

INVESTIGATION OF THE ROLE OF cGAMP IN DIFFERENTIATION OF T LYMPHOCYTES

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By

Begüm YILDIZ

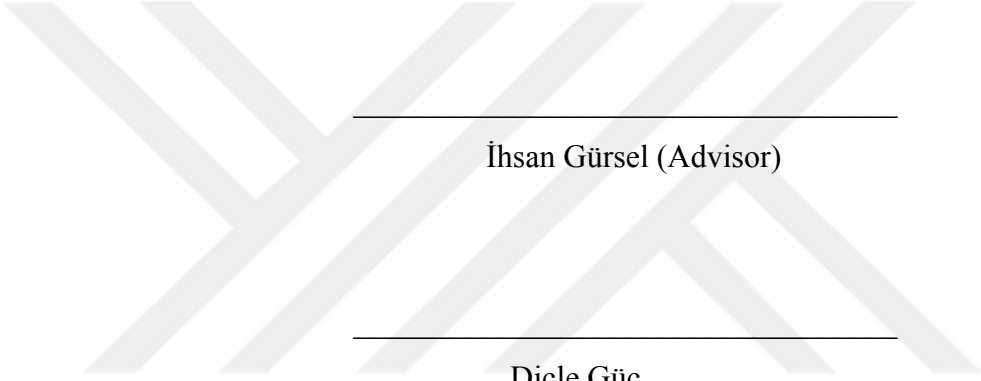
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We certify that we have read this thesis and that in our opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.



İhsan Gürsel (Advisor)

Dicle Güç

Ali Osmay Güre

Approved for the Graduate School of Engineering and Science:

Ezhan Karaşan

Director of the Graduate School

Abstract

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Begüm YILDIZ

M.S. in Molecular Biology and Genetics

Advisor: İhsan Gürsel

October, 2016

STING is the pivotal mediator for the recognition of host and pathogenic cytosolic dsDNA as well as cyclic di-nucleotides metabolites from microbes. STING can either recognize DNA itself or sense the presence of cGAMP, which is converted from ATP and GTP upon DNA binding to cGAS enzyme. Not only strategy against intracellular pathogens makes STING an ideal target, but also the recognition of DNA from host cells has a significant role in tumor immunity. Previous studies demonstrated that DNA released from cancerous cells are internalized by innate immune cells such as macrophages and dendritic cells in tumor microenvironment and trigger the production of IFN- β and other pro-inflammatory cytokines including IL-6, TNF- α , and IL-12 through STING triggered signaling pathway. These cytokines then enhance cytotoxic activity of CD8⁺ T cells by further increasing IFN γ production. Since enhanced T cell immunity is the hallmark of vaccine adjuvants, cyclic di-nucleotides such as cGAMP become an important and effective vaccine adjuvants against intracellular pathogens and malignant cells. Although STING activating cyclic di-nucleotides are envisioned as novel and

potent vaccine adjuvants, more thorough research is needed to unearth the mechanism of action of STING on different immune cells. Therefore, it will pave the way for the initiation of successful human trials. The important criteria while developing vaccine adjuvant are the magnitude, and the quality of an immune response and its toxic side effects. To identify these, members of the both innate and adaptive immune system should be taken into account. However, previous studies merely focus on the function and effect of cGAMP in innate immune cells such as macrophages, monocytes and dendritic cells. However, to date there is no explicit study investigating the effect of STING signaling cascade on T-cells. In the light of these findings, we aimed to investigate the direct effect and function of cGAMP on T lymphocytes. Since there were not any preliminary data, we firstly stimulated Pan T cells with cGAMP alone or together with various TLR ligands and then, checked the cytokine profiles and the viability of cells. Surprisingly, 2.5µg/ml dose of cGAMP had a toxic effect on T cell but not on bone marrow derived dendritic cells and macrophages. While cGAMP triggered cell death, interestingly IL-17 secretion from both CD4⁺ and CD8⁺ T cells was dramatically increased. Beside, cGAMP stimulation drastically increased CD4⁺/CD8⁺ T cells ratio of Pan T cells population. Next, we sought to identify the source of IL-17. The IL17 inductive capacity of cGAMP was investigated on purified CD4⁺ T cells from mice. Unexpectedly, data revealed that cGAMP elicited apoptosis of CD4⁺ T cells. Moreover, there was no significant induction of IL-17 secretion. Next, we aimed to find a condition that will reduce the toxic effect of cGAMP, while maintaining IL-17 secretion. When Pan T cells were stimulated with cGAMP and R848 (a TLR7 ligand), the toxic action of cGAMP decreased while IL-17 secretion was enhanced. Lastly, the potency of T cells stimulated with cGAMP was investigated. According to our results, macrophages were activated in the presence of conditioned medium obtained from T cells stimulated with cGAMP. When taken together our findings point out that STING dependent direct activation of T-cells via cGAMP and its subsequent effect on macrophages might be utilized as an immunotherapeutic approach where IL17 induction is important and could be harnessed as vaccine adjuvants against mucosal infections or against cancer.

Keywords: cGAMP, STING, T lymphocytes, apoptosis, immune response, adjuvant

Özet

T LENFOSİT FARKLILAŞMASI ÜZERİNE cGAMP'IN ROLÜNÜN İNCELENMESİ

Begüm YILDIZ

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Yöneticisi: İhsan Gürsel

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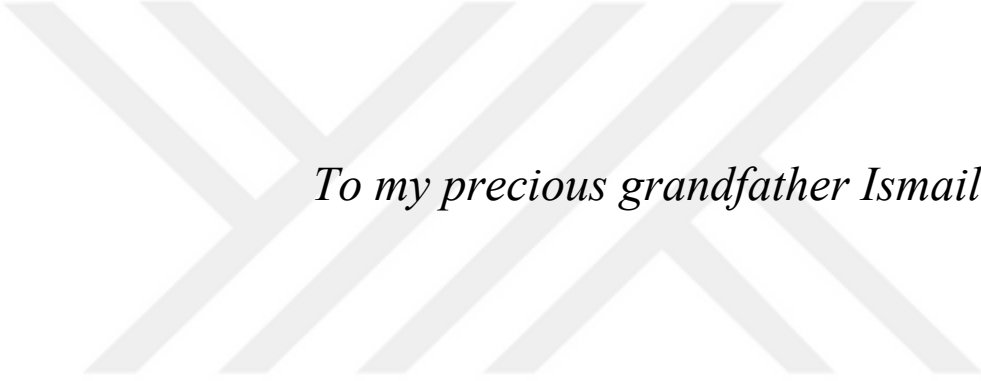
STING, hem patojen ve konağın kendi sitozolik çift iplikli DNA (çİDNA) sının hem de mikrop kaynaklı siklik di-nükleotidlerin tanınmasında önemli bir mediyatördür. STING ya DNA'nın kendisini ya da cGAMP molekülünü tanır. cGAMP molekülü DNA'nın cGAS enzimine bağlanması sırasında ATP ve GTP'nin dönüştürülmesiyle oluşmaktadır. Hücre içi patojenlere karşı geliştirilen strateji STING'i ideal bir hedef yaptığı gibi, konağın kendi DNA'sının tanınması sonucu verilen tepki tümöre karşı geliştirilen immün yanıt açısından önemlidir. Daha önceki çalışmalar göstermiştir ki; kanser hücrelerinden salınan DNA, makrofaj ve dendritik hücreler gibi doğal bağışıklık sistemi hücreleri tarafından tümör mikro çevresinde hücre içine alınır. Bu internalizasyon STING'in tetiklediği yolak üzerinden IFN- β ve IL-6, TNF- α , ve IL-12 gibi diğer pro-enflammatuar sitokinlerin salınımını tetikler. Daha sonra bu sitokinler IFN γ üretimini artırarak CD8⁺ T hücrelerinin sitotoksik aktivitelerini artırır. Aşı adjuvanlarının öne çıkan özelliklerinden biri de T hücre bağışıklığının artırılmasını sağlamaktır. Bu sebeple cGAMP gibi siklik di-nükleotidler hücre içi patojen ve kanser hücrelerine karşı önemli ve

etkili bir aşı adjuvanıdır. STING'i aktive eden siklik di-nükleotidler özgün ve potansiyel aşı adjuvanları olarak öngörülse de, STING mekanizmasının farklı bağışıklık sistemi hücreleri üzerindeki etkilerinin açığa çıkarılması için daha kapsamlı bir çalışma gerekmektedir. Bu çalışmalar, başarılı insan deneylerinin başlamasının önünü açacaklardır. Aşı adjuvan geliştirirken, immün tepkinin niteliği, şiddeti ve toksik yan etkileri önemli kriterler arasında yer almaktadır. Bu kriterleri belirlemek için doğal ve sonradan kazanılan bağışıklık sistemi hücrelerinin tümü göz önünde bulundurulmalıdır. Fakat, şu ana kadar yapılan tüm çalışmalar cGAMP etkisinin sadece doğal bağışıklık sistemi üzerine etkilerini araştırmaktadır. Bugüne dek, T hücrelerinde STING yolağının aktivasyon şelalesini açıkça gösteren hiç bir çalışma bulunmamaktadır. Bu bulgular ışığında, biz bu çalışmada cGAMP'ın T hücreleri üzerindeki direkt etkisini ve fonksiyonunu araştırmayı amaçladık. Bu alanda herhangi bir ön veri olmadığı için ilk olarak Pan T hücreleri sadece cGAMP veya cGAMP ile birlikte bazı TLR ligandlarıyla stimüle edildiler. Bu stimülasyon sonucunda, sitokin profilleri ve hücrelerin durumu kontrol edildi. Şaşırtıcı bir şekilde, cGAMP 2.5µg/ml dozu makrofaj ya da dendritik hücreler üzerinde hiç bir etki göstermezken, T hücreleri üzerine toksik etkisi olmuştur. Hücre ölümü etkisi yanı sıra, cGAMP, Pan T hücreleri içindeki hem CD4⁺ hem de CD8⁺ T hücrelerinin IL-17 salınımı ilginç şekilde artırmıştır. Bunun yanı sıra; Pan T hücreleri içinde CD4⁺/CD8⁺ oranı ciddi şekilde artmıştır. Bu sonuçlardan sonra, IL-17 kaynağını aramayı hedefledik. cGAMP'ın IL-17 indüklemeye kapasitesi farelerden pürifiye edilen CD4⁺ T hücreleri üzerinde araştırılmıştır. Beklenmedik bir şekilde, cGAMP CD4⁺ T hücreleri üzerinde toksik etki gösterip, IL-17 salınımını etkilememiştir. Bundan sonra, cGAMP'ın Pan T üzerine olan toksik etkisini IL-17 salınımını etkilemeyecek şekilde azaltan bir durum bulmayı amaçladık. Pan T hücreleri cGAMP ve R848 (TLR7 ligandı) ile stimüle edildiğinde IL-17 salınımı artarken, toksik etkinin azaldığı gözlemlenmiştir. Son olarak, cGAMP ile stimüle edilmiş T hücrelerinin potansiyeli araştırılmıştır. Sonuçlarımıza göre, cGAMP ile stimüle edilmiş T hücrelerinin salgıları makrofaj aktivasyonuna yol açmıştır. Tüm bunlar göz önüne alındığında, sonuçlarımız T hücrelerinin cGAMP kullanılarak STING'e bağlı direkt aktivasyonları ve buna bağlı makrofajlar üzerine etkilerinin immünoterapötik amaçlar üzerine kullanılmasına dikkat

çekmektedir. Ayrıca, IL-17 salınımı önemli bir bulgu olmakla beraber, mukozal enfeksiyonlara ve kansere karşı aşı adjuvanı olarak koşullanabilir.

Anahtar kelimeler: cGAMP, STING, T lenfositleri, apoptoz, immün cevap, adjuvan





To my precious grandfather Ismail Seyhan...

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Abbreviations

Ab	Antibody
AIM2	Absent in melanoma 2
ALR	AIM2 like receptor
AP-1	Activator Protein 1
APC	Antigen presenting cell
Bcl-2	B-cell lymphoma 2
BM	Bone marrow
BSA	Bovine serum albumin
C/EBP β	CCAAT/enhancer-binding protein- β
cGAMP	Cyclic guanosine adenosine monophosphate
cGAS	Cyclic GMP-AMP Synthase
CLR	C-type lectin receptor
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
ddH ₂ O	Double-distilled water
DMSO	Dimethyl sulfoxide
DN	Double negative
DNA	Deoxyribonucleic acids

DP	Double positive
dsRNA	Double stranded RNA
EtOH	Ethanol
FBS	Fetal bovine serum
FOXP3	Forkhead box P3
IBD	Inflammatory bowel disease
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF3	Interferon regulatory factor 3
iTreg	Induced regulatory T cell
JAK	Janus Kinase
KIR	Killer inhibitory receptor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
NF- κ B	Nuclear factor- κ B
NK	Natural killer
NLR	Nucleotide oligomerization receptor
ODN	Oligonucleotide
PAMP	Pathogen Associated Molecular Pattern

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death 1
PRR	Pattern Recognition Receptor
RA	Rheumatoid arthritis
RLR	RIG-1 like receptor
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SOCS	Suppressor of cytokine signalling
ssRNA	Single stranded RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
T-bet	T-box transcription factor
TBK1	Tank binding kinase 1
TCR	T-cell receptor
TGF- β	Transforming growth factor beta
Th	T helper
TLR	Toll-like Receptor
TNF	Tumor necrosis factor
TRAF	TNF receptor associated factor
PMA	Phorbol 12-myristate 13-acetate

Chapter 1

Introduction

1.1 The immune system

The general and the main goal of the immune system are to guard the body from wide range of microorganism. During recognition and elimination of the microbes, the first discernment mechanism is its ability to distinguish self from non-self. This ability gives the immune system to demolish not only microbes but also toxins and allergens [1]. The immune system has two arms including innate and adaptive immunity. These two arms of the immune system implement the main mechanism, which is the discrimination of self from non-self to protect host organism. Although the innate and adaptive immunity are based on the same mechanism, they differ in terms of rapidity and specificity. The innate immune system is the first line of defense of the body and consists of both physical, chemical barriers and specific type of cells such as monocytes, macrophages, and neutrophils. The basic virtue of the innate immune system is an immediate defense [2]. The members of the innate immune system react against pathogens at the same extent no matter how many times they encounter. On the other hand, adaptive immune system possesses the antigen specific response, which gives the specificity to T and B-lymphocytes. In other words, the hallmark of the adaptive immunity is an increase

response in the presence of repeated infectious and the development of a memory against specific antigen [3].

