T Lymphocyte Activation, Proliferation, and Apoptosis in Corning® BioCoat™ T-Cell Activation Anti-human CD3 96-well Flat-bottom Assay Microplates

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Application Note

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Introduction

Mature T lymphocytes (T cells) recognize and respond to the major histocompatibility complex (MHC) on antigen-presenting cells through T cell receptor (TCR) activation. Upon activation, signaling pathways are initiated that lead to clonal expansion of T cells, up-regulation of activation markers on the cell surface, differentiation into effector cells, induction of cytotoxicity or cytokine secretion, and induction of apoptosis. With a rapidly expanding focus on adoptive cell therapy utilizing TCR-engineered T cells or autologous tumor-infiltrating lymphocytes (TILs), the ability to expand and characterize T cells *in vitro* without loss of effector function is of growing importance[1]. It is well known that activation of T cells through the TCR complex can be accomplished *in vitro* using immobilized antibodies in the presence of cytokines such as interleukin-2 (IL-2). Corning BioCoat T-Cell activation microplates are pre-coated with high quality BD Pharmingen™ CD3 antibodies supplied by BD Biosciences that are available for use with human or mouse T cells. Offered in a 96-well format, the T-Cell activation microplates are ideal for assay condition and parameter optimization and are amenable to high throughput screening (HTS). Here, we demonstrate the activation of human Pan T cells using the Corning BioCoat T-Cell activation anti-human CD3 96-well assay microplates in the presence and absence of CD28 antibody and/or human recombinant IL-2, as well as subsequent characterization via flow cytometry. Proliferation, apoptosis, and IL-2 production assays were then performed using the activated T cells.

Materials/Methods

T Cell Activation

Fresh Pan T cells from multiple donors were obtained from AllCells® (Cat. No. PB009-1) and transferred into culture medium containing RPMI-1640 (Corning Cat. No. 15-040-CM) with 2 mM L-glutamine (Corning Cat. No. 25-005-CI) and 10% heat-inactivated Fetal Bovine Serum (HI-FBS) (Corning Cat. No. 35-011-CV). T cells were seeded in 100 µL of culture medium per well in Corning BioCoat T-Cell activation anti-human CD3 96-well flat-bottom assay microplates (Corning Cat. No. 354725) or 96-well tissue-culture (TC)-treated flat-bottom microplates (Corning Cat. No. 3997) for comparison. Cells were seeded at densities ranging from 5 x 10^4 to 2 x 10^5 cells/well.

For activation marker expression, proliferation assay, and apoptosis assay studies, T cells were seeded in culture medium; culture medium supplemented with 5 µg/mL purified NA/LE anti-human CD28 antibody (BD Biosciences Cat. No. 555725); or culture medium supplemented with 5 µg/mL CD28 antibody and 50 U/mL IL-2 (Corning Cat. No. 354043). For IL-2 production studies, T cells were seeded in culture medium supplemented with 5 µg/mL CD28 antibody. Microplates were sealed with breathable sealing tape (Corning Cat. No. 3345), lidded, and cultured in a humidified, 5% CO_2, 37°C incubator for up to 72 hours.

Surface Marker Analysis by Flow Cytometry

After 72 hours, T cells from 3 donors initially seeded at 1 x 10^5 cells/well were assessed for surface marker expression of activation markers CD95 and CD25 using flow cytometry. Cells were transferred to 96-well V-bottom microplates (Corning Cat. No. 3357) and centrifuged at 200 x *g* for 5 minutes. The supernatant was aspirated and replaced with 100 µL/well of flow stain buffer [PBS (Corning Cat. No. 21-031-CM) with 0.5% BSA (Sigma Cat. No. A9576) and 2 mM EDTA (Corning Cat. No. 46-034-CI)]. The wash was repeated, replacing the supernatant with 100 µL/well of fresh flow stain buffer. To each well, 10 µL of antibody or isotype control was added (Table 1). Samples were incubated at 4°C in the dark for 10 minutes, washed once with flow stain buffer, and resuspended in a final volume of 100 µL/well of flow stain buffer for analysis using a MACSQuant® flow cytometer (Miltenyi Biotec).

