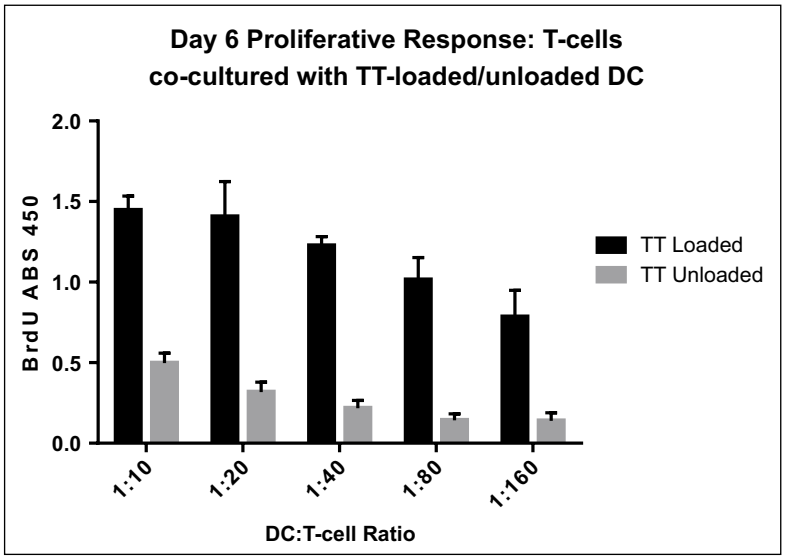
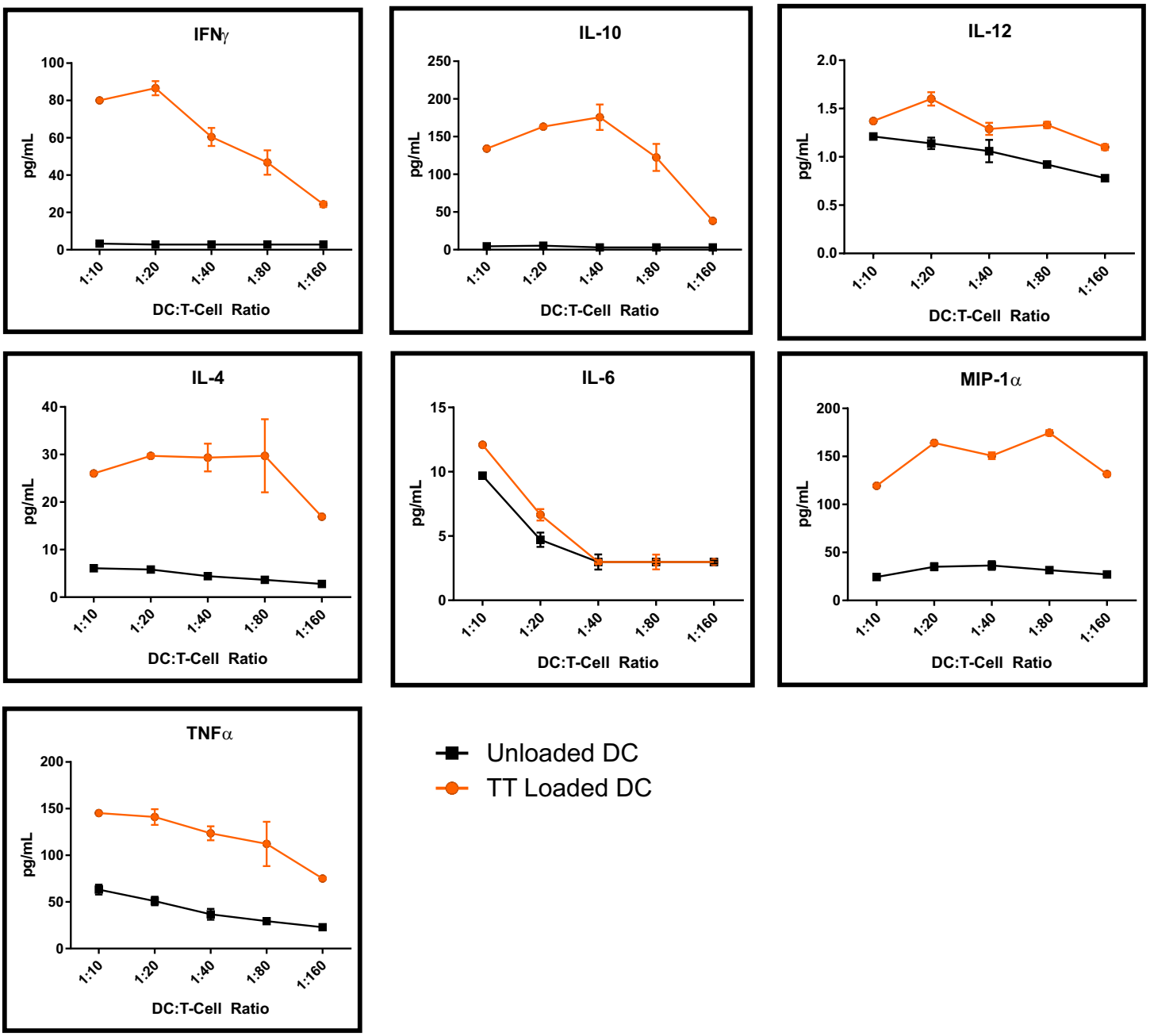