1.1.1 Innate immune system

Innate immune system, which comprises of various types of cells including monocytes, dendritic cells, macrophages, neutrophils, basophils, eosinophils, mast cells and natural killer cells, provides the first and immediate response against pathogens. Although innate immune system does not have any capabilities to develop memory against specific type of antigens, the most of the infectious that the host encounter are cleared by innate immune cells without any interaction with adaptive immunity. Innate immune system has three distinct phases to eliminate pathogens. In the first phases, physical barriers and soluble factors such as antimicrobial peptides or complement system are implemented for the clearance of pathogens. After releasing soluble factors upon microbes, pathogen-sensing mechanism by innate immune cells become part of an activity. Pathogen associated molecular patterns (PAMPs) are molecules from pathogens that are sensed by the members of the innate immune system. They are recognized by pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), Nucleotide oligomerization receptors (NLRs), C-type lectin receptors (CLRs) and RIG-1 like receptors (RLRs), AIM2 like receptors (ALRs) and cytosolic DNA sensors. As a third phase, specific type of innate immune cells called antigen presenting cells (APCs) present pathogen specific antigens to T lymphocytes for the expansion of antigen specific lymphocytes and long term memory [4].

1.1.1.1 Pattern recognition receptors (PRRs)

The pathogen sensing mechanism of innate immune system is mostly based on pattern recognition receptors (PRRs), which can identify the different part of pathogens. PRRs consist of different type of receptors including TLRs, NLRs, CLRs, RLRs, ALRs and cytosolic DNA sensors. They can be either at the cell surface or inside a cell [5]. When pathogen associated molecules are sensed by PRRs, pro-inflammatory cytokines or chemokines start to be secreted by innate immune cells by activating several signaling

transduction pathways. Upon secretion of several cytokines and chemokines, other immune system members are recruited the site of infection to strengthen the response [6].

1.1.1.1.1 Toll-like receptors (TLRs)

TLRs, which are the most well-known and studied receptors among PRRs, are glycoproteins characterized by extracellular ligand binding domain or Toll/interleukin-1 (IL-1) receptor homology (TIR) domain. They can be found either at the cell surface or embedded in endosomal membranes. The recruitment of the adaptor proteins upon recognition is the first step of the signaling pathway. TLRs have 2 main adaptor proteins including MYD88 and TRIF. Once TLRs bind their ligands, adaptor proteins recruit other proteins such as kinases to activate MAP kinase, NF κ B or IRF pathway by providing secretion of pro-inflammatory cytokines [6]. TLRs can recognize various parts including

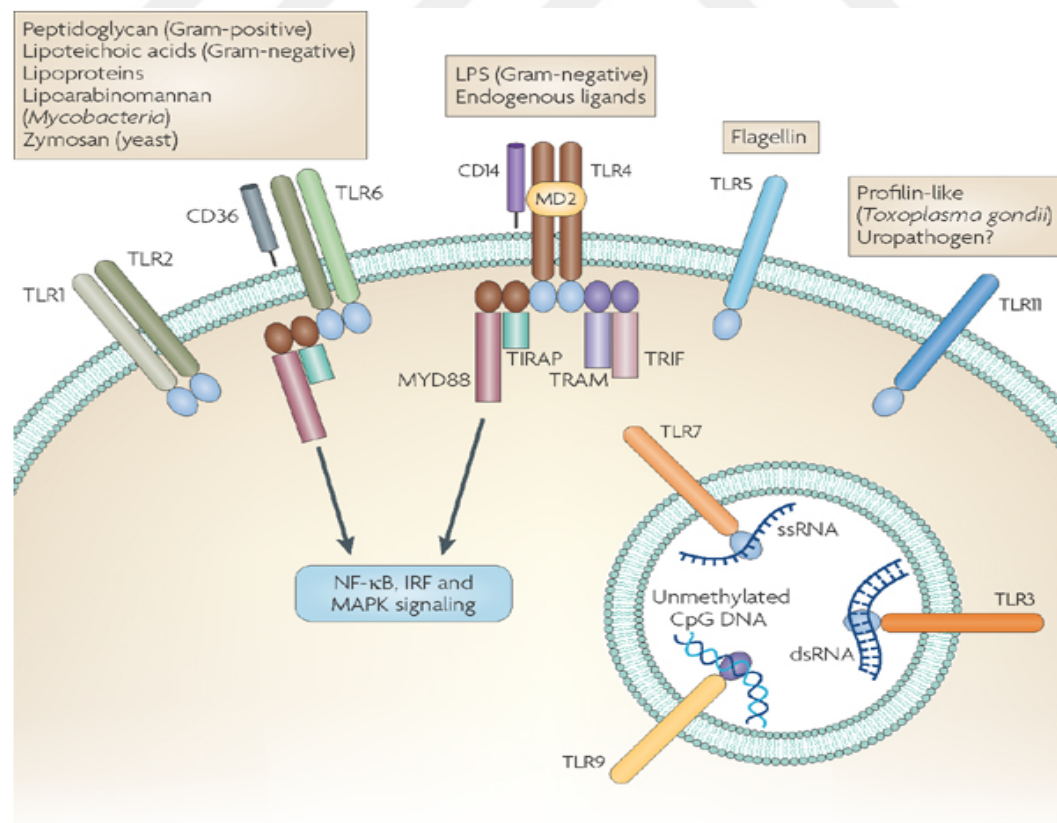


Figure 1.1 Physiological functions of toll-like receptors (TLRs) [11]

lipids, saccharides and nucleic acids of virus, bacteria, fungi or parasites and divided into subgroups. TLR1, 2, 4, 5, 6 and 10 which are found at the cell surface mainly recognize bacterial products, whereas TLR3, 7, 8, 9, which are found in endosomes, sense nucleic acids [7][8]. TLR1/2 and TLR4 sense triacetylated lipopeptides and lipopolysaccharides, respectively. On the other hand, as nucleic acid sensors, TLR3, TLR7/8, and TLR9 directly bind to double stranded RNA (dsRNA), single stranded RNA (ssRNA) and CpG DNA, respectively [9][10].

1.1.1.1.2 Cytosolic DNA sensors

The recognition of pathogen-associated deoxyribonucleic acids (DNA) is the essential and spectacular mechanism by which innate immune cells detect microbes to trigger the second response for the protection. However, one of the most important criteria for this mechanism is the differentiation of self-DNA and DNA from microbes. If this differentiation mechanism is interrupted, various types of autoimmune diseases arise. This is why the molecular mechanism of DNA sensors should be investigated and understood [12].

There are several cytosolic DNA sensors including TLRs, ALRs and other DNA sensors. However, the major difference between TLRs and other cytosolic DNA sensors is the specificity of the sequence. TLR9 only senses CpG motif by inducing Type I IFN response whereas other DNA sensors including AIM2, cGAS (Cyclic GMP-AMP synthase) and STING (Stimulator of interferon genes) recognize DNA in a sequence-independent manner and provide huge amount of inflammatory cytokine secretion from innate immune cells. AIM2 is one of the cytosolic DNA sensors and leads the formation and oligomerization of the inflammasome complex by triggering caspase-1 cleavage and secretion of pro-inflammatory cytokines such as IL-1b and IL-18 unlike cGAS and STING [13].

cGAS and STING are the key DNA sensors to boost both innate and adaptive immune system in the presence of virus or bacteria. cGAS which is an enzyme, recognize dsDNA in cytosol in a sequence-independent manner and leads the secretion of Type I interferon from innate immune cells. Upon activation of cGAS by binding dsDNA, it converts ATP and GTP to cyclic guanosine adenosine monophosphate (cGAMP). cGAMP activates STING which is an endoplasmic DNA sensor by leading phosphorylation of IRF3 and Type I interferon secretion [14-15].

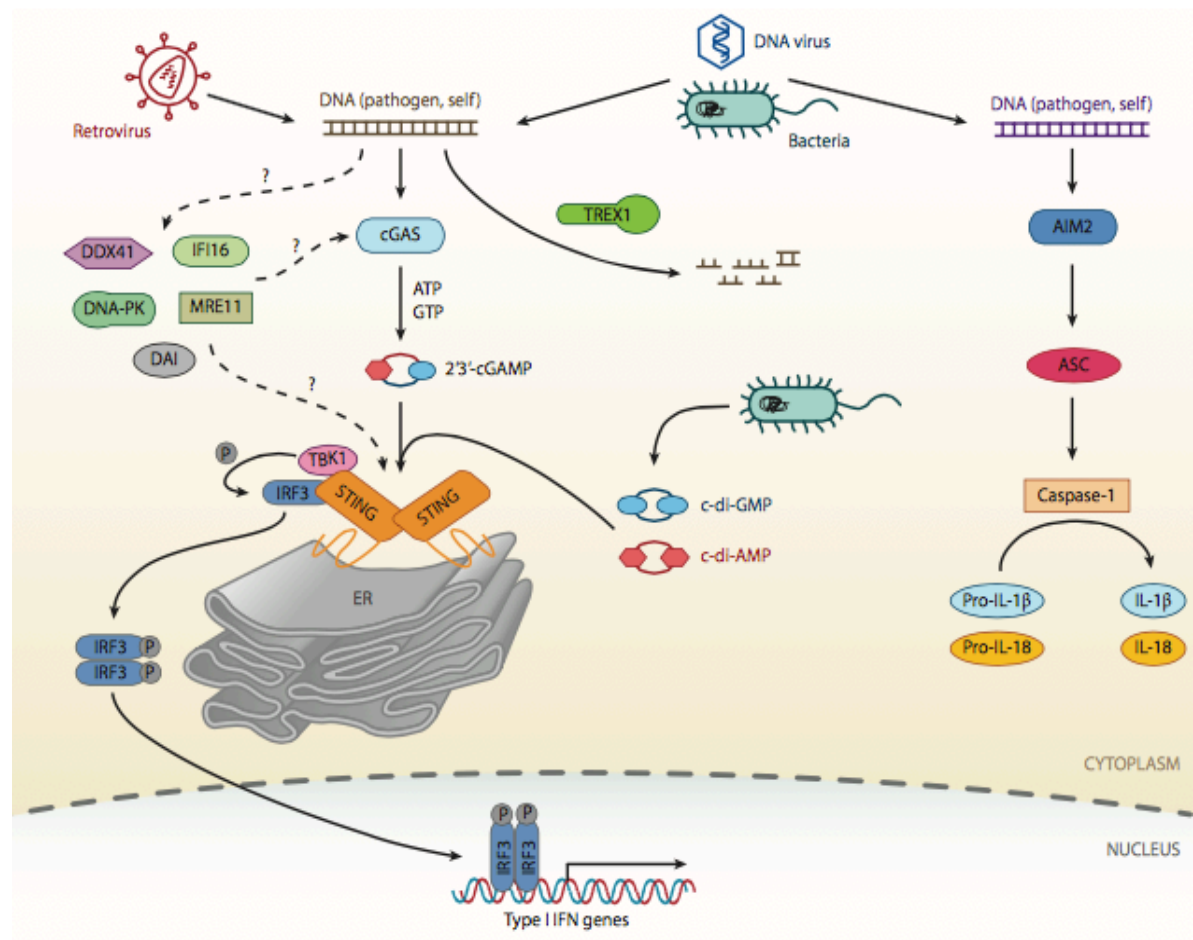


Figure 1.2: Cytosolic DNA-sensing system [16]

STING (Stimulator of interferon genes; also known as TMEM173, MPYS, MITA and ERIS) has a critical and important role as a DNA sensor since it can sense various type of pathogens such as bacteria, virus and eukaryotic [17]. Moreover, recent studies showed that over-expression of STING causes severe autoimmune diseases. On the other hand, other studies showed that it triggers activation of the adaptive immune cells in response to DNA vaccines [18]. Thus, understanding DNA sensing mechanism of STING through innate and adaptive immunity is vital. STING is expressed mainly in the thymus, peripheral leukocytes, spleen, lung, heart and placenta but is poorly expressed in the brain, skeletal muscle, colon, small intestines, liver and kidney [19-20]. STING either binds DNA including cyclic dinucleotides directly or is activated through cGAS to then spark TBK1 (Tank binding kinase 1) and phosphorylate IRF3 (Interferon regulatory factor 3). Upon IRF3 phosphorylation, Type I IFN genes are up-regulated and innate immune cells such as macrophages, dendritic cells and monocytes abundantly secrete IFN- β , which is crucial for activation of adaptive immune system by inducing cytotoxic T lymphocytes [21-22].

1.1.2 Adaptive immune system

Adaptive immune system, also called acquired immune system steps in at the last phase of innate immune system by which innate immune cells cannot eliminate pathogens from the system. Unlike innate immunity, adaptive immune system has a capable of generating antigen-specific responses and long-term memory against specific type of pathogens. B and T lymphocytes are the members of the adaptive immune system and provide long lasting protection from infectious [23].

B-lymphocytes are responsible from humoral immunity as a precursor of plasma cells, which secrete antibodies specific to pathogens. Once they move to secondary lymphoid organs as a naïve form, they are activated by either antigens or T helper cells and start to release antigen specific antibodies for the elimination of microbes [24].

T-lymphocytes form the cell mediated immunity arm of the adaptive immune system. Naïve T cells which do not encounter any antigen yet are activated upon antigen recognition and differentiate into different subsets. The general mechanism including

both CD4⁺ and CD8⁺ T cells is based on the recognition of peptide part of an antigen that are already processed by antigen presenting cells (APCs). These peptide fragments were presented by binding to MHC (major histocompatibility complex) molecules. Naïve CD4⁺ T cells recognize peptide-MHCII complex to become effector CD4⁺ T cells and the major role of CD4⁺ T cells is to aid antibody production of B cells. On the other hand, naïve CD8⁺ T cells recognize peptide-MHCI complex to become effector or memory CD8⁺ T cells. Activated CD8⁺ T cells are mostly responsible from clearance of infection by killing infected cells via apoptosis [4,25].