Table 1. Activation Markers

Antibody/Isotype Control	Supplier/Cat. No.
CD25 Phycoerythrin mouse IgG2b	Miltenyi/130-091-024
Phycoerythrin Isotype Control mouse IgG2b	Miltenyi/130-092-215
CD95 Fluorescein mouse IgG1κ	Miltenyi/130-092-415
Fluorescein Isotype Control mouse IgG1	Miltenyi/130-092-213

Proliferation Assay

To demonstrate T cell proliferation in the T-Cell activation microplate, relative ATP content was measured using a CellTiter-Glo® Luminescent cell viability assay (Promega Cat. No. G7570). T cells from 2 donors initially seeded at 5 x 10^4 cells/well in 100 µL in 96-well T-Cell activation microplates and TC-treated microplates were assessed at 24, 48, or 72 hours post-seeding. The luminescent assay was performed following vendor's protocol. Briefly, 100 µL of prepared reagents were added to each well, the microplates were agitated on a shaker platform for 2 minutes, and then incubated for 30 minutes at room temperature. The samples from each well were transferred to 96-well black/clear-bottom microplates (Corning Cat. No. 3694) and luminescence was detected using a PerkinElmer EnVision™ microplate reader.

Apoptosis Assay

For apoptosis assays, T cells initially seeded at 1 x 10^5 cells/well in 100 µL in 96-well T-Cell activation microplates and TC-treated microplates were assessed at 5 or 24 hours post-seeding. Relative levels of apoptosis were determined using a Caspase-Glo® 3/7 assay (Promega Cat. No. G8091). The microplates were centrifuged at 200 x *g* for 5 minutes and nearly all supernatant was aspirated from each well. Culture medium was replaced with 80 µL/well of PBS, as the presence of sera can contribute to high background signals in the assay. The Caspase-Glo 3/7 assay was performed following vendor's protocols directly in the assay microplates and the samples from each well were transferred to 96-well black/clear-bottom microplates for luminescence detection using a PerkinElmer EnVision™ microplate reader.

IL-2 Production Assay

To measure the IL-2 production from T cells cultured in the T-Cell activation microplate with soluble CD28, a human IL-2 SimpleStep® ELISA kit (Abcam Cat. No. ab174444) was used. T cells initially seeded at 5 x 10^4, 1 x 10^5, and 2 x 10^5 cells/well in 100 µL per well in 96-well T-Cell activation microplates and TC-treated microplates were assessed after incubation for 48 hours. The culture microplates were centrifuged at 2,000 x *g* for 10 minutes, and the supernatant was transferred to 96-well V-bottom microplates which were then foil-sealed (Corning Cat. No. 6569) and stored at -20°C until characterization via the ELISA assay. Prior to the ELISA assay, samples were thawed at room temperature. The vendor's protocols were followed for sample and standard curve preparation, the colorimetric assay was performed, and absorbance at 450 nm was measured using a PerkinElmer EnVision microplate reader.

Results/Discussion

T Cell Activation

Pan T cells from 3 donors were cultured in T-Cell activation anti-human CD3 96-well microplates or 96-well TC-treated microplates in culture medium supplemented with or without soluble CD28 and IL-2. After 72 hours, the T cells were analyzed for the presence of T cell activation surface markers CD25 and CD95 using flow cytometry (Figure 1). T cells cultured in T-Cell activation microplates with and without CD28 and IL-2 supplementation displayed an upregulation of CD25 and CD95 surface expression that was >80% for all three donors, demonstrating successful activation of T cells using the CD3-coated microplate. There were no significant differences in expression level between cells cultured in the anti-human CD3 microplate alone and cells cultured in the anti-human CD3 microplate with media supplementation of CD28 or CD28 and IL-2. In comparison, T cells cultured in TC-treated microplates displayed CD25 and CD95 surface expression that was below 12%, in both the presence and absence of CD28. With CD28 and IL-2 supplementation, T cells cultured in TC-treated microplates displayed CD25 surface expression that was <6% and CD95 surface expression that was 25% to 30%. This data supports optimal T cell activation, as characterized by CD25 and CD95 surface marker expression, is achieved when culturing T cells in the presence of immobilized anti-CD3 *in vitro*.

