



Research Article

CD3/CD28 costimulation-induced NF-κB activation, is not mediated by protein metallothionein 2A and FAS associated death domain

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Abstract

Objectives: The immune response depends on T cell activation, which is triggered by signals from receptors such as CD28 and TCR/CD3, resulting in T cell proliferation and programmed cell death (AICD). This control avoids disorders like immunity and cancer. These receptors are stimulated by Antigen-Presenting Cells (APCs), which set off a signaling cascade that activates the important transcription factor NF-κB. Degrading inhibitory proteins is necessary for NF-κB activation, which permits it to reach the nucleus and regulate gene expression.

Methods: Semi-quantitative RT-PCR was used to assess the levels of metallothionein 2A mRNA in primary T cells to validate the results of the microarray analysis. Five- to six-week-old male C57BL/6 wild-type mice were used in the investigation. Mouse primary T cells from lymph nodes were suspended aseptically, and anti-CD3/CD28 was used to activate the cells. The cells were transfected with plasmid DNA using a Gen Pulser, and the T cells were separated by magnetic cell sorting. Following the synthesis of cDNA from total RNA, microarray analysis was used to assess variations in gene expression. The microarray results were validated by RT-PCR. Western blotting was used to confirm protein expression, and flow cytometry with CD69 was used to measure cell death. Immunoreactive bands were visible in co-immunoprecipitation assays using monoclonal anti-MT2A and FADD antibodies.

Results: This work focused on FAS-associated Death Domain (FADD), MT2A, and NF-κB gene expression profiles in mouse primary T cells before and after anti-CD3/CD28 stimulation, utilizing microarray analysis. The results shed light on these genes' functions in AICD and T cell activation. The cascade triggers the activation of transcription factors necessary for T cell proliferation and cytokine production, such as NF-κB. Degradation of inhibitory IκB proteins is necessary for NF-κB activation, which permits NF-κB to reach the nucleus and control gene transcription. The involvement of PLC-γ1 in CD3/CD28-induced NF-κB activation has been highlighted by recent studies.

Conclusion: The processes that determine whether T cells divide or undergo apoptosis are still unknown, despite advances. To compare the gene expression of FADD, MT2A, and NF-κB in mouse primary T cells before and after anti-CD3/CD28 stimulation, this work used microarray analysis. The purpose of our research is to shed light on these genes' functions in T cell activation and activation-induced cell death (AICD).

Keywords: Apoptosis, CD28, NF-κB, T cell activation, T-cell receptor (TCR/CD3)

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The process of activating T cells is intricate and requires close collaboration. It is among the immunological response's most significant occurrences. In a physiologically normal state, lymphocytes are at rest. The potency of signals received

by the T-cell receptor (TCR/CD3) and a series of co-stimulatory receptors, the most notable of which is CD28, determines the capacity of naïve T cells to multiply and acquire potential roles [1]. Concurrent ligation of these receptors sets off signals that

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promote T cell differentiation and proliferation. Recent data have shown that the signals naïve T cells respond to when T cell receptors are ligated not only cause effector cell production and differentiation, but also cause immature thymocytes to undergo apoptosis and mature T cells to experience activation-induced cell death (AICD) [2, 3].

Apoptotic cell death is a crucial process that regulates cellular homeostasis during development, differentiation, and other pathophysiological conditions; dysregulation of this process is linked to a number of diseases, such as cancer, stroke, autoimmunity, tumors that develop drug resistance, and the advancement of some degenerative diseases [4, 5]. After activation and multiplication, T cells must reduce in number to mount an effective immune response. This is because unregulated T cell proliferation increases the risk of cancer and several autoimmune disorders [6].