1.2 T lymphocytes

Progenitors of T lymphocytes form in the bone marrow and then migrate to the thymus unlike other immune cells. When they reside in a sub-capsular region of the thymus, they are called double negative (DN) thymocytes due to lack of TCR, CD4 and CD8 expression. Until double positive (DP) stage, they experience 4 different subgroups of double negative stages, which differ the expression of CD44 and CD25 receptors.

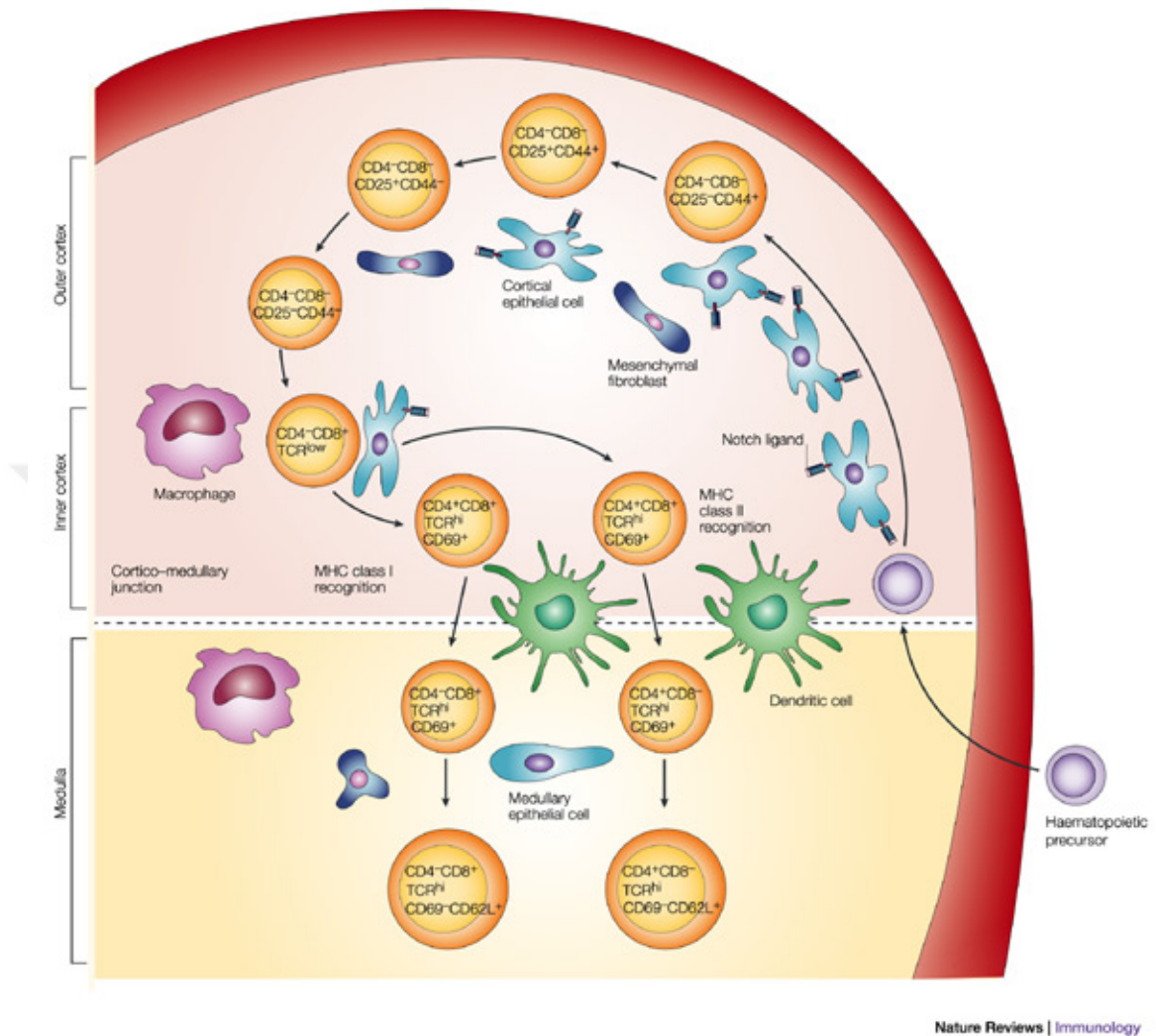


Figure 1.3: T cell development in thymus [27]

At the first stage, which is called DN1, cells express kit and CD44. CD25 expression starts to take place from DN1 to DN2 stage. When expressions of Kit and CD44 reduce, cells move to DN3, which is a critical stage in terms of TCR beta rearrangement. If cells cannot complete the rearrangement of TCR beta, they undergo apoptosis rather than progressing to DN4 stage by decreasing CD25 expression. In other words, when cells reach the DN4 stage, it express rearranged TCR-beta and non-rearranging pre-TCR alpha chain. Once TCR alpha chain rearranges and forms complete TCR alpha-beta chain, cells transit from double negative to double positive stage by passing through cortex and start to express both CD4 and CD8. The cortex of the thymus composes of epithelial cells,

which highly express MHC class I and class II molecules bound with self-peptide. The destiny of double positive thymocytes are determined by strengthen of signaling during interaction of TCR and MHC-peptide complex. T cells that are only activated with an intermediate signal can survive and become mature. If TCR of the double positive thymocytes encounter and bind to MHC class I-self peptide complex, they become CD8⁺ T cells. However, an interaction between TCR and MHC Class II-self peptide complex leads generation of CD4⁺ T cells. When the fate of the T cells are determined and they are educated against self-antigens, they travel through secondary lymphoid organs such as spleen and lymph nodes and become ready to differentiate into effector cells [26-28].

Despite the fact that CD4⁺ and CD8⁺ T cells differ for the recognition of the MHC class molecules, they have a common phenomenon in terms of activation. Dendritic cells as an APC have a key role for the activation of naïve T cells. They encounter with an antigen, process and present the peptide fragment with MHC class II or I at the cell surface for the interaction with CD4 or CD8⁺ T cells, respectively. However, TCR-MHC-peptide complexes are not enough for the activation of both CD4⁺ or CD8⁺ T cells. In addition to that, co-stimulation signal is necessary for the activation, proliferation and differentiation after TCR activation. The family of B7 proteins (B7-1 and B7-2) that are expressed at the cell surface of APCs provide co-stimulation signals to T cells by interacting with CD28 which is found only in naïve T cells. Interaction of B7 families and CD28 prevents anergy state of T cells, conducts secretion of survival factors such as IL-2 and induces the expression of anti-apoptotic BCL-2 (B cell lymphoma-2) family members. On the other hand, when T cells are activated by these 2 signals, they start to express inhibitory receptors such as cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed death 1 (PD-1) and killer inhibitory receptors (KIRs) as well. CTLA-4 competes with CD28 to bind the B7-1 and B7-2 by inhibiting T cell proliferation and causes suppression of T cells by arresting cell cycle at the G1 phase [29-31].

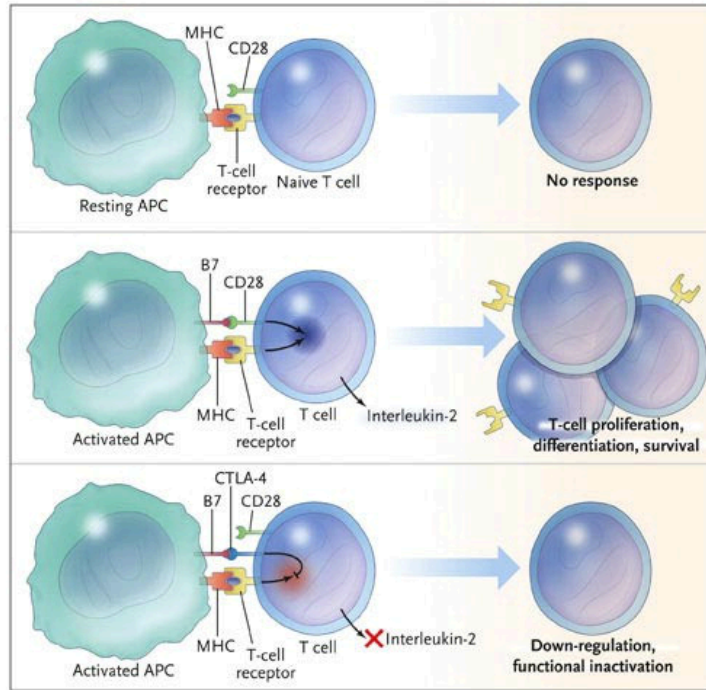


Figure 1.4: Roles of the B7–CD28/CTLA-4 pathway in regulating T-cell activation [29]

1.2.1 CD8⁺ T lymphocytes

Naïve CD8⁺ T lymphocytes (Cytotoxic T cells) enter into secondary lymphoid organs such as lymph node to check whether there is an infection. At this stage, they highly express homing marker CD62L. However, if there is an infection, they encounter with dendritic cells, which present a non-self antigen on their MHC class I and they are activated by the interaction of TCR-MHC class I- peptide complex and lose the expression of homing ligand, CD62L [32]. Upon activation, activated CD8⁺ T cell become antigen specific and start to undergo clonal expansion to become effector cells. The clonal expansion of the CD8⁺ T cells result in reduction and obliteration of the virus. After then, CD8⁺ T cells enter to contraction phase, only 5-20% of them stay alive and become memory cells [33,34].

Cytotoxic T cells (CTLs) are of capital importance to respond to malignant cells and intracellular pathogens such as viruses. When CTLs are activated and trained against infectious such as virus, they induce apoptosis in infected cells by implementing different mechanisms. The most well known mechanism is the release of enzymes including

perforin and granzyme. These two proteins are found in the cytoplasmic granules in CTLs. Upon their activation, granzyme specifically granzyme B and perforin are released on target cell. Perforin causes pore formation on the cell membrane by facilitating the penetrance of granzyme B into the cell [35,36]. Granzyme B affects several pathways and proteins to trigger cell death. It provokes the cleavage of a BAX-like BH3 protein, BID which is a member of pro-apoptotic member of the Bcl-2 family by causing cytochrome c release into the cytosol with ensuing the activation of caspase cascade [37]. Beside, it directly acts on caspase 3 and 7 resulting in death of the target cell. The other affection mechanism of granzyme B is based on cleavage of ICAD, the inhibitor of a DNase, which can provide DNA hydrolysis by causing cell death [38]. Death mechanisms involving granzyme B and perforin are literally calcium dependent. However, still CD8⁺ T cells show cytotoxic activity during the depletion of calcium. This state can be explained by another cell death mechanism, which is perforin independent [39,40]. This mechanism is based on ligand-receptor interaction. CTLs express Fas ligand, which binds Fas in the target cell membrane. Fas-Fas ligand interaction promotes the activation of several cascades of caspases by leading to apoptosis in a target cell [41].

Not only the mechanism of killing but also the secretion of specific cytokines put CTLs forward in immune system. Activated CTLs secrete huge amount of IFN- γ and TNF- α to inhibit viral replication and to recruit the other members of the immune system such as macrophages [42].

1.2.2 CD4⁺ T lymphocytes

CD4⁺ T lymphocytes also called helper T cells (Ths) are of prime importance to centralize both innate and adaptive immune cells. The one of the most significant role of helper T cells is to help B cells make antibodies [43]. Beside, they improve and sustain responses of CTLs. For the innate arm, they promote the recruitment of cells such as neutrophils, basophils and eosinophils to sites of infection and inflammation through the secretion of several cytokines and chemokines. Also, they enhanced the activation status of macrophages by inducing their microbicidal activity [44]. Actually, they behave like a

maestro to control and balance the whole immune system. Hence, they have a role in elimination of various pathogens, regulation of autoimmune diseases and malignancies.

Like CTLs, naïve $CD4^+$ T cells, which exit from thymus, enter into secondary lymphoid organs such as spleen, lymph nodes or the mucosa-associated lymphoid tissue. Here, they can be activated by APCs, which present an antigen- MHCII complex on their membranes. Beside TCR-antigen-MHCII interaction, co-stimulatory signal such as CD28 as a second signal is necessary for complete activation of $CD4^+$ T cells. Upon the interaction of TCR-antigen-MHC II complex with co-stimulatory signal, $CD4^+$ T cells are activated and started to differentiate into effector cells [45,46]. The differentiation processes of $CD4^+$ T cells are a little bit different from CTLs. They differentiate into several subsets of $CD4^+$ T cells. The cytokine milieu of the environment affects the subset differentiation and they become Th1, Th2, Th17, iTreg or other minor subsets [47].