Proliferation Assay

To perform a proliferation assay, Pan T cells from 3 donors were cultured in T-Cell activation anti-human CD3 96-well microplates or 96-well TC-treated microplates in culture medium supplemented with or without soluble CD28 and IL-2 for 24, 48, or 72 hours. Multiple donors were tested because T cell response

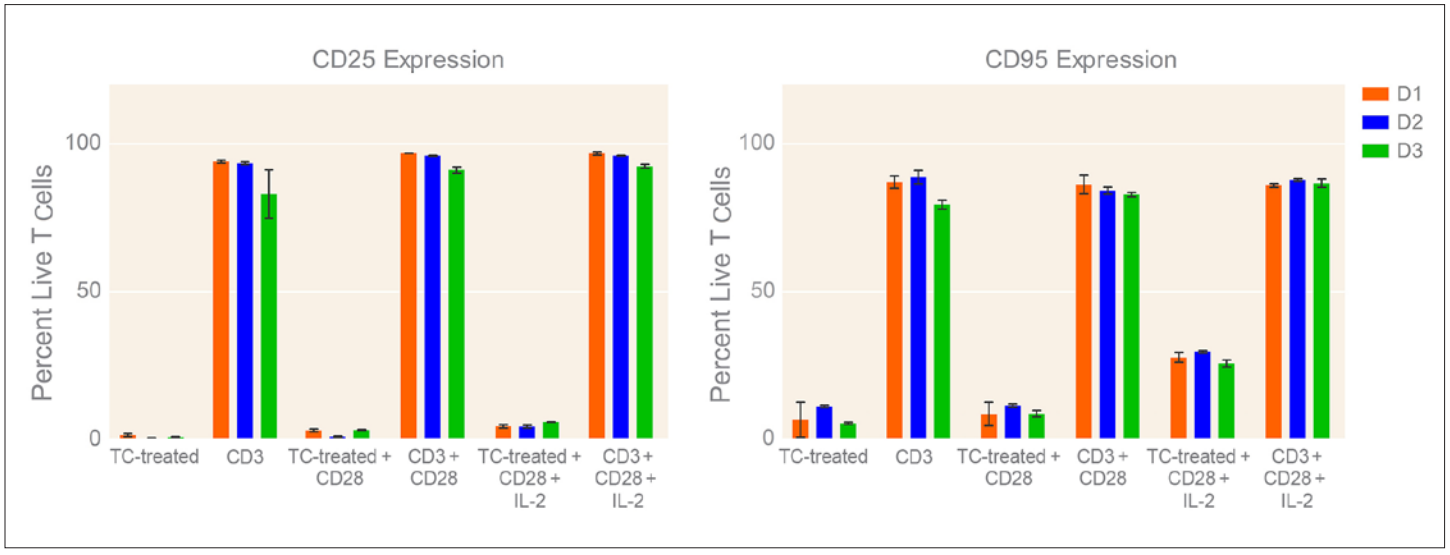

Figure 1. T cell activation surface marker expression is upregulated in primary human T cells cultured in the Corning T-Cell activation microplate. Pan T cells from three donors were cultured in T-Cell activation microplates (CD3) or TC-treated microplates for 72 hours prior to analysis for surface marker expression via flow cytometry. The results from donor 1 (D1), donor 2 (D2), and donor 3 (D3) are displayed. The activation markers CD25 and CD95 demonstrated significantly higher expression in T cells cultured in the T-Cell activation microplate with or without co-stimulatory reagents CD28 antibody and IL-2. Each data point is from 3 donors analyzed in duplicate. Error bars represent standard deviation (SD).

to stimulatory factors varies between individual donors, which is attributed to the initial T cell population[2]. At each time point, viable cells were quantified using a CellTiter-Glo® Luminescent cell viability assay, which resulted in a luminescent signal that is directly proportional to ATP content. T cells cultured in the TC-treated microplate did not display an increase in luminescent signal over 72 hours of culture (data not shown); however, a 2-fold expansion of T cells was observed when T cells from donors 1 and 3 were cultured in the T-Cell activation microplate (Figure 2). Cells from donor 2 did not demonstrate proliferation under any of the conditions tested (data not shown). After 72 hours of culture, T cells cultured in Corning® BioCoat™ T-Cell activation CD3-coated microplates displayed an increase in signal and thus cell number that is >2-fold the signal from T cells cultured in TC-treated microplates (Figure 2). This fold-expansion over cells cultured in TC-treated microplates increases with CD28 and IL-2 supplementation (Figure 2). With CD28 supplementation alone, T cells cultured in CD3-coated microplates displayed an increase in signal that is 2- to 5-fold the signal from T cells cultured in TC-treated microplates. With both CD28 and IL-2 supplementation, T cells cultured in CD3-coated microplates displayed an increase in signal that is 3- to 6-fold greater than the signal from T cells cultured in TC-treated microplates.