Antigen-presenting cells (APCs) simultaneously stimulate costimulatory receptors like CD28 and the T-cell receptor (TCR)/CD3 complexes, which activate T cells. Lck, ZAP70, and Syk are examples of cytosolic tyrosine kinases that get activated in response to TCR/CD3 stimulation [7]. LAT, SLP76, Vav, and Grb2 are among the adaptor proteins that these tyrosine kinases phosphorylate in turn [8–10]. The effector proteins, which include small GTPases, phospholipase C- γ 1 (PLC- γ 1), and protein kinases/phosphatases, are further recruited by the phosphorylated adapter proteins. This results in the activation of several transcription factors, such as NF-AT, AP-1, and NF- κ B, which ultimately regulate the transcription of cytokines and T-cell proliferation. Crucially, activation of NF- κ B in T cells requires more than just TCR/CD3 complex stimulation. For the best activation of CD28, costimulation through its ligand, B7, is necessary for NF- κ B activation, leading to T cell activation and optimal production of interleukin-2 (IL-2) and other cytokines [11].

The Rel homology DNA-binding domains found in the NF- κ B family of transcription factors can exist as different homo- or heterodimers [12]. Their interactions with a group of cytoplasmic inhibitory proteins known as I κ B govern their function. I κ B proteins sequester NF- κ B in the cytoplasm by hiding NF- κ B's nuclear localization signal. I κ B kinase (IKK) is activated when cells are treated with different stimuli, such as tumor necrosis factor alpha (TNF- α), IL-1 ϵ , phorbol myristate acetate (PMA), or costimulation of TCR/CD3 and CD28 (CD3/CD28 costimulation). After IKK phosphorylates I κ B, I κ B in the 26S proteasome complex is quickly ubiquitinated and proteolyzed [13]. The nuclear localization signal of NF- κ B is then revealed by the degradation of I κ B. After making a quick translocation into the nucleus, NF- κ B dimers interact with cognate κ B enhancer regions to alter the transcription of several genes related to the immunological and inflammatory responses [14]. While the exact mechanism by which CD3/CD28 costimulation-induced signaling pathways result in NF- κ B activation remains unclear, current research suggests that PLC- γ 1 is critical for this process [15].

The signal transduction pathways responsible for T cell activation or apoptosis induced by TCR/CD3 have been better understood in recent times. Nevertheless, the switch that dic-

tates whether primary T cells undergo activation-induced proliferation or apoptosis remains poorly understood, despite being of great interest. In the meantime, a number of significant findings on the post-activation gene expression profiles of human T cells were obtained by the use of microarray analysis [16, 17]. Therefore, in this work, we report the use of an Affymetrix Mouse Genome 430 2.0 Array to evaluate the variations in the gene expression patterns of mouse primary T cells prior to and following stimulation with anti-CD3/CD28 for either 0 or 18 hours. The changes in FADD, MT2A, and NF- κ B expression before and after T cell activation are of particular interest to us. The results of this study might provide more molecular evidence for understanding the function of FADD, MT2A, and NF- κ B in T cell activation and its associated AICD.

Materials and Methods

BD Pharmingen (San Diego, CA) supplied the antibodies against CD3, CD28, Th1 (FITC-conjugated), and CD69 (FITC-conjugated). Trizol came from Invitrogen, whereas the antibody against mouse MT2A was acquired from Zymed (Zymed, CA). The supplier of CFSE was Sigma in St. Louis, MO.

Cell culture and plasmid transfection

Five- to six-week-old male wild-type C57BL/6 mice were acquired from SIPPR-BK Experimental Animal Centre in Shanghai, China, and were kept in an environment devoid of pathogens. Fresh RPMI 1640 medium (Gibco, USA) supplemented with 20% FBS, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, 1% non-essential amino acids, penicillin (100 U/ml), and streptomycin (100 mg/ml) was used to prepare an aseptic suspension of mouse primary T cells from lymph nodes, which were then diluted to a density of 1×10^7 cells/ml. The cells were then kept in an incubator set at 37°C with 95% room air and 5% CO₂. The primary T cells were activated by the addition of anti-CD3/CD28. At 37°C and 5% CO₂, Jurkat cells were grown in RPMI 1640 medium enhanced with 10% fetal calf serum [18]. Transfection was done using a Gene Pulser (Bio-Rad, Hercules, CA) at 250 V (950 μ F) with pRK5-MT2A plasmid DNA.