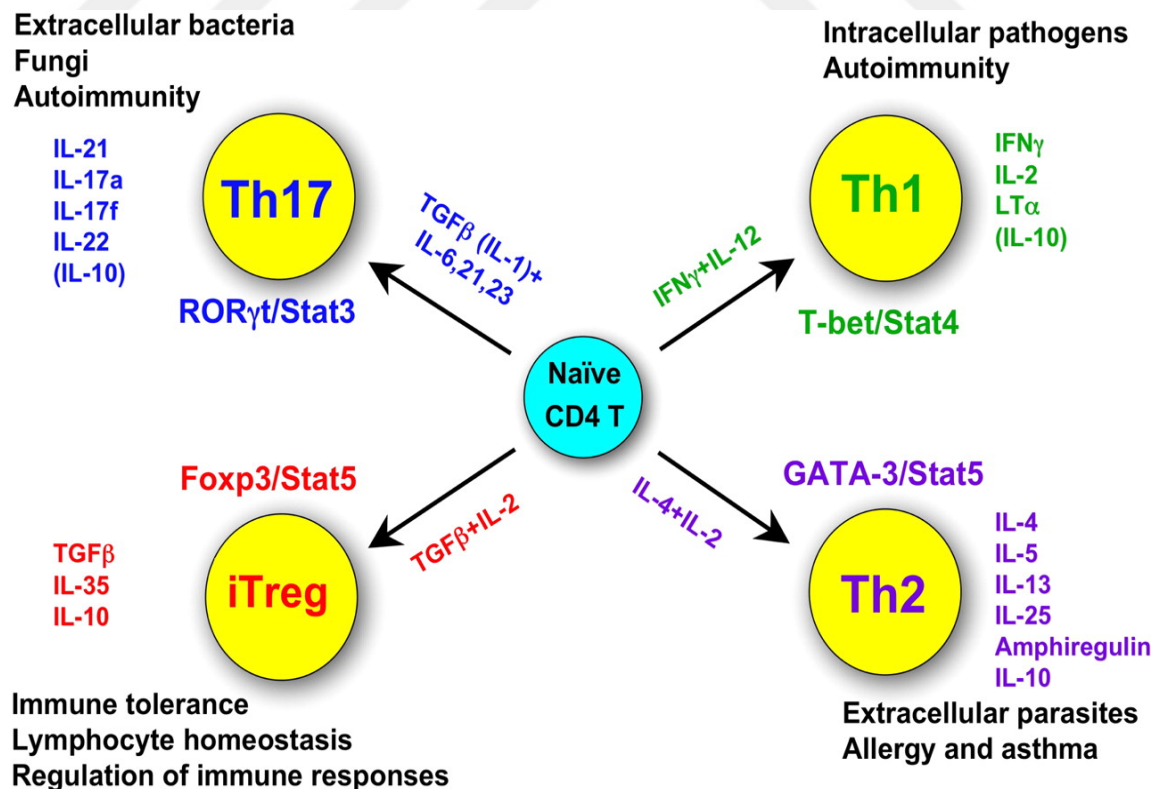


Figure 1.5: Summary of the $CD4^+$ T helper cell fates [48]

When naïve CD4⁺ T cells are exposed to IL-12 and IFN γ during activation by APCs, these cytokines trigger signaling pathways for differentiation of Th1 cells [49]. The most significant transcription factor for Th1 differentiation is T-box transcription factor (T-bet). T-bet not only promotes the activation of various genes related with Th1 differentiation but also prevent the development of other helper T cell subsets. T-bet as a transcription factor strongly augments the secretion of IFN γ by binding its promoter. On the other hand, expression of T-bet is connected with the phosphorylation of signal transducer and activator of transcription 1 (STAT1). Once, STAT1 is phosphorylated, T-bet starts to be activated which binds the promoter of IFN γ . Secreted IFN γ activates STAT1 which triggers positive feedback loop by promoting the expression of T-bet. This positive loop enhances the differentiation of helper T cells into Th1 [50-53]. In order to prevent the development of other subsets, T-bet inhibits the production of IL-4 and GATA3 which are the most important cytokine and transcription factor for development of Th2, respectively [54,55]. Beside, it binds to promoter region of ROR γ c (ROR γ t) by suppressing the development of Th17 [56]. Th1 cells are crucial for the clearance of intracellular pathogens [57]. However, their unrestrained proliferation causes several autoimmune diseases [58]. They generally secrete IFN γ and IL-2. For the elimination of intracellular pathogens, IFN γ triggers the phagocytic features of cells such as macrophages and microglial cells, thereby killing infected cells [59]. Also, it enhances the response of CD8⁺ T cells during viral infectious. Besides its role as T cell growth factor, IL-2 is of capital importance for CD8⁺ T cells since it augments the proliferation of CD8⁺T cells and the development of CD8⁺ memory T cells [60-62].

During TCR-MHCII-peptide interaction, IL-4 and IL-2 in the environment lead the differentiation of naïve CD4⁺ T cells into Th2 [48]. The crucial transcriptional regulator of Th2 is GATA3, which is induced by signal transducer and activator of transcription 6 (STAT6). GATA3 affects the differentiation by either up regulating the production of Th2 cytokines or suppressing T-bet and STAT4 [63-65]. Beside STAT6, STAT5 also regulates the differentiation of Th2 cells by binding IL-4 together with GATA3 [66]. Th2 immunity is vital for the elimination of extracellular parasites such as helminthes,

bacteria, allergens and toxins. The elimination mechanism is mediated by cytokines including IL-4, IL-5, IL-6, IL-9, IL-13, and IL-25 [67,68]. IL-4 mostly takes part in allergic inflammation by promoting IgE switching. Beside, metabolites such as histamine and serotonin are released from mast cells and basophils as a result of IL-4 secretion. IL-5 provides the recruitment of eosinophils to the site of inflammation. On the other hand, IL-9 plays a critical role in asthma. One of the most significant roles of IL-13 is to fight intestinal helminthes and intracellular pathogens, especially *Leishmania* [69,70].

In the presence of cytokines including IL6, IL21, IL23, and TGF- β , naïve CD4⁺ T cells are differentiate into Th17 cells by activating the specific transcriptional factor, ROR γ t. Th17 differentiation is more complex compared with the differentiation of other subsets. TGF- β at low concentration with IL-6 are crucial for the differentiation step by inducing the production of IL-21 [71-73]. IL-6 and IL-21 directly activate STAT-3 while TGF- β is repressing SOCS3, which is the negative regulator of STAT3. Both, the activation of STAT3 and the expression of ROR γ t enhance the secretion of IL-17A, IL-17F, IL-21 and IL-22 from Th17 cells [74]. Also, ROR α (RORa) takes place in Th17 differentiation process by collaborating with ROR γ t [75]. Th17 cells are of prime importance for the elimination of extracellular bacteria and fungi. However, they can also cause several autoimmune diseases. Since the IL-17 receptor is expressed in multiple tissues including skin, intestine, lung, hematopoietic tissue, IL-17 secreted from Th17 affects the whole system. IL-21 has an effect on T cells, B cells and Natural Killer (NK) cells in term of their activation and differentiation. On the other hand, IL-22 maintains the protection of mucosal immunity by triggering secretion of antimicrobial peptides [76,77].

Induced regulatory T cells (iTregs) are differentiated from naïve CD4⁺ T cells in the presence of TGF- β and IL-2. TGF- β signaling pathway induces expression of Forkhead transcription factor FOXP3 that is vital for iTreg differentiation [78]. Beside, IL-2 signaling pathway enhances the phosphorylation of STAT5, which further increases FOXP3 expression [79]. iTregs provide immunological tolerance and neutralize the inflammatory environment by secreting TGF- β , IL-10 and IL-35. IL-10 inhibits tissue damage by suppressing pro-inflammatory cytokines. IL-10 together with TGF- β prevent

production of IgE which causes allergic reactions. FOXP3 not only promotes iTreg development but also suppress Th17 differentiation by blocking ROR γ t [80,81].

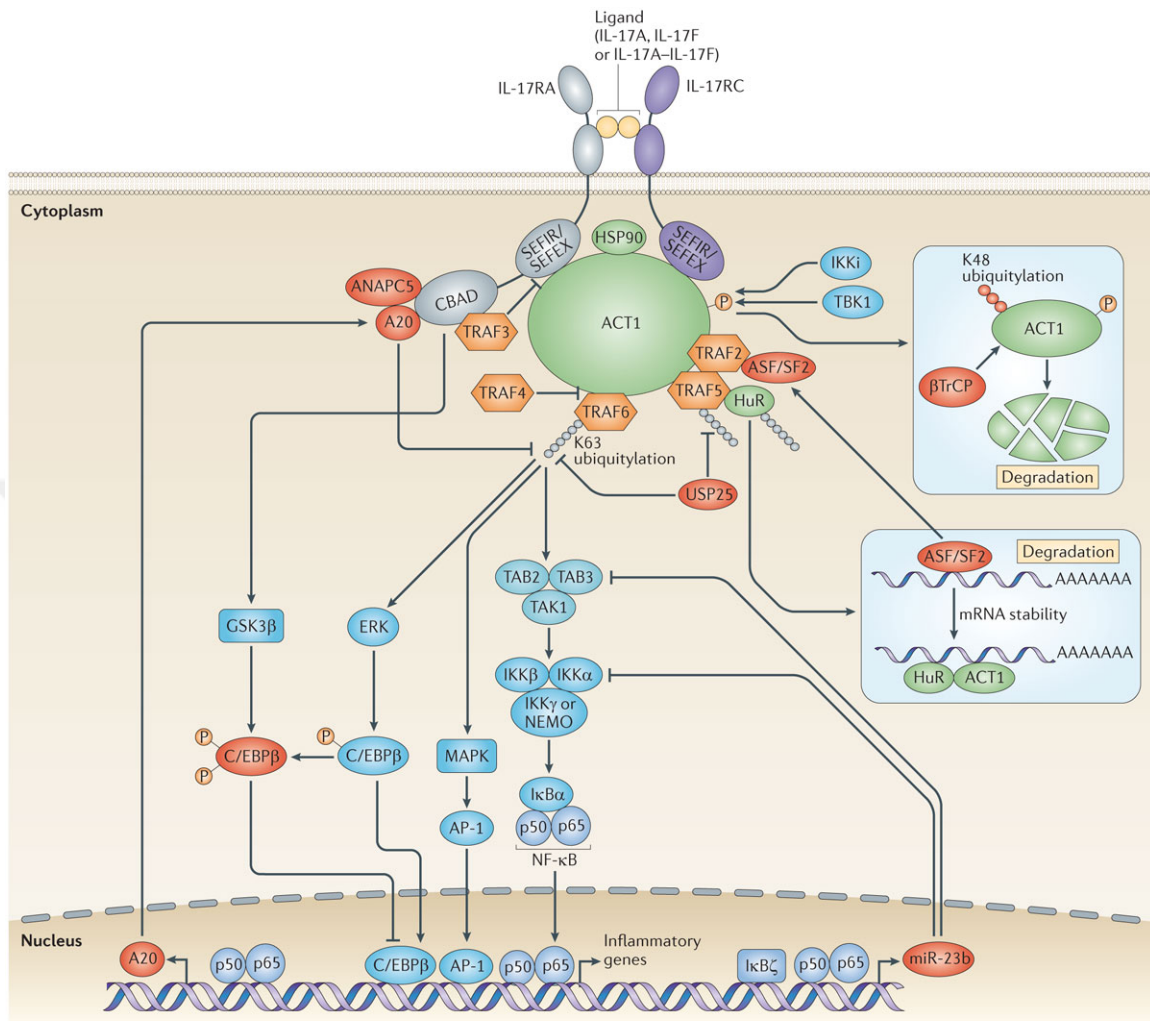
1.3 Interleukin-17 (IL-17A)

1.3.1 IL-17A producing T cells

The most potent IL-17 producing T cells are Th17 cells. TGF- β at low concentration with IL-6, IL-21 and IL-23 provide differentiation of naïve CD4⁺ T cells into Th17. The activation of transcription factor, ROR γ t and JAK-STAT3 pathway promote IL-17 secretion from Th17 cells [71-73]. Beside Th17 cells, a recent study showed that CD8⁺ T cells can also secrete IL-17 and they are named as Tc17 cells. When naïve CD8⁺ T cells are activated by TCR-MHCI-peptide under Th17 culture conditions, they start to secrete IL-17. However, they both lose their cytotoxicity and inhibit the expression of cytotoxic factors. The most important feature of Tc17 cells is that they have a positive impact on the generation of Th17 cells. Like Th17 cells, Tc17 cells can cause autoimmunity or amplify the severity of autoimmune diseases [82]. In addition to Tc17 cells, $\gamma\delta$ T cells, which compose 1-5% of T lymphocytes and reside in mucosal tissues, can secrete IL-17. In mucosal tissues, when they encounter with an antigen, they respond like innate immunity by secreting IL-17 mostly during *Listeria monocytogenes* and *Mycobacterium* infection [83]. Also, Hirota et. al. demonstrated that IL-17 producing $\gamma\delta$ T cells collaborates with Th17 cells to fight against *C. albicans* [84].

1.3.2 Mechanism of IL-17 signaling

IL17 that can be secreted by T cells including Th17, Tc17 and $\gamma\delta$ T cells acts on IL17 receptor [71,82,83]. Although IL17 receptor mostly express on non-hematopoietic cells such as epithelial, endothelial and fibroblast cells, both innate and adaptive immune cells such as macrophages, neutrophils and T cells express this receptor [85]. IL-17 binding to its receptor triggers the activation of several signaling pathways including NF κ B, AP1, MAPK and C/EBP [86].



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Figure 1.6: IL-17 receptor signal transduction [87]

Upon IL-17 binding, the adaptor protein ACT1 is recruited to site to sustain the K63-linked ubiquitylation of TRAF6, which further activates the non-canonical pathway of NFκB. In addition, IL-17 binding triggers MAPK pathways by inducing the expression of inflammatory genes. ERK from MAPK pathway phosphorylates the CCAAT/enhancer-binding protein-β (C/EBPβ) transcription factor. Alternatively, recruited protein ACT1 is phosphorylated by inducible IκB kinase and serine/threonine-protein kinase TBK1. ACT1 phosphorylation recruits TRAF2 and TRAF5 by calling HuR to stabilize mRNA

transcripts. As a result, activation of several signaling pathways enable cells to secrete inflammatory cytokines and chemokines [87].

1.3.3 IL-17 role in host defense and autoimmunity

IL-17 is the hallmark cytokine for the elimination of extracellular bacteria and the generation of autoimmunity. *S. aureus*, *C. rodentium*, and *Klebsiella pneumoniae* are the most sensitive strains against IL-17 which promotes the recruitment of macrophages, neutrophils to the site of infection [88,89]. Some intracellular bacteria such as *L.monocytogenes* , *S.typhimurium* can also be cleared by IL-17 production. In addition to bacterial infectious, fungal infectious are susceptible to IL-17. In fact, some fungi developed a strategy, which is based on the production of neutralizing antibody against IL-17 to escape from the effect of IL-17 [90]. The overall effects of IL-17 arise from induction of inflammatory mediators such as cytokines, chemokines and antimicrobial peptides.

Excessive production and secretion of IL-17 causes tissue damage and autoimmunity by enhancing the secretion of pro-inflammatory cytokines. Unstrained IL-17 secretion can be related with several autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), inflammatory bowel disease (IBD). Since it affects broad spectrum of autoimmune diseases, IL-17 blockade strategy became hot topic as a treatment strategy [91].