Figure 3: Autologous T-Cell Proliferation with Matched TT Loaded and Unloaded DC cultured with CD40L

		DC: T-Cell	IFNγ	IL-10	IL-12	IL-4	IL-6	MP-1α	TNFα
Plate 1 (CD40L)	Loaded	**1:10**	80.06	134.14	1.37	26	12.09	119.41	145.23
		1:20	86.61	163.17	1.6	29.73	6.65	164.13	141.03
		1:40	60.47	175.75	1.29	29.36	2.98	150.78	123.57
		1:80	46.77	122.43	1.33	29.73	2.98	174.59	112.18
		1:160	24.34	38.25	1.1	16.91	2.98	131.6	75.13
		20k DCs	2.82	3.02	1.21	13.77	13.34	202.37	54.46
		200k TCs	2.82	3.02	1.06	2.78	2.98	16.51	25.42
	Unloaded	**20k DCs**	2.82	3.02	1.14	14.8	14.66	162.4	53.22
		1:10	3.33	4.43	1.21	6.08	9.7	24.4	63.18
		1:20	2.82	5.23	1.14	5.8	4.71	35.13	50.98
		1:40	2.82	3.02	1.06	4.42	2.98	36.46	36.81
		1:80	2.82	3.02	0.92	3.62	2.98	31.63	29.45
		1:160	2.82	3.02	0.78	2.78	2.98	27.11	22.95

Table 1: Quantitative cytokine measurements from tetanus toxoid loaded dendritic cell & autologous T-Cell culture stimulated with sCD40L. Blue cells were below the lower limit of detection (LLOD).

Figure 4: Quantitative cytokine measurements from tetanus toxoid loaded dendritic cell & autologous T-Cell culture stimulated with sDC40L.



Effect of IL-2 and IL-7 on T-Cells co-cultured with DC Loaded with Tetanus Toxoid

To test whether DC activated autologous T-cells could be expanded, IL-2 and IL-7 were added in addition to sCD40L, on culture Day 4 to a separate set of tetanus toxoid (TT) loaded DC:T-cell ratios (1:10, 1:20, 1:40, 1:80, and 1:160). BrdU was added on Day 5 and total BrdU incorporation (proliferation) was measured by ELISA on Day 6.