Apoptosis Assay

T-Cell activation anti-human CD3 96-well microplates were used to perform an apoptosis assay with T cells in an HTS-compatible manner using a Caspase-Glo® 3/7 Assay. When seeded at 1 x 10^5 cells/well in culture medium supplemented with and without CD28 and IL-2 and cultured for 5 or 24 hours, up to a 3.4-fold increase in apoptosis was observed over T cells cultured in TC-treated microplates with the same media and supplements (Figure 3).

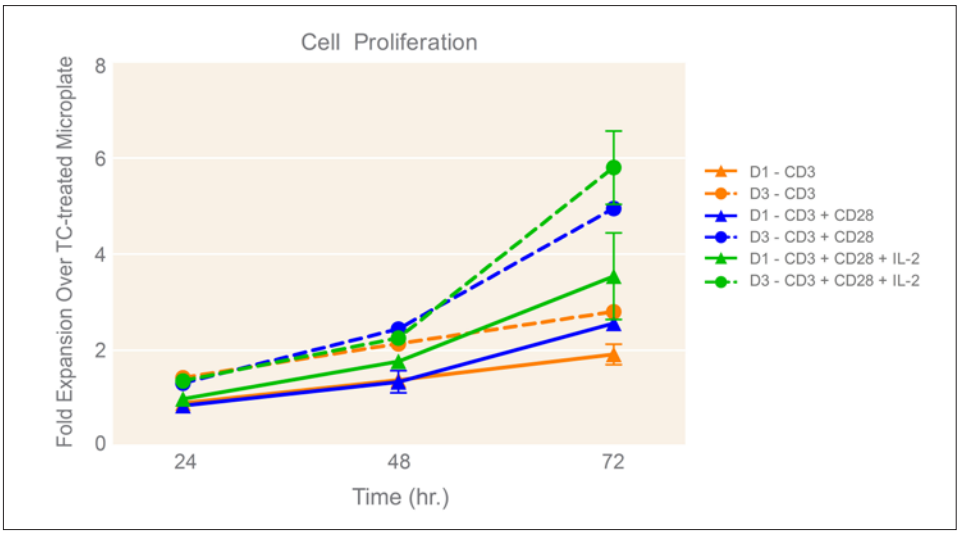

Figure 2. Representative fold expansion of human Pan T cells cultured in Corning T-Cell activation microplates relative to the cells cultured in TC-treated microplates increased over 72 hours and with CD28 and IL-2 supplementation. Cells from three donors were seeded at 5 x 10^4 cells/well and expanded over the course of 72 hours. Results from donor 1 (D1) and donor 3 (D3) are displayed. Cells from donor 2 did not demonstrate significant proliferation under any of the conditions tested. Cells were cultured in culture medium supplemented with or without soluble CD28 and IL-2, and at each time point relative viable cell amount was determined indirectly using a CellTiter-Glo luminescent cell viability assay. N = 2. Error bars represent SD.