1.4 Aim of the study

Recent studies demonstrated that STING activating cyclic di nucleotides such as cGAMP promote tumor shrinkage when they are used as vaccine adjuvants [92]. The effect of tumor reduction is based on IFN beta secretion through STING pathway by which adaptive immune members are recruited the site to secrete inflammatory cytokines such as IFN γ . By this way, cytotoxic effects of CD8⁺ T cells against tumor cells are enhanced. This makes cyclic dinucleotides an important candidate for effective vaccine adjuvants. Studies investigating vaccine adjuvant therapies merely focus on the effect of cGAMP on myeloid cells such as macrophages and dendritic cells. However, the role or the effect of cGAMP in T lymphocytes is still unknown. Hence, we aimed to understand the direct effect of cGAMP in T lymphocytes. Since we do not have any preliminary data from the literature, our first approach is to stimulate Pan T cells with cGAMP alone or together with various TLR ligands for general screening. That screening strategy helped to discover the cytotoxic effect of cGAMP for Pan T cells. In addition to toxic effect, functional screening showed that IL-17 secretion is increased from Pan T cells stimulated with cGAMP. Then, all assays performed for Pan T cells were also conducted for CD4⁺ T cells to understand the source of IL-17. Surprisingly, cGAMP alone could not trigger IL-17 secretion from CD4⁺ T cells, unlike Pan T cells. As a second approach, we sought a way to decrease toxic effect of cGAMP while maintaining IL-17 secretion. To do that, Pan T cells were stimulated with cGAMP alone or combining with R848. According to our results, cGAMP together with R848 can be a better vaccine adjuvant rather than cGAMP. Lastly, the effects of IL-17 secretion on innate immune cells such as macrophages were investigated to understand the overall effect of cGAMP stimulation.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. General laboratory & cell culture reagents and materials

RPMT-1640 medium with PBS, FBS, L-glutamine and 2-mercaptoethanol were purchased from Gibco, USA. Zap-OGLOBIN® II Lytic reagent and Z-PAK Isoton II diluent which were used for cell counting purchased from Beckman Coulter, USA. Other cell counting reagent Trypan Blue alongside Hypure Molecular Biology Grade Water were purchased from Hyclone. Another cell culture reagents including Pen-Strep, Na-Pyruvate, HEPES, NEAA and Trypsin were purchased from Lonza, Switzerland. For PanT and CD4⁺ T cell isolation, mouse Pan T, CD4⁺ T cell negative magnetic isolation kits and other equipments including magnet, MS columns were purchased from Miltenyi Biotech, Germany. Container for freezing cells was purchased from Thermo Scientific, USA. Beside, 2 ml screw cap cryo vials and tissue culture dishes were purchased from Greiner bio-one, Austria.

2.1.2 Recombinants and other agents

Mouse IL-4, which was used for both ELISA and bone marrow, derived dendritic cell generation, alongside GM-CSF were purchased from Tonbo, USA. Beside, in vivo ready mouse anti-CD3 and anti-CD28 were purchased from Tonbo, USA for T cell activation.

The other recombinants used in ELISA such as mouse IL-10, mouse IFN- γ , mouse IL-17 and mouse IL-2, mouse IL-6 which were used for T cell survival and differentiation, as well as Brefeldin A were purchased from Biolegend, USA. PMA (phorbol 12-myristate 13-acetate) and Ionomycin were supplied from Sigma, USA.

2.1.3 CpG ODNs

K3-PS and 1466 Acore-PO were purchased from Alpha DNA, Canada. Beside, A151 was supplied from NIH, USA. Their length and sequence are listed as the followings;

Table 2.1: Commercial name of the CpG ODN and their properties

ODN	Brand	Length	Sequence
K3	Alpha-DNA, Canada	12mer	TCGAG <u>CGTT</u> CTC
1466-Acore-	Alpha-DNA, Canada	16mer	tcaacg <u>ttgatt</u> caaa
A151	NIH, USA	24mer	TTAGGGTTAGGGTTAGGGTTAGGG

*uppercase letters denotes phosphothioate linkages (PS) and lowercase letters denotes phosphodiester (PO) linkages between bases. Underlined bases denote CpG motifs.

2.1.4 PRR ligands

Ligands used for cell stimulations were listed in Table 2.2.

Table 2.2: Commercial name of the ligands and their sources

Ligand	Source	Brand
LPS	<i>E. coli</i>	Sigma, USA
R848	Synthetic	Invivogen, USA
3'3' cGAMP	Synthetic	Invivogen, USA
8-Fluo-AET-cGAMP(2'-5')	Synthetic	Biolog, Germany

2.1.5 Flow cytometry

Medium A and Medium B, which were used during cell fixation and permeabilization, respectively were purchased from Invitrogen, USA. In order to perform CD4⁺ T cell phenotyping, mouse Th1/Th2/Th17 kit were purchased from BD, USA. Antibodies used in cell surface or intracellular staining were listed in Table 2.3.

Table 2.3: Commercial name of the antibodies and their properties

Dye	Species	Brand	Cat #
Anti-CD3-PE	Mouse	Biolegend, USA	100206
Anti-CD4-FITC	Mouse	Biolegend, USA	100406

Anti-CD8a-Pe/Cy5	Mouse	Biolegend, USA	100710
Anti-IL17-PE	Mouse	Biolegend, USA	506903
Anti-CD80-FITC	Mouse	Biolegend, USA	104706
Anti-CD86-Pe/Cy5	Mouse	Biolegend, USA	105016
Anti-I-A/I-E (MHC-II)-PE	Mouse	Biolegend, USA	107608

2.1.6 Determination of gene expression

Trizol, which was used for the first step of RNA isolation, was purchased from Life Technologies, USA. In order to conversion of RNA into cDNA, ProtoScript M-MuLV cDNA Synthesis Kit together with OneTaq Quick-Load 2x Master mix with Standard Buffer were purchased from NEB, USA. Reagents used for the visualization of the PCR products, 6x gel loading dye and DNA ladders were purchased from Fermentas, USA. Quantitative Real Time PCR was performed by using Light Cycler 480 Sybr Green I Master, which was purchased from Roche, USA.

2.1.6.1 Primers

Primers that were used in both PCR and qRT-PCR are listed in Table 2.4. By obtaining sequences of the specific genes from NCBI database, primers were designed with Primer3 Input v.0.4.0 program (<http://frodo.wi.mit.edu/primer3/input.htm>). In order to check their specificity, each primer pair was blasted

(<http://www.ncbi.nlm.nih.gov/BLAST/> or <http://genome.ucsc.edu/>) against the mouse genome.

Table 2.4: Primers used in PCR

Gene Name	Direction	Sequence	Product size (bp)
<i>rorc</i>	F	TGCAAGACTCATCGACAAGGC	86
<i>rorc</i>	R	AGCTTTTCCACATGTTGGCTG	
<i>rora</i>	F	GAACACCTTGCCCAGAACAT	145
<i>rora</i>	R	AGCTGCCACATCACCTCTCT	
<i>il-17</i>	F	AAGGCAGCAGCGATCATCC	150
<i>il-17</i>	R	GGAACGGTTGAGGTAGTCTGAG	
<i>socs1</i>	F	ACCTTCTTGGTGCGCGAC	60
<i>socs1</i>	R	AAGCCATCTTCACGCTGAGC	
<i>sting</i>	F	TGGCTGCTGATGCCATACTC	107
<i>sting</i>	R	TCGAGACTCGGGGACATCTT	
<i>casp-3</i>	F	TCTACAGCACCTGGTTACTATTCCTGG	130
<i>casp-3</i>	R	TCCTGTTAACGCGAGTGAGAATGTG	
<i>bax</i>	F	AACTGGTGCTCAAGGCCCTGTG	226
<i>bax</i>	R	GCCACAAAGATGGTCACTGTCTGC	
<i>β-actin</i>	F	GAAGATCAAGATCATTGCTCCTCCTG	120
<i>β-actin</i>	R	CTCATCGTACTCCTGCTTGCTGATCC	

2.1.7 Reagents for ELISA

2HB ELISA plates were obtained from SPL Life Sciences, Korea. ELISA antibodies except IL-4 were purchased from Biolegend, USA. Mouse Anti-IL4 and IL-4 Biotinylated antibody were obtained from Endogen, USA. Besides, SA-ALP was purchased from MabTech, Sweden and PNPP Tablets and 5X Diethanolamine Buffer were purchased from Thermo Scientific, USA. The features of the antibodies including coating, biotinylated and recombinant are listed in detail on Table 2.5.

Table 2.5: Antibodies used in ELISA

Antibody Name	Brand	Cat #	Working concentration
Anti-IFN- γ Ab	Mabtech, Sweden	3321-1a-20	1 μ g/ml in PBS
IFN- γ Biotinylated Ab	Mabtech, Sweden	3321-1a-20	1:1000 diluted in T cell buffer
Recombinant IFN- γ	Biolegend, USA	575309	80 ng/ml in Blocker
Anti-IL-10 Ab	Biolegend, USA	504902	4 μ g/ml in PBS
IL-10 Biotinylated Ab	Biolegend, USA	505004	1:1000 diluted in T cell buffer
Recombinant IL-10	Biolegend, USA	575804	100 ng/ml in Blocker
Anti-IL-4 Ab	Endogen, USA	MM450C	4 μ g/ml in PBS
IL-4 Biotinylated Ab	Endogen, USA	5MM450D B	1:1000 diluted in T cell buffer
Recombinant IL-4	Endogen, USA	RMIL4I	1 μ g/ml in Blocker
Anti-IL-17a Ab	Biolegend, USA	506902	4 μ g/ml in PBS
IL-17a Biotinylated Ab	Biolegend, USA	507002	1:1000 diluted in T cell buffer
Recombinant IL-17A	Biolegend, USA	576009	5 ng/ml in Blocker
Anti-IL-2 Ab	Biolegend, USA	503702	4 μ g/ml in PBS
IL-2 Biotinylated Ab	Biolegend, USA	503804	1:1000 diluted in T cell buffer
Recombinant IL-2	Biolegend, USA	575402	100 ng/ml in Blocker

2.1.8 BCA

In order to measure the concentration of unknown proteins, BCA was used and purchased from Pierce, USA. The contents of the kit are listed on Table 2.6.

Table 2.6: BCA reagents

Name	Ingredient
BCA Reagent A	sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide
BCA Reagent B	4% cupric sulfate
Albumin Standards Ampuls	bovine serum albumin (BSA) at 2mg/mL in 0.9% saline and 0.05% sodium azide

2.1.9 Western blot

Prestained protein ladder was purchased from Fermentas, USA and PVDF transfer membranes were purchased from Thermo Scientific, USA. As a substrate, SuperSignal West Femto Kit was purchased from Thermo Scientific, USA. The visualization of the membranes was performed by Amersham Imager 600. All Western Blot antibodies were purchased from Cell Signalling, USA and listed on Table 2.7.

Table 2.7: Antibodies used in Western blot

Antibody Name	Brand	Cat #	Working concentration
β -actin	Cell Signaling,	3700	1:5000 diluted in 5% BSA in BSA

	USA		
p-STAT3 (SER727)	Cell Signaling, USA	9134	1:1000 diluted in 5% BSA in BSA
p-STAT3(TYR705)	Cell Signaling, USA	9145	1:1000 diluted in 5% BSA in BSA
CASPASE-3	Cell Signaling, USA	9665	1:1000 diluted in 5% BSA in BSA
CLEAVED CASPASE-3	Cell Signaling, USA	9664	1:1000 diluted in 5% BSA in BSA
PARP	Cell Signaling, USA	9542	1:1000 diluted in 5% BSA in BSA
p-IRF3(TYR)	Cell Signaling, USA	29047	1:1000 diluted in 5% BSA in BSA
IRF3	Cell Signaling, USA	4947	1:1000 diluted in 5% BSA in BSA
CASPASE-9	Cell Signaling, USA	9508	1:1000 diluted in 5% BSA in BSA
Anti-Rabbit IgG-HRP Linked	Cell Signaling, USA	7074	1:10,000 diluted in 5% milk in TBS-T
Anti-Mouse IgG-HRP Linked	Cell Signaling, USA	7076	1:10,000 diluted in 5% milk in TBS-T

2.1.10 Determination of cell viability

In order to determine the viabilities of cells, Cell Counting Kit-8 was used and purchased from Dojindo Molecular Technologies, Japanese.

2.2 Solutions, Buffers and Culture Media

2.2.1 Cell culture media

High Glucose RPMI-1640 (Lonza)

- 2, 5 or 10% FBS inactivated at 55 °C
- 50 g/ml Penicillin/Streptomycin
- 10 mM HEPES
- 0,11 mg/ml Na Pyruvate
- 1% Non-Essential Amino Acids Solution
- 2 mM L-Glutamine

All ingredients were added into 500 ml medium and stored at +4 °C.

2.2.2 Flow cytometry buffers

PBS-BSA-Na azide Buffer (FACS Buffer)

- 500 ml 1x PBS
- 5g BSA (1%)
- 125 mg Sodium Azide (0.25%)

The solution was stored at +4 °C.

%0.3 Saponin Buffer

- 0.3 g Saponin
- 100 ml FACS Buffer

2.2.3 Agarose gel electrophoresis

50X TAE (Tris-Acetate-EDTA)

- 242 g Tris ($\text{C}_4\text{H}_{11}\text{NO}_3$)
- 37.2 g Tritiplex 3 (EDTA= $\text{C}_{10}\text{H}_{14}\text{N}_2\text{Na}_2\text{O}_2 \cdot 2\text{H}_2\text{O}$)
- 57.1 ml Glacial acetic acid

All ingredients were dissolved in 1 lt ddH₂O, autoclaved and stored at RT.

1X TAE is prepared as a working concentration.

1%Agarose gel

- 1 g Agarose
- 100 ml 1X TAE

The mixture was boiled at microwave and poured into tray after adding 3ul ethidium bromide.

2.2.4 ELISA buffers

Blocking buffer

- 500 ml 1x PBS
- 25 g BSA (5%)
- 250 µl Tween20 (0,025%)

The mixture was stored at -20°C.

T-cell buffer

- 500 ml 1x PBS
- 25 ml FBS (5%)
- 250 µl Tween20 (0,025%)

The mixture was stored at -20°C.

Wash buffer

- 500 ml 10x PBS
- 2.5 ml Tween20
- 4.5 lt ddH₂O

10X PBS (Phosphate Buffered Saline)

- 80 g NaCl
- 2 g KCl
- 8,01 g Na₂HPO₄ · 2H₂O
- 2 g KH₂PO₄
- 1 lt ddH₂O

After adjusting pH to 6.8, it was stored at RT.

2.2.5 Western blot buffers

RIPA buffer (Radio Immuno Precipitation Assay Buffer)

- 150 mM Sodium chloride
- 1.0% NP-40 or Triton X-100
- 0.5% Sodium deoxycholate
- 0.1% SDS
- 50 mM Tris, pH 8.0

Buffer was prepared freshly, kept at +4 °C and used immediately.