Preparing an aseptic suspension of mouse primary T cells from lymph nodes

To prepare an aseptic suspension of mouse primary T cells from lymph nodes, start by euthanizing the mouse using an approved method and sterilize the area with 70% ethanol. Carefully dissect the lymph nodes, typically from the cervical, axillary, and inguinal regions, using sterile instruments. Place the lymph nodes in a Petri dish containing sterile, cold PBS (phosphate-buffered saline). Mince the lymph nodes into small pieces and gently grind them using a sterile glass slide or plunger to release the cells into the PBS. Filter the cell suspension through a 70 μ m cell strainer to remove debris and obtain a single-cell suspension. Centrifuge the suspension at 300xg for 5–10 minutes at 4°C, discard the supernatant, and resuspend the cell pellet in a suitable volume of sterile culture medium. Count the cells using a hemocytometer and adjust the concentration as

needed for downstream applications. Maintain aseptic techniques throughout the process to prevent contamination.

Western blot analysis

The protocol for the immunoblot analysis was followed. On 12% SDS-PAGE gels, equal volumes of protein (50 μ g) were separated. Next, the protein was blotted onto PVDF (polyvinylidene fluoride) membranes using an electrophoretic technique. The membrane was exposed to Kodak X-Omat Blue film (NEN Life Science Products) after being developed using enhanced chemiluminescence reagent (Amersham Life Science Inc.).

Isolation of T cells by macs magnetic cell sorting

Following the manufacturer's instructions, MACS magnetic cell sorting was used to isolate T cells (Miltenyi Biotec, Germany). In short, biotinylated anti-Thy1.2 antibody was used to mark the primary cells after they were extracted. MicroBeads containing streptavidin were used for secondary labeling. MACS LS columns were used to separate the cell suspension following magnetic labeling. To isolate RNA, cell pellets were again put in Trizol reagent.

Preparation of biotin-labeled cDNA probes and hybridization

Primary T cells were treated with an RNeasy Kit (Qiagen Crawley, UK) to extract total cellular RNA. According to the manufacturer's instructions (Genetech Inc., CA), double-stranded cDNA probes were made from total RNA, purified using Phase Lock Gel (PLG)-phenol/chloroform extraction, and then concentrated using the ethanol precipitation method. Using a BioArray High Yield RNA Transcript Labeling Kit (Affymetrix, P/N 900182), probes were biotin-labeled. Following labeling, the probes were once again quantified and purified before being hybridized for 16 hours with Mouse Genome 430 2.0 Array chips (Affymetrix). Following hybridization, the array chips underwent staining, cleaning, and scanning using an Affymetrix G2500A GeneArray Scanner. Software called Affymetrix Microarray Suite (MAS) was used to analyze the data.

RT-PCR Analysis

Using oligo (dT)18 as a primer and AMV Reverse Transcriptase (Promega), total cellular RNA from primary T cells was reverse transcribed into cDNA. The cDNA products were amplified using PCR, ensuring the templates were equal. The sequence of the oligonucleotide primers was as follows: (MT2A) antisense, 5'-GTCGCGTTTCTACATC-3', and sense, 5'-ATGACTGGTGGACAGCAA-3'.

Detection of CD69 expression level and cell death

After being stimulated with anti-CD3/anti-CD28 for a predetermined amount of time, the cells were collected, twice-washed in staining buffer (PBS+1% BSA+0.2% Sodium Azide+1% HEPES), and simultaneously labeled with FITC-anti-CD69 on ice for 30 minutes to measure CD69. Afterwards, flow cytometry (Becton Dickinson, USA) was used to examine the cells. For every sample, a total of 10,000 events were analyzed.