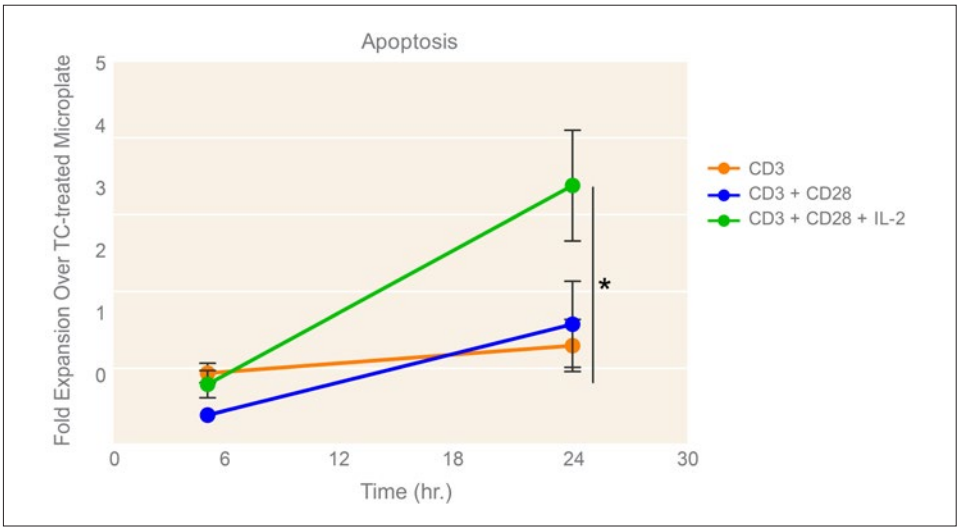

Figure 3. Representative fold signal from Caspase 3/7 of human Pan T cells cultured in Corning T-Cell activation microplates relative to the cells cultured in TC-treated microplates significantly increased over 24 hours with CD28 and IL-2 supplementation. Cells were cultured in culture medium supplemented with or without soluble CD28 and IL-2, and at each time point relative amount of apoptosis indicators Caspase-3 and -7 was determined using a Caspase-Glo 3/7 luminescent assay. N = 2. *p<0.05, Two-way ANOVA with Bonferroni post-test. Error bars represent SD.

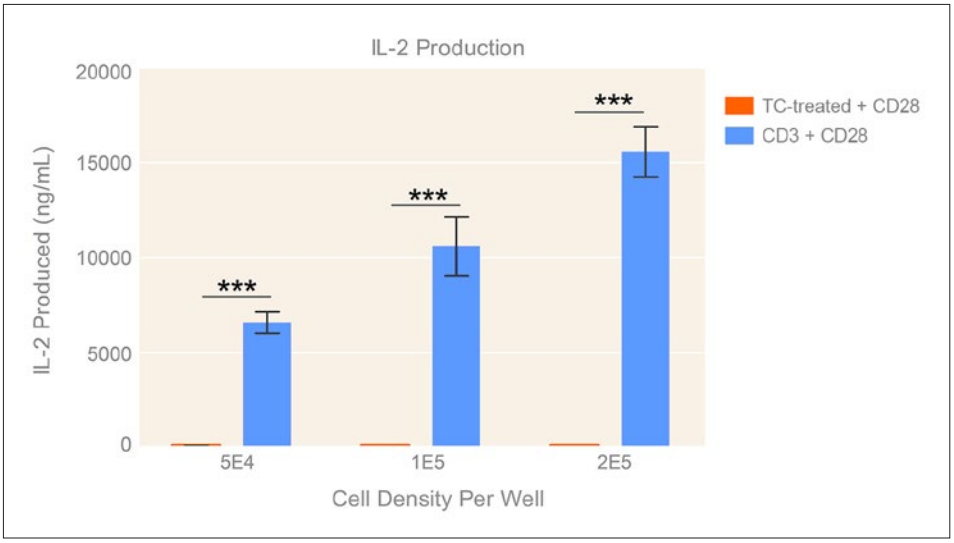

Figure 4. Primary human T cells cultured in the Corning T-Cell activation microplate with supplementation of soluble CD28 antibody for 48 hours produced IL-2. Representative IL-2 production was measured in the supernatant of cells using an ELISA for human IL-2. The combination of the T-Cell activation microplate and CD28 (CD3 + CD28) induced cells to produce >6,000 ng/mL IL-2 with an increase in IL-2 production that correlates with an increase in cell density. T cells cultured in TC-treated microplates with CD28 (TC-treated + CD28) did not produce detectable levels of IL-2. N = 2. ***p<0.001, Two-way ANOVA with Bonferroni post-test. Error bars represent SD.

IL-2 Production Assay

T cell IL-2 production was assessed after seeding at densities ranging from 5 x 10^4 to 2 x 10^5 cells/well and culturing for 48 hours in culture medium supplemented with CD28 in T-Cell activation 96-well microplates and TC-treated 96-well microplates. The combination of using the T-Cell activation microplate and CD28 induced cells to produce >6,000 ng/mL IL-2 (Figure 4). This increase in IL-2 production is supported by the observed increase in cell density upon proliferation also observed under these culture conditions. T cells cultured in a TC-treated microplate with the same media and CD28 supplement did not produce significant levels of IL-2 (Figure 4).

Conclusions

- The Corning® BioCoat™ T-Cell activation anti-human CD3 96-well flat-bottom assay microplate can be used to activate primary T cells with or without the addition of soluble co-factors CD28 and IL-2.
- Following the activation of primary T cells, the 96-well T-Cell activation microplate can be used for proliferation, apoptosis, and IL-2 production assays.

References

1. Ping Y, Liu C and Zhang Y. Protein Cell (2017) 1-13.
2. Wölfl M and Greenberg PD. Nat Protoc. (2014) 9(4):950-966.

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