4X Laemmli buffer

- 4% SDS
- 10% 2-mercaptoethanol
- 20% Glycerol
- 0.004% Bromophenol blue
- 0.125 M Tris-HCl

Buffer pH was adjusted to 6.8 and stored at -20 °C.

TBS 10x

- 24.23 g Trizma HCl
- 80.06 g NaCl

Solution was prepared with 1 lt ddH₂O. Its pH was adjusted to 7.6 with pure HCl and stored at RT. It was diluted to 1X before use.

TBS-T

- 100 ml of 10X TBS
- 900 ml ddH₂O
- 1 ml Tween20

Solution was mixed well and stored at RT.

10X Running buffer

- 25 mM Tris base
- 195 mM Glycine
- 0.1% SDS

Its pH was adjusted to 8.3 and stored at RT. It was diluted to 1X before use.

Transfer buffer

- 25 mM Tris base
- 195 mM Glycine
- 20% Methanol

Its pH was adjusted to 8.3 and stored at +4 °C.

Blocking and antibody incubation buffer

- 50 ml TBS-T
- 2.5 g Milk powder or BSA

Solution was mixed well and stored at +4 °C.

12% Resolving gel

- 2.5 ml 1.5 M Tris (pH 8.8)
- 100 µl 10% SDS
- 3.96 ml 30% Acrylamide
- 100 µl 10% APS
- 10 µl TEMED
- 3.44 ml ddH₂O

Solution was mixed well by vortexing and used immediately.

8% Resolving gel

- 2.5 ml 1.5 M Tris (pH 8.8)
- 100 µl 10% SDS
- 2.64 ml 30% Acrylamide
- 100 µl 10% APS
- 10 µl TEMED
- 4.76 ml ddH₂O

Solution was mixed well by vortexing and used immediately.

5% Stacking gel

- 2 ml 0.5 M Tris (pH 6.8)
- 80 µl 10% SDS
- 1.3 ml 30% Acrylamide
- 80 µl 10% APS

- 16 µl TEMED
- 4.5 ml ddH₂O

Solution was mixed well by vortexing and used immediately.

2.2.6 MACS buffer

- 0.5% BSA
- 2mM EDTA

Ingredients were dissolved in 1x PBS. Buffer was freshly prepared and stored at +4 °C during isolation process.

2.3 Methods

2.3.1 Maintenance of cell lines

2.3.1.1 RAW264.7

RAW 264.7 (ATCC®, TIB-71) is an Abelson murine leukemia virus transformed mouse macrophage cell line. Cells are sustained in RMI1640 media with 10% regular FBS. For sub-culturing, when they reach around 90% confluence, cells are scraped and centrifuged at 300g for 5 min. After counting by haemocytometer, they are seeded into a new plate according to their appropriate concentration.

2.3.2 Cryopreservation and thawing of cells

Cryotubes that are taken from liquid nitrogen are heated to 37 °C by using water bath. Once they are melted, cells were transferred into 15 ml falcon and additional proper medium is added until the volume reaches 15 ml to get rid of DMSO. Hereafter, cells are centrifuged at 300 g for 5 min and re-suspended with 1 ml appropriate medium. Then, cells are seeded into T-25 flask and their volume is completed to 5 ml and put into cell culture incubator, which is adjust to 37 °C with 5% CO₂.

Once cells reach around 80-90% viability, they can be frozen for further experiments. When the confluency of cell is around 80%, cells are scraped and centrifuged at 300 g for 5 min. The cell pellet is re-suspended with cold FBS in such a way that 5×10^6 cells will be in 500 ul. Then, they are transferred into cryotubes which are already labeled and 500 ul FBS with 20% DMSO is added. Cryotubes with final 10%DMSO concentration are placed into Ms. Frosty Freezing Container and stored at -80 °C. For immediate use, they can be stored at -80 °C. However; for long-term storage, they should be put into liquid nitrogen.

2.3.3 Cell counting

2.3.3.1 Haemocytometer

After cell line or primary cells are taken and centrifuged, pellet is resuspended with 1 ml appropriate medium. 10 ul from suspended pellet is mixed with 490 ul medium for 50X dilution. Afterwards, 10 ul from 50X diluted sample is mixed with 10 ul trypan blue and 10 ul from Trypan Blue-cell mixture is loaded on haemocytometer and cells are counted under light microscope. Counted cell number is multiplied with 10^6 to calculate actual cell number.

2.3.3.2 Flow cytometer

After cell line or primary cells are taken and centrifuged, pellet is resuspended with medium in appropriate volume (generally between 1-10 ml). 20 ul cell pellet is put into cell cuvette containing 10 ml Isoton II Diluent buffer. If necessary, 3 drops of ZAP-OGLOBIN II Lytic Reagent is added. 30 ul from the mixture is analyzed by Accuri C6 Flow Cytometry. After arranging threshold to get rid of dead cells, the numbers of gated cells are multiplied with appropriate dilution rate to obtain actual number of cells.

2.3.4 Primary murine cell suspension preparation

2.3.4.1 Mouse spleen cell suspension preparation

Spleens from C57/BL6 mice are removed by cervical dislocation. Removed spleen is put into ice in 5 ml %2 FBS supplemented complete RPMI-1640 media. Afterwards, spleen is squeezed with the backside of the 5 ml syringe plunger and squeezed cells are transferred into 15 ml falcon completed with 10 ml fresh 2% RPMI-1640 media and centrifuge at 300 g for 5 min. After centrifugation, pellet is resuspended with 1 ml ACK Lysis Buffer and incubated for 2 min at RT to remove red blood cells. Then, fresh 2% RPMI media is added and centrifuged at 300 g for 5 min. Finally, cells are resuspended in 10% RPMI media and counted in flow cytometer.

2.3.4.2 Isolation of bone marrow cells

After cervical dislocation of C57/BL6 mice, femur and tibia bones were removed. By using 26G insulin syringe, bone marrow cells were washed out from bones and washed with 2% FBS supplemented RPMI media for 3 times. Collected cells were centrifuged and suspended with 1 ml ACK lysis buffer to blow up red blood cells and incubated for 2

min. Centrifugation step was repeated and cells were resuspended with %10 FBS supplemented RPMI-1640 media to count and use further applications.

2.3.5 Bone marrow derived dendritic cell generation

After arranging concentration of bone marrow cells to 1×10^6 cells/ml, they were seeded into 10cm² plate in RPMI-1640 supplemented with 10% FBS including 20ng/ml mGM-CSF and 10ng/ml mIL-4 recombinant proteins. The half of the medium was aspirated and replenished with fresh 10% FBS supplemented RPMI-1640 media after three days. Six days after initial seeding, cells were collected with 1X cold PBS and centrifuged and counted by flow cytometry. For their quality control, CD11b/CD11c staining was performed before their usage.

2.3.6 Pan T and CD4⁺ T cell isolation from mouse spleen

After counting spleen cells with flow cytometry, centrifuged spleen cells were re-suspended in MACS buffer as 40 ul per 10^7 cells. Then, 10 ul Pan T or CD4⁺ T cell Biotin-Antibody cocktail was added per 10^7 cells and incubated for 10 min at 4 °C. 30 ul MACS buffer and 20 ul Pan T or CD4⁺ T cell Microbead Coctail were included and cells were incubated at 4 °C for an additional 15 min. In the mean time, MS column was washed with 500 ul MACS buffer. After incubation, the volume of the cells was completed to 10 ml and cells were centrifuged at 300 g for 5 min. Cell pellet was re-suspended with 500 ul MACS buffer and loaded into the MS column attached to a magnet. Flow-through was collected in a 15 ml falcon and columns were washed two times with 500 ul MACS Buffer to increase the efficiency of the isolation. Collected cells were additionally washed with 10 ml MACS buffer and centrifuged at 300 g for 10 min. Pellet was re-suspended in 1ml of RPMI-1640 supplemented with 50μM 2-

mercaptoethanol. To check the efficiency of the isolation, CD3 and CD4 markers were used during cell surface staining.

2.3.7 T cell activation

Since both Pan T and CD4⁺ T cell can not survive in the absence of antigen presenting cells without any activation signal, artificial activation should be performed. That activation was provided with anti-CD3 and anti-CD28 antibodies. For anti-CD3, plates were coated with 2 ug/ml anti-CD3 antibodies for 2h at 37 °C or overnight +4 °C and washed twice with 1X PBS. On the other hand, anti-CD28 was provided in a soluble form at 1ug/ml concentration.

2.3.8 Cytokine ELISA

After stimulation of RAW, Pan T or CD4⁺ T cells, their supernatants were collected and stored at -20 °C for usage in ELIS experiments. The first step of ELISA is coating of the plates. 96 well plates were coating with 50 ul/well monoclonal antibodies which were prepared with 1X PBS and incubated for overnight at +4 °C. Following day, monoclonal antibody solution was discarded by inverting the plates and 200 ul blocking buffer was added onto each wells and incubated for 2h at RT. After blocking, plates were washed 5 times with washing buffer. 3 minutes incubation was done between each washing. Plates were additionally washed 5 times with ddH₂O. Meanwhile, frozen supernatants were thawed at RT. After washing, plates were dried and 50ul/well supernatants were added. Also, recombinant cytokines were prepared in blocker at desired concentration and diluted serially to obtain concentration of cytokine in cell supernatants. Beside, 50 ul PBS was put into each plate as a blank. Plates were incubated for overnight at +4 °C. On the following day, plates were washed as it is indicated above, dried and 50 ul biotin conjugated antibodies which were diluted at 1:1000 in T cell buffer were added into each

well and incubated for 2 h at RT. After that, washing step was applied again and streptavidin-AKP solution, which was diluted at 1:1000 in T cell buffer, was added into wells and incubated for 1 h at RT. For the last time, plates were washed and dried. As a substrate of AKP, PNPP was prepared as it is indicated in datasheet. 50 ul PNPP was added into each well and read at ELISA reader at 405 nm with time intervals.

2.3.9 The measurement of cell viability

For the measurement of cell viability, Cell Counting Kit-8 (Dojindo) was used. After stimulation and incubation of cells in a 96 well plate, their supernatants were discarded. Meanwhile, for each well, 100 ul 10% FBS supplemented RPMI media was mixed with 10 ul CCK-8 and 110 ul mixture was added to each well of the plate. They were incubated for 1-4 h in the incubator and their OD were measured at 450 nm at intervals of 1 h by using BioTek.

2.3.10 CFSE labeling of Pan T cells

Isolated Pan T cells were re-suspended in 1 ml of 5% FBS supplemented with RPMI media with a cell concentration of between 0.5×10^6 - 10×10^7 /ml. Once 1 ul 5mM CFSE was added, tube was inverted for several times and incubated for 15 min at 37 °C. After that, cells were washed with 10 ml of 20 °C PBS containing 5 % FBS and centrifuged at 300 g for 5 min. Cell pellets were resuspended in RPMI-1640 supplemented with 50µM 2-mercaptoethanol.

2.3.11 Flow cytometry

2.3.11.1 Surface marker staining of cells

Collected cells were transferred to 1.5 ml eppendorf tubes, centrifuged at 300 g for 5 min and washed twice with 1X PBS. Meanwhile, antibody cocktails were prepared at 1ug/ml concentration in FACS buffer. 100 ul from cocktail was added to cells and incubated for 30 min on ice at dark. After incubation, 1 ml FACS buffer was added and centrifuged at 300 g for 5 min at +4 °C. Cell pellets were suspended in 200 ul PBS for flow cytometry analysis or they could be suspended in 50 ul Fixation Medium A for fixation.

2.3.11.2 Fixation of cells

Cell pellets were suspended with 50 ul Fixation Medium A per 10^6 cells while they were mixed vigorously by vortexing. Then, cells were incubated for 15 min at RT. Fixation Medium A was completed with 1 ml FACS buffer and centrifugation step was repeated. Cell pellets were resuspended in 1X PBS for flow cytometry or FACS buffer containing 0.3% Saponin for intracellular cytokine staining.

2.3.11.3 Intracellular cytokine staining

5 hours before the end of the stimulation, cells were treated with PMA (50ng/ml), Ionomycin (1ug/ml) and Brefeldin-A (1X). After those treatments, cells were collected with centrifugation at 300 g for 5 min and cell surface staining and fixation procedure were performed. After fixation, cell pellets were resuspended in 1 ml FACS buffer containing %0.3 Saponin and incubated for 20 min at RT. Then, centrifugation was performed. Cells were resuspended with 100 ul Fixation Medium B including antibody cocktails at 1ug/ml concentration and incubated for 30 min at dark. Once the incubation

was finished, 1 ml FACS buffer was added and cells were centrifuged at the same conditions. Cell pellets were resuspended in 100 ul 1X PBS for flow cytometry analysis.

2.3.12 Binding & uptake and internalization assays

After isolation and activation of T cells, they were stimulated with 2'3 cGAMP-FAM and incubated for 1-4 hours. Then, cells were collected at different time points and centrifuged at 300 g for 5 min. Firstly, cell pellets were resuspended with 100 ul 1X PBS and analyze by using flow cytometer. That analysis showed the binding and uptake capacity of cGAMP. To measure the internalization efficiency, trypan blue (0.01% final concentration) was added and cells were analyzed again by flow cytometry.

2.3.13 Determination of gene expression

2.3.13.1 Total RNA isolation

After the stimulation of cells, they were collected and transferred into 1.5 ml eppendorf tubes. Cells were washed with 1X PBS and centrifuged at 300 g for 5 min. Then, cell pellets were resuspended in 1 ml of TRIzol. After this step, tubes can be stored at -80 °C for further isolation. If tubes are kept at -80 °C, they can be thawed at RT. After suspension of cells in TRIzol, 200 ul chloroform was added to each eppendorf tubes and shaken vigorously for 15 seconds and incubated for 3 min at RT. Then, cells were centrifuged at 13200 rpm for 17 min. After centrifugation, 3 phases including aqueous, intermediate and organic phases. For the RNA isolation, only aqueous phase, which was the upper layer, was transferred into another 1.5 ml eppendorf tube. 500 ul isopropanol was added to aqueous phase and the solution was mixed gently by inverting tubes for several times and incubated for 10 min at RT. Then, tubes were centrifuged at 13200 rpm

for 12 min. Supernatant was discarded and pellet was resuspended in 1 ml of 75% EtOH as a first washing step. After 5 min centrifugation at 8000 rpm, pellet was washed with 1 ml of >99,8% EtOH again and suspension was subjected to centrifugation at the same conditions. Supernatants were taken by pipet tips and let them dry for 5-10 min. Dried pellets were resuspended in 15 μ l of RNase/DNase free water. Their qualities in terms of purity and clarity were determined by using NanoDrop® ND-1000 spectrophotometer. Isolated RNAs were stored at -80 °C.