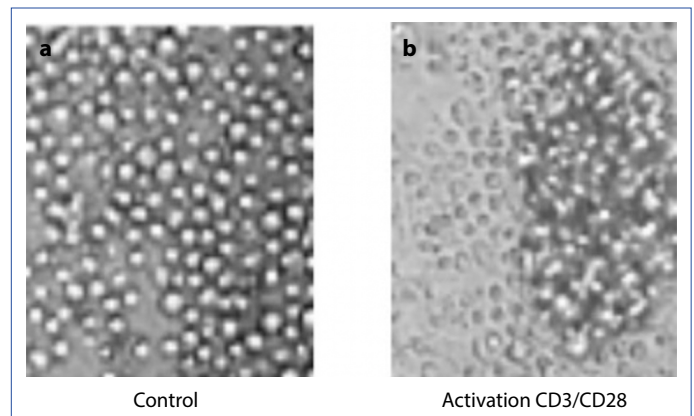


Figure 1. Activation of primary T cells with anti-CD3/CD28. Primary lymphocytes were aseptically taken from mice lymph nodes, separated and diluted into 1×10^7 cells/ml, further cultured in RPMI 1640 for 18 h with (a) or without anti-CD3/CD28 (b), cells were photographed with a KODAK camera (DX4330) (magnification 100 \times).

Co-immunoprecipitation

After 18 hours of maintenance in 1640 Medium, isolated B6 mouse primary T cells, CD3+, and CD28+ cells were incubated for an additional 18 hours in a cell incubator with regulated CO₂. Following this, a lysis buffer was used to lyse the cells. After 30 seconds of sonication, cell lysates were centrifuged for 10 minutes at 4°C at 14,000 rpm. After mixing 5 μ l of anti-FADD antibody (Cell Signaling Technology, Beverly, MA) with protein lysate in 500 μ l lysis buffer, which served as the precipitating antibody, the mixture was incubated at 4°C for two hours on a rocking platform. Protein was immunoprecipitated using Protein A agarose beads. Following SDS-PAGE protein separation and immunoblotting with a monoclonal anti-MT2A antibody (Zymed, USA), the membrane was cleaned and subjected to horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using enhanced chemiluminescence reagents (Pierce, Rockford, IL).

Electrophoretic mobility shift assay

The DNA binding activity of MT2A was measured in the primary T cell extract from lymphosarcoma cells following a published protocol [19].

Results

Activation of primary T cells

The primary lymphocytes from mouse lymph nodes exposed to anti-CD3/CD28 for 18 hours formed large cell aggregates (Fig. 1), which is characteristic of T cell proliferation. In contrast, the control cells did not exhibit this change (Fig. 1), indicating that anti-CD3/CD28 was successful in activating primary lymphocytes.

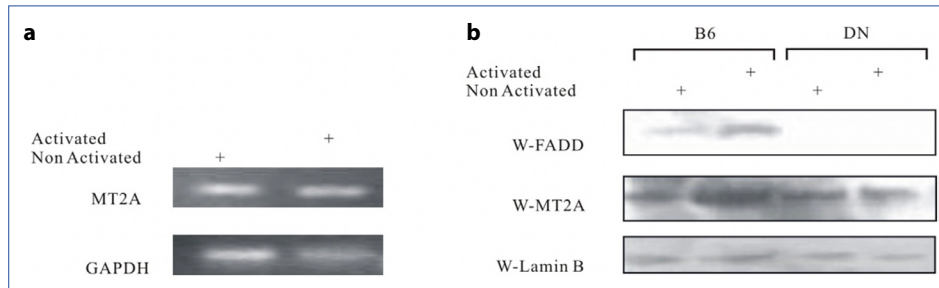
Differential gene expression profiles in mouse T cells after stimulation for 18 H

Using biotin-anti-Thy1.2 labeling and MACS magnetic cell sorting, naive T cells (purity >90%) were separated from cul-

Table 1. The differentially expressed MT, FADD and NFkB genes in primary T cells after stimulation for 18 H (at least 2-fold)

Gene name	Genebank ID	Ratio – 18h
1 Metallothionein 2A	NM_005953	28.5
2 Caspase 8 and FADD like apoptosis regulator	NM_009805	4.3
3 NFkB p105	NM_003998	20.2

MT: Metallothionein; FADD: FAS-associated Death Domain; NFkB: Nuclear factor kappa B.