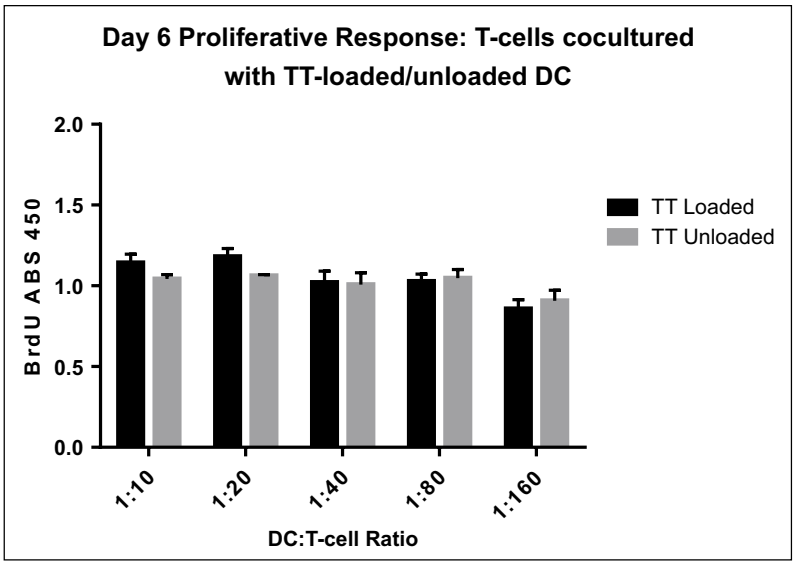


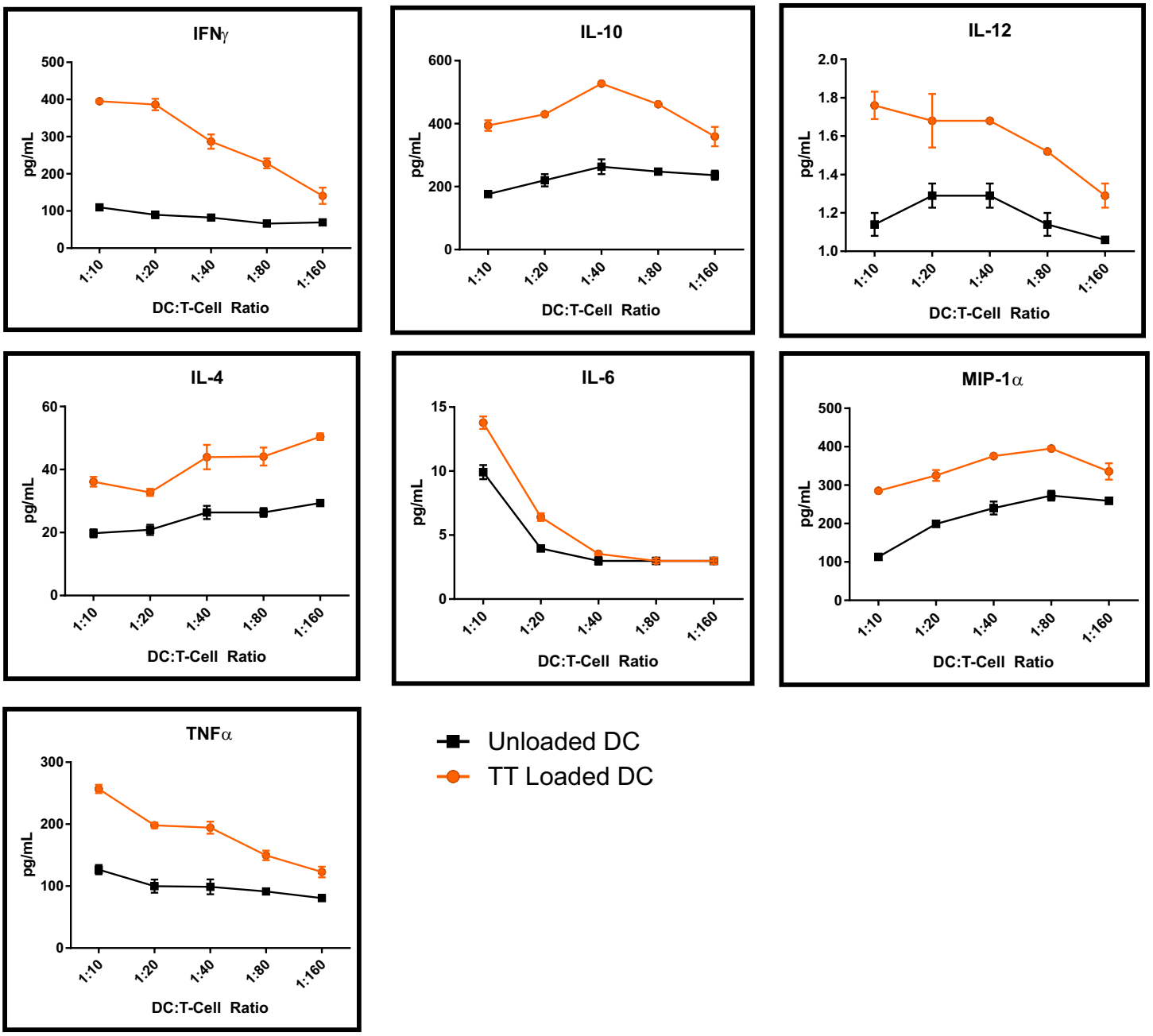
Figure 5: Autologous T-Cell proliferation with matched TT loaded and unloaded DC cultured sCD40L, IL-2, IL-7