2.3.13.2 cDNA synthesis

RNAs from -80 °C were thawed on ice. According to experiment, RNA concentrations were equalized (1 ng-1 μ g). 2 μ l oligo dT was mixed with equal amounts of RNA and the volumes were completed to 8 μ l. Those mix were exposed to 70 °C for 5 min and put on ice immediately. After that, 10 μ l M-MuLV reaction mix and 2 μ l M-MuLV enzyme mix were added and cDNA synthesis was completed by incubating at 42 °C for 60 min, 80 °C for 5 min. After isolation, cDNA samples were stored at -20 °C.

2.3.13.3 PCR

For a STING gene, a specific primer pairs (Table ?) and optimal PCR conditions were set and the reaction was performed on MJ Mini thermocycler (Bio-Rad, USA). The protocol and the conditions were listed on Table 2.8 and Table 2.9, respectively.

Table 2.8: Sample PCR Reaction

Reagent	Volume (μ l)

Quick Load Taq 2x Master Mix	12,5
Nuclease-free H ₂ O	9.5
10pmol Forward Primer	1
10pmol Reverse Primer	1
cDNA	1
Total Volume	25

Table 2.9: PCR Protocol

Step	Temperature (°C)	Duration	Cycle
Initial Denaturation	94	2 min	1x
Denaturation	94	20 sec	35x
Annealing	60	30 sec	
Elongation	68	30 sec	
Final Extension	68	5 min	1x
Hold	4	∞	

2.3.13.4 qPCR

Primer pairs for a specific gene were listed on Table 2.4. Each primer had the same annealing temperature. The reaction was performed on STRATAGEN. Additionally, the protocol and the conditions were listed on Table 2.10 and Table 2.11, respectively.

Table 2.10: Sample qPCR reaction

Reagent	Volume (μl)
Light Cycler 480 Sybr Green I Master	5
Nuclease-free H ₂ O	3
10pmol Forward Primer	0.5
10pmol Reverse Primer	0.5
cDNA	1
Total Volume	10

Table 2.11: qPCR protocol

Step	Temperature (°C)	Duration	Cycle
Initial Denaturation	95	5 min	1x
Denaturation	95	15 sec	45x
Annealing	60	20 sec	
Elongation	72	20 sec	
Final Extension	72	5 min	1x
Hold	4	∞	

2.3.13.5 Agarose gel electrophoresis

After performing PCR, the visualization of STING gene was done by running 1% agarose gel. 1 g agarose was added to 100 ml of 1X TAE and heated until agarose dissolved. Then 3 ul ethidium bromide solution was added to solution and pour into the tray. The comb was chosen according to number of samples and sinked in agarose gel. After

cooling, 15 μ l PCR products were put into the wells. Beside PCR products, 100 bp DNA ladders were loaded as a marker and the gels were ran for 45 min at 120 V power. Visualization of the gels were performed under UV transilluminator (Vilber Lourmat, France) with BIO-PROFILE Bio-1D V 11.9 software.

2.3.14 BCA protein assay

To determine protein concentration of samples, BCA protein assay was used according to the manufacturer's protocol. Generally, BSA standards were prepared at various dilutions to determine the concentration of unknown proteins. 25 μ l standard was mixed with 200 μ l working reagent, which was prepared according to kit manual. Beside, 5 μ l of samples were mixed with 200 μ l working reagent and they were incubated at 37 °C for 30 min. After incubation, the absorbance of samples measured at 562 nm by spectrophotometer (BioTek) and their concentrations were measured by machine.

2.3.15 Western blot

2.3.15.1 Preparation of protein samples

Cell were collected from culture plates and pelleted with centrifugation at 300 g for 5 min. Cell pellet were resuspended in freshly prepared RIPA buffer. Cells were left on ice for 30 min to be lysed. When lysis was over, samples were centrifuged at 13,200 rpm for 20 min at 4 °C. At the end of centrifugation supernatant of the tubes were taken into new 1.5 ml eppendorf tubes. The samples were labeled and stored at -80 °C. Before starting to a western blot, total protein concentrations were determined using BCA protein assay. 15-30 μ g protein was prepared with 5 x loading dye in 20-30 μ l for loading. A mixture of protein and loading dye was put in 1.5 ml eppendorf tubes and placed in heater, which

was pre-heated to 95 °C and incubated for 5 min. Then samples were spun down for 5-10 sec and loaded into SDS gel.

2.3.15.2 SDS-PAGE running

The concentration of a gel depends on the molecular weight of the proteins to be used, in this study the proteins had various molecular weight between 17-130 kDA, therefore 12% and 8% resolving gel and 5% Stacking gel was prepared. For the preparation and running procedures, Bio-Rad Mini PROTEAN Tetra Cell system was used. Completing the preparation of the gels, they were transferred into mini tank and upon transfer, the tank was filled with 1X running buffer, after which the comb was removed. Previously prepared samples were loaded into gel and the power-supply was connected and set to 80 V until the samples escaped the stacking gel. Then the voltage was raised to 120 V until the desired time. After running, gels were put in transfer directly.

2.3.15.3 Transfer to the membrane

The transfer was performed by using a wet transfer system and the apparatus of Bio-Rad Mini Transblot Cell. Complete transfer buffer was prepared freshly. PVDF membranes and Whatman papers were prepared in size of the SDS gel. Two sponges, 4 Whatman papers, membrane and gel were placed properly into the sandwich apparatus, which was then enclosed and placed into transfer tank filled with complete transfer buffer. Transfer was done at 100 V for 90 min.

2.3.15.3 Blotting and development

Blocking was done with 5% non-fat dry milk dissolved in 0.2% Tween-20 in TBS-T and was performed as 1 h at RT by gently shaking. Primary antibody was prepared with 1:1000 dilution in 5% BSA dissolved in 0.2% Tween-20 in TBS-T. It was incubated overnight at +4 °C by gently shaking. After primary antibody incubation, the membranes were washed with 0.2% Tween-20 in TBS-T as 10-10-10 min. Then, secondary antibody was prepared with 1:10,000 dilution in 5% non-fat dry milk dissolved in 0.2% Tween-20 in TBS-T. It was incubated 1 h at RT by gently shaking. After secondary antibody incubation, the membranes were washed with 0.2% Tween-20 in TBS-T as 10-10-10 min. The last two steps were immunodetection of the membrane and its visualization. For the preceding step, ECL Prime chemiluminescent detection kit was used and incubated for 5 min at dark. Then the membrane was visualized by using Amersham Imager 600.

2.3.16 Statistical analyses

All statistical analyses were performed in GraphPad Prism 6.0 software, if not otherwise stated. Student t-test was used for analysis. $p < 0.05$ was considered as statistically significant.

Chapter 3

Results

3.1 Preliminary studies to optimize the culture conditions of T cells in culture

The improvement of T cells from resting to fully activated state; proliferation of cells is a vital part to initiate a proper immune response [93]. Since the role of cGAMP in T lymphocytes was not described before, the activation of T cells in in-vitro conditions underpinned our study. Thus, our first effort was the optimization of culture conditions before investigating the role of cGAMP. Pan T cells were magnetically isolated from female C57BL/6 spleens by using Pan T MACS isolation kit. Since it was so important not to mix myeloid cells into Pan T population, isolation efficiency and the purity of cells were measured (Figure 3.1).

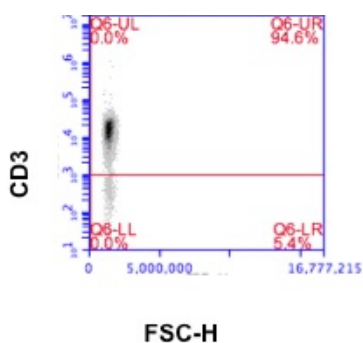


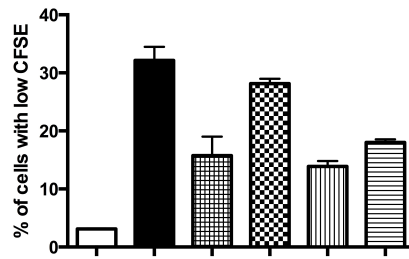
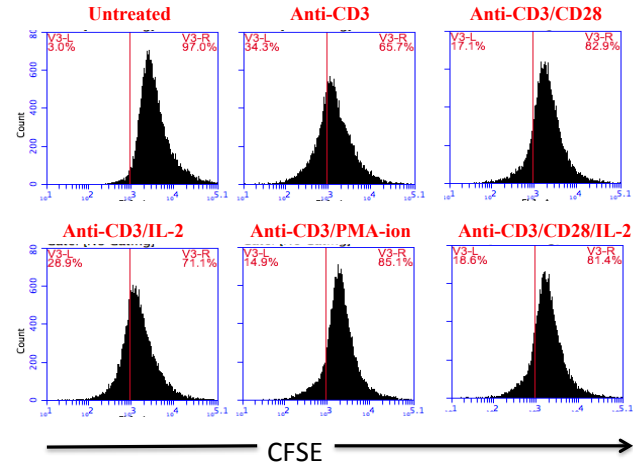
Figure 3.1: Isolation efficiency of Pan T cells. *Pan T cells isolated from BL/6 spleen were stained with CD3 to determine the efficiency of the isolation and purity of cells. A representative plot following purification is presented.*

Our initial trials revealed that it was possible to obtain highly pure Pan T cell population ($95.43 \pm 1.99\%$ CD3⁺ population was obtained). The next step was the application of different conditions for T cells. In the literature, different antibodies or chemicals were used to activate T cells [94]. Anti-CD3 antibody was the common for all conditions because this is the first and irreplaceable signal for T cells [95]. We preferred to add growth factor, chemical or an antibody with anti-CD3 for T cell culture.

IL-2 cytokine is a crucial cytokine for T cell proliferation and differentiation. Activated T cells start to secrete IL-2 and secreted IL-2 binds the IL-2 receptor on T lymphocytes, and then activates several signaling pathways such as the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, the phosphoinositide 3-kinase (PI3K)–AKT pathway and the mitogen-activated protein kinase (MAPK) pathway [96]. On the other hand, IFN γ is produced by activated T cells and has fundamental roles in basic immunological processes such as cell-mediated immunity. Because of that, IL-2 and IFN γ ELISA were performed to compare the functional effects of different conditions on Pan T cells. According to our observations, all conditions provided the secretion of huge amount of IFN γ . However, only two conditions including anti-CD3/anti-CD28 and anti-CD3/PMA-ionomycin promoted IL-2 secretion (Figure 3.2.B). Beside that, proliferation status of Pan T cells was determined. According to our results, anti-CD3 and anti-CD3/IL-2 supplementations increased proliferation of T cells compared with other conditions (Figure 3.2.A). However, since IL-2 has pivotal roles to activate fundamental signaling pathways, it ranked in priority. Thus, only two conditions i) anti-CD3/anti-CD28 and ii) anti-CD3/PMA-ionomycin combinations were selected. In literature, Majowicz et. al. showed that anti-CD3 with PMA/ionomycin leads to generation of induced regulatory T cells which could lead to an undesirable outcome from our perspective [97]. Also, anti-CD3/anti-CD28 are more close to physiological

activation of T cells compared with anti-CD3/PMA-ionomycin. Hence, we decided to use anti-CD3/anti-CD28 for further experiments unless indicated otherwise.

A.



Pan T cells :	+	+	+	+	+	+
Anti-CD3 Ab:	-	+	+	+	+	+
Anti-CD28 Ab:	-	-	+	-	-	+
IL-2:	-	-	-	+	-	+
PMA/ion:	-	-	-	-	+	-

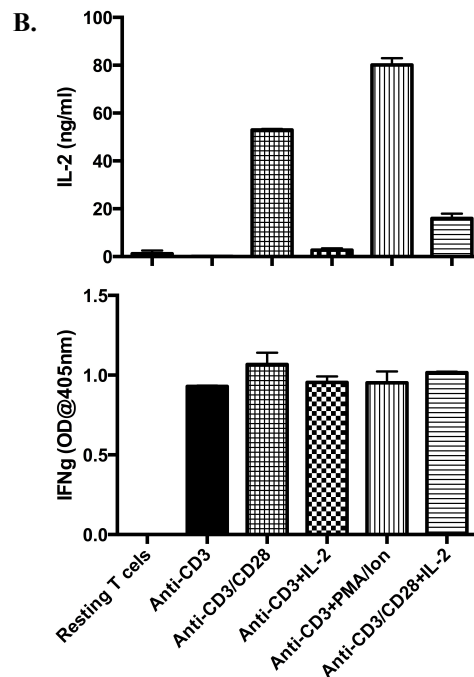


Figure 3.2: The activation and proliferation of T cells were analyzed by comparing different conditions (A) Pan T cells that were stained with CFSE were incubated for 3 days and their CFSE signals were measured by flow cytometry. (B) Cytokines that are necessary for T cell proliferation and differentiation were analyzed by ELISA.

3.2 Studies to understand the effect of cGAMP on Pan T cells

According to literature, STING is expressed in spleen and thymus, which mostly composed of T lymphocytes. Before functional analysis of cGAMP as a STING ligand, we checked the expression of STING in T lymphocytes (Figure 3.3). Macrophages were used as a positive control. We confirmed that STING is expressed in T lymphocytes at similar levels compared to macrophages.