**Figure 2.** Activation-related genes differently expressed in primary T cells after stimulation. Cells were stimulated with anti-CD3/CD28 for 18 h. Verification of microarray results with semi-quantitative RT-PCR (a), and western blot (b) At least 3 independent experiments were performed.

MT: Metallothionein; FADD: Fas-associated death domain protein; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR: Reverse transcription polymerase chain reaction.

tured lymph node cells (data not shown). After 18 hours of activation, the gene expression pattern in T cells was evaluated using the mouse Genome 430 2.0 Array (Affymetrix), which includes over 39,000 transcripts. After stimulation for eighteen hours, a significant variation in the gene expression patterns of T cells was discovered. Specifically, in T cells activated for 18 hours, 4543 genes were found to be up-regulated and 5797 genes down-regulated. Table 1 provides an overview of the general data about genes that were differentially expressed in T cells during an 18-hour stimulation period.

Genes related to activation of primary T cells

Consistent with previous studies [20–22], our findings demonstrated that numerous genes, including Metallothionein 2A, directly implicated in the activation of primary T cells had notable alterations in their expression levels. Metallothionein 2A mRNA levels in primary T cells were measured using semi-quantitative RT-PCR to confirm the microarray analysis findings. Western blot was used to confirm protein expression. The outcome, as shown in Figure 2, aligned with the microarray findings. The current work suggests that one of the most important steps in the activation of T cells is the disruption of MT2A in naïve T cells. Figure 2 shows the alterations in these genes in primary T cells during an 18-hour stimulation period.

FADD-DN inhibits mitogen-induced proliferation of mature T cells

Flow cytometry analysis in Figure 3 indicated that CD69 was up-regulated in a time-dependent manner. The precise roles of these two genes—MT and NF- κ B—in primary T cell

activation are still unknown, despite their being widely documented as functionally significant to cell development.

To explore this, control and pure T cell stimulation via CD3+CD28 cross-linking were examined. Mitogenic antibodies against CD3 and CD28 were used *in vitro* to activate control and FADD-DN transgenic animals. When exposed to CD3/CD28 stimuli, T cells from FADD-DN transgenic mice reacted inappropriately. DN had approximately 28% of CD69 activation compared to B6's 85%. All things considered, these findings demonstrate that disruption of FADD/MORT1's regular function results in a malfunction in the mitogen-induced proliferation of T cells.

FADD-DN downregulates NF- κ B activation

We recently discovered that MT interacts directly with NF- κ B in our study. According to the data, there is a correlation between the downregulated NF- κ B activity of DN T cells and their lower activation and proliferation (Fig. 4).

Based on the aforementioned findings, it is evident that dominant negative significantly impairs FADD's ability to promote T cell proliferation in FADD-DN T cells. Dysregulation of NF- κ B activation and MT2A expression in activated T cells coexist with this phenomenon.