As shown in Figure 5, the addition of IL-2 and IL-7 enhanced the proliferation of those co-cultures with unloaded DC to a level roughly equivalent to the proliferation occurring in cultures with loaded DC. This response indicates that T cells in co-cultures with mature TT-loaded DC are already maximally stimulated and that proliferation cannot be enhanced with IL-2 and IL-7, in contrast to co-cultures without unloaded DC. As shown in Table 2 and Figure 4, the relative cytokine secretion profile was similar to that observed in cultures without IL-2 and IL-7 (IL-10>MIP-1α>TNF-α> IFN-γ>IL-4 and IL-6), however, the levels of each individual cytokine (except IL-6) were markedly enhanced in wells supplemented with IL-2 and IL-6.

		DC: T-Cell	IFNγ	IL-10	IL-12	IL-4	IL-6	MP-1α	TNFα
Plate 2 (CD40L, IL-2, IL-7)	Loaded	**1:10**	395.41	394.19	1.76	36.14	13.78	285.15	256.86
		1:20	386.52	429.91	1.68	32.74	6.41	325.22	198.16
		1:40	286.74	527.25	1.68	43.94	3.53	375.8	194.41
		1:80	228.06	461.71	1.52	44.13	2.98	395.37	149.56
		1:160	140.81	359.02	1.29	50.42	2.98	335.56	122.75
		20k DCs	2.82	3.02	1.18	18.69	15.67	197.15	66.38
		200k TCs	61.16	219.87	1.21	38.8	2.98	275.33	73.24
	Unloaded	**20k DCs**	2.82	3.02	0.99	14.8	11.66	218.55	58.87
		1:10	109.72	176.44	1.14	19.77	9.92	113.3	126.67
		1:20	89.64	220.09	1.29	20.86	3.96	198.81	99.98
		1:40	82.19	263.27	1.29	26.37	2.98	240.32	98.86
		1:80	66.02	247.76	1.14	26.37	2.98	272.67	91.29
		1:160	69.49	236.13	1.06	29.36	2.98	258.77	80.69

Table 2: Quantitative cytokine measurements from tetanus toxoid loaded/unloaded dendritic cell & autologous T-cell culture stimulated with sCD40L, IL-2, and IL-7. Blue cells were below the lower limit of detection (LLOD).

Figure 6: Quantitative cytokine measurements from tetanus toxoid loaded/unloaded dendritic cell & autologous T-cell culture stimulated with sCD40L, IL-2, and IL-7



Conclusion

Overall, the Xeno Diagnostics in vitro method for accelerating the differentiation and maturation of human DC produces functional antigen presenting cells (APC) that are capable of activating T-cells in response to DC loaded with tumor cell protein homogenate.

References:

Dauer, M., Obermaier, B., Herten, J., Haerle, C., Pohl, K., Rothenfusser, S., Schnurr, M., Endres, S., & Eigler, A. (2003). Mature Dendritic Cells Derived from Human Monocytes Within 48 Hours: A Novel Strategy for Dendritic Cell Differentiation from Blood Precursors. *The Journal of Immunology, 170,* 4069-4076.