Discussion

Physiologically, T cell activation is a prolonged process that takes at least a few days, even though the early stage event is crucial for both T cell activation and the initiation of the immune response. Therefore, it is biologically significant to clarify the late phase of T cell activation. Regrettably, there

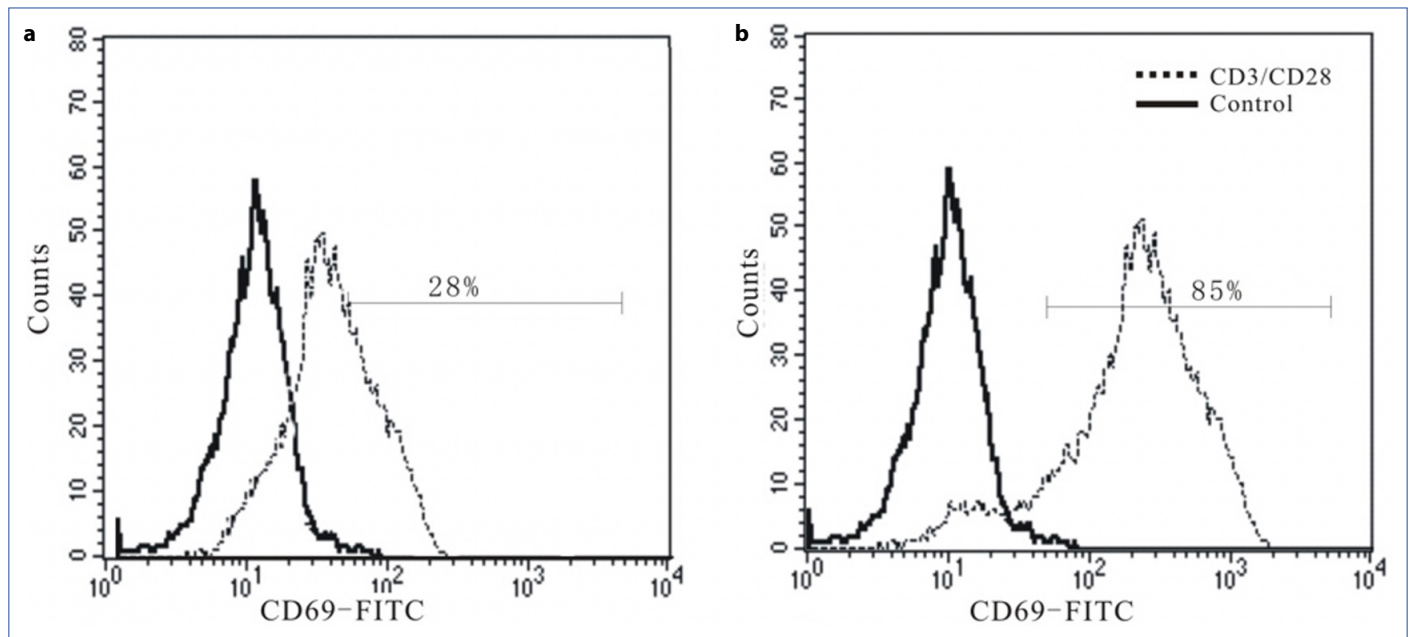
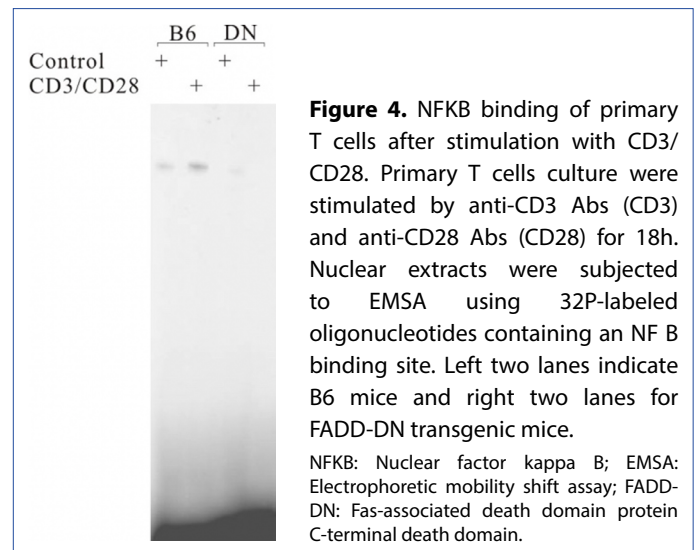


Figure 3. Primary T cells were stimulated with anti-CD3/CD28-FITC 0h (a), or 18h (b). At least 3 independent experiments were performed. FITC: Fluorescein isothiocyanate.

have not been many reports on this issue until now. In the current study, we compared the differentially expressed genes in mouse primary T cells after stimulation for 0 and 18 hours using a Mouse Genome 430 2.0 Array. The results showed that the gene expression profiles of primary T cells were completely different after stimulation for 18 hours. Lastly, a few genes, whose functions are unknown in T cell proliferation, were found to be preferentially regulated in activated T cells, such as FADD.

Three reports on the interaction between MT and NF- κ B have recently been published [23–25]. According to Abdel-Mageed et al. [23] MT and NF- κ B interact directly, as shown by EMSA supershift analysis with an anti-MT antibody. However, Sakurai et al. [24] found that MT inhibits the degradation of I κ B produced by tumor necrosis factor and suppresses the expression of genes that are dependent on NF- κ B. Crowthers et al. [25] observed that activated splenocytes from MT-null animals had increased NF- κ B activity. To bind zinc, NF- κ B's cysteine residues need to be free of thioredoxin [26], although the exact mechanism is yet unknown.

MT2A's mRNA level increased threefold *in vitro* after eighteen hours. A transcription factor called NF- κ B is activated by TCR signaling and may control the transcription machinery that activates Fas L [27]. Throughout T cells' lifetime, the two opposing biological processes of cell growth and death are frequently combined, though they sometimes occur separately. In actuality, numerous genes, including JNK and FADD, are involved in the transmission of both proliferation and death signals. Although FADD's phosphorylated form is increasingly understood to be a crucial regulator of cell proliferation, it has long been known to function as an adapter in the traditional CD95-mediated cell apoptosis [28]. Thus, these mole-



cules, including p-FADD and MT2A, may be the crucial factors at the crossroads that will either save or destroy primordial T cells after activation. Our study identified that, after activation, these molecules produce a high amount of proteins. These differentially expressed genes are important for T cell proliferation because they have been linked to a number of cellular functions, such as anti-apoptotic response, cell cycle regulation, and carcinogenesis.

The results of the analysis of the primary T cell proliferation process using FACS protein level, gene expression mRNA level, and EMSA all pointed to the correlation between FADD, MT2A, and NF- κ B in primary T cells and their necessity for the process of T cell activation and proliferation.

Conclusion

The processes governing whether T cells divide or undergo apoptosis are still unknown, despite advances. Through the use of microarray analysis, the gene expression profiles of FADD, MT2A, and NF- κ B in mouse primary T cells were compared before and after anti-CD3/CD28 stimulation in an effort to shed light on these processes. Important discoveries emphasize these genes' functions in T cell activation and activation-induced cell death (AICD). Degradation of inhibitory I κ B proteins is necessary for the activation of transcription factors such as NF- κ B, which are critical for T cell proliferation and cytokine production. The involvement of PLC- γ 1 in CD3/CD28-induced NF- κ B activation has also been highlighted by recent investigations. By shedding light on the molecular processes underlying T cell responses, our work advances knowledge of immune control and possible targets for treatment.

Ethics Committee Approval: The study was approved by The State Key Laboratory of Pharmaceutical Biotechnology, College of Life Sciences, Nanjing University, Nanjing, China Ethics Committee (No: RP/S/2022/10, Date: 22/09/2022).

Authorship Contributions: Concept – H.Z.C., F.M.; Design – F.M.; Supervision – H.Z.C.; Funding – H.Z.C.; Materials – F.M.; Data collection &/or processing – F.M.; Analysis and/or interpretation – H.Z.C.; Literature search – F.M.; Writing – F.M., H.Z.C.; Critical review – H.Z.C.

Conflict of Interest: The authors declare that there is no conflict of interest.

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