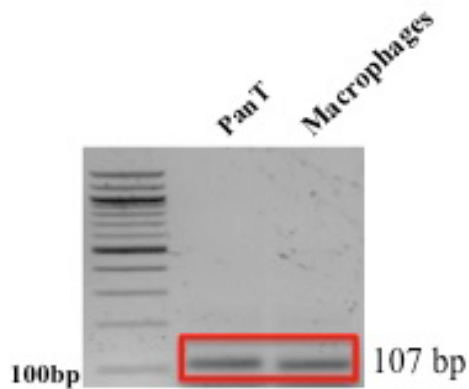


Figure 3.3: Expression analysis of STING on Pan T cells. *RNA was isolated from Pan T, which was isolated from murine spleen. After cDNA conversion, PCR analysis was performed to check whether STING is expressed on T cells. Macrophages used as a positive control.*

In order to delineate the effect of cGAMP on T-cells we sought to understand the internalization level and kinetics to T-cells. Strikingly, there was no data that show the internalization of cGAMP by T cells. Thus, we incubated Pan T cells with fluorescently labeled cGAMP and analyzed their internalization at different time points. According to our observations, Pan T cells easily and readily internalized cGAMP (Figure 3.4). Majority of the internalization occurred within 30 minutes. After 30 minutes, the internalization started to decrease in time but sustained at a constant level within the cells. Thus, since STING was expressed on T lymphocytes and cGAMP was internalized by Pan T cells, the role of cGAMP could be investigated by stimulating Pan T cells.

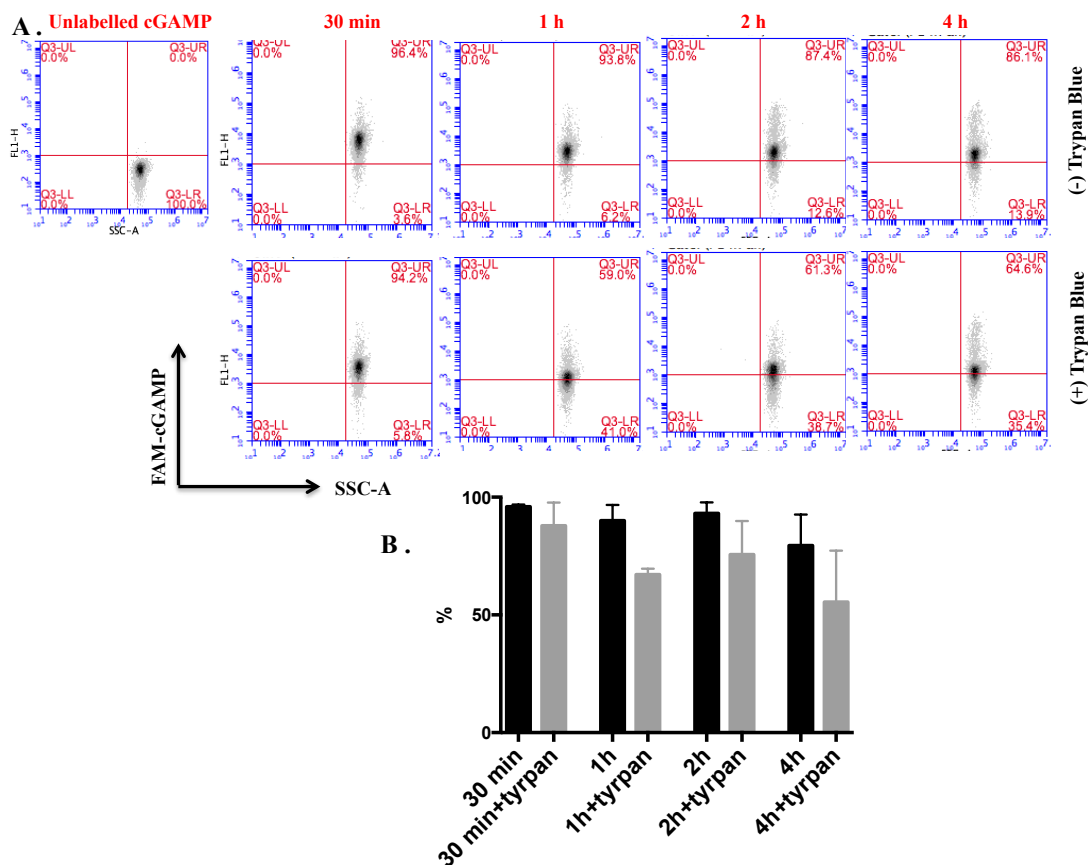


Figure 3.4: Activated Pan T cells internalize cGAMP. (A) Pan T cells were stimulated with cGAMP (2.5 $\mu\text{g/ml}$) or FAM labelled cGAMP (2.5 $\mu\text{g/ml}$) and their fluorescence signals were measured at different time points. (B) Percentage of binding, uptake and internalization of cGAMP. Black bars show binding, uptake and internalization efficiency. On the other hand, gray bars show only internalization efficiency.

3.2.1 cGAMP has a cytotoxic effect on Pan T cells

In the literature, there was no data regarding the effect of cGAMP on T cells. Previous studies focused on its effect on myeloid cells. Since we did not have any prior knowledge, we performed screening experiment. Isolated Pan T cells were stimulated with cGAMP in addition to various TLR ligands for 3 days. Then, cytokines that were mostly secreted by T cells were screened. According to our observation, when cGAMP mixed with different types of TLR ligands, the amount of secreted cytokine dramatically

decreased (Figure 3.5). At the same time, when the cell morphologies were checked under microscope after 3 days, cells stimulated with cGAMP alone or together with TLR ligands were either dead or dying. Thus, we suspected that cGAMP had a cytotoxic effect on T cells and could trigger signaling pathways related with apoptosis.

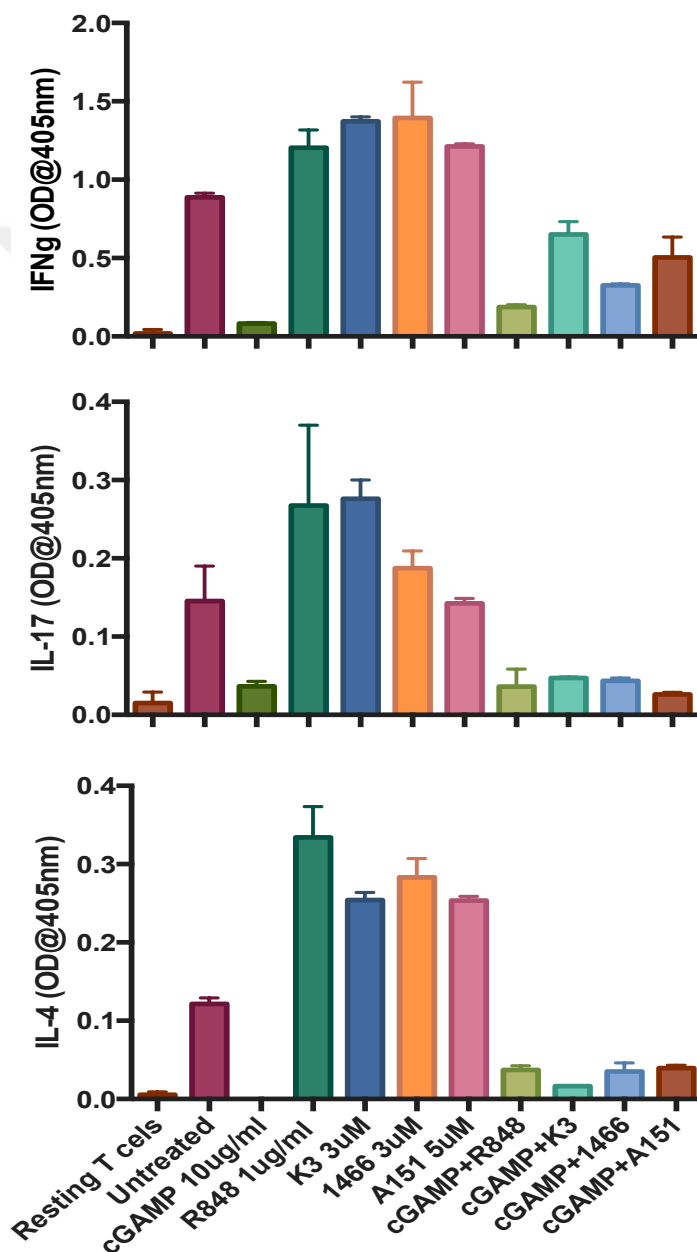


Figure 3.5: Screening of the general effect of cGAMP on Pan T cells. *Pan T cells were stimulated with cGAMP and different PRR ligands for 3 days to screen the effect of cGAMP on Pan T cells. After 3days stimulation, IFNγ, IL-17, and IL-4 ELISA were performed.*

Consequently, we wanted to check the effect of cGAMP on T cells' viability. In order to do that, isolated Pan T cells were stimulated with cGAMP at different concentrations for 3 days. Each day, their viabilities were analyzed by cell counting kit-8. To see whether apoptosis was derived from high concentration of cGAMP, bone marrow derived dendritic cells and RAW cells were used as controls. The results revealed that cGAMP had a dramatic effect on Pan T cell survival. Neither dendritic cells nor macrophages were affected by cGAMP stimulation (Figure 3.6). That was the first and novel finding about cGAMP effect on Pan T cells.

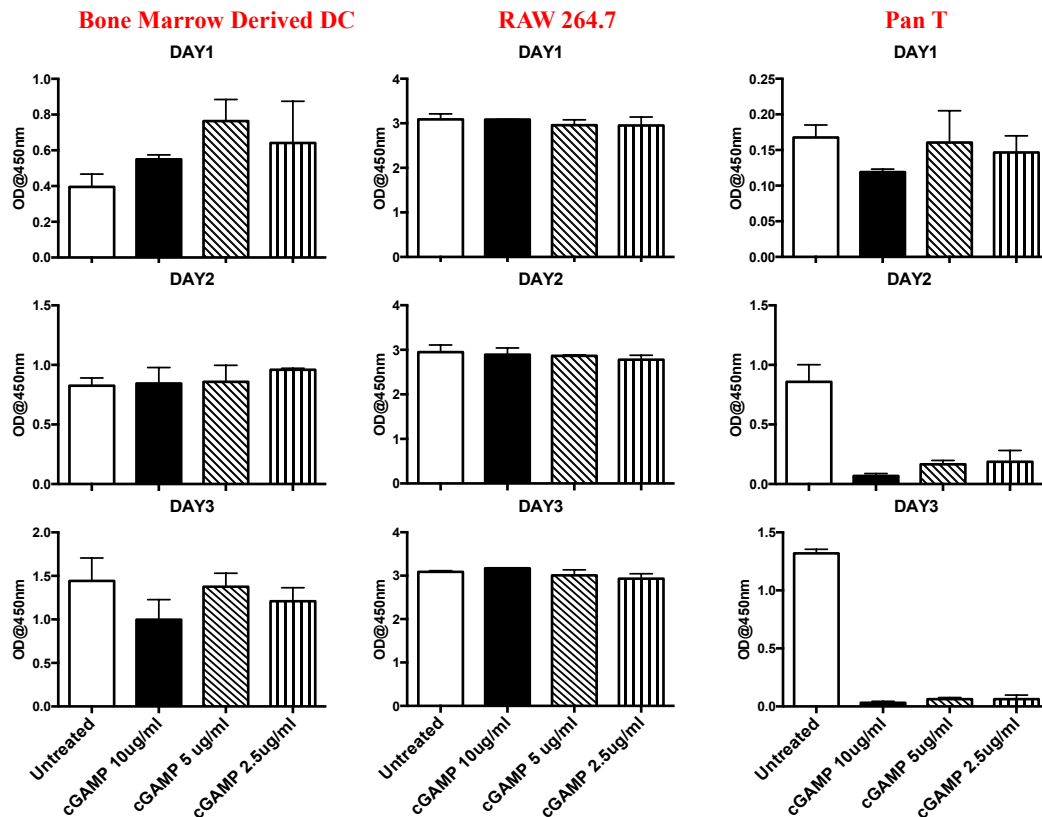
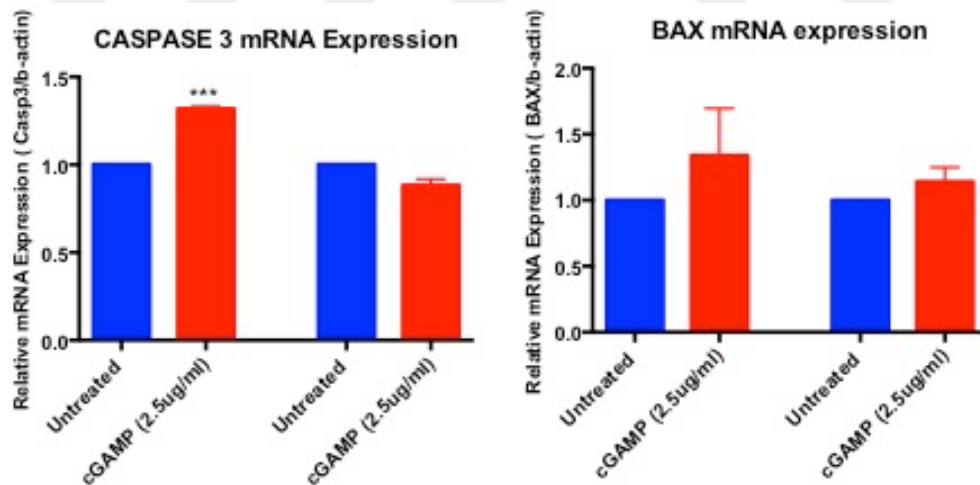


Figure 3.6: Determination of the toxic effect of cGAMP on Pan T cells. Bone marrow derived dendritic cells, RAW cells and Pan T cells were stimulated with cGAMP. For each day, their viabilities were measured by Cell Counting Kit-8.

We observed that 10 and 5 $\mu\text{g/ml}$ concentrations were highly detrimental for Pan T cells. Because of that, we focused on 2.5 $\mu\text{g/ml}$ for further experiments to confirm apoptosis. In order to determine whether cGAMP caused apoptosis in Pan T cells, several assays were performed. First, we attempted to see the change in mRNA levels. 4 and 8 hours after stimulation, the expression levels for caspase-3 and BAX, which are the important players of apoptosis, were determined by qPCR. As we expected, cGAMP stimulation increased the expression of Caspase-3 and BAX (Figure 3.7.A). Beside, those findings were also verified with western blot. As seen in Figure 3.7, cGAMP stimulation caused an increase in protein level of cleaved caspase-3 and cleaved PARP (Figure 3.7.B). Also, there was an increase in phosphorylated IRF-3, which was the downstream element of STING. Although IRF3 phosphorylation was shown in myeloid cells because of cGAMP stimulation in several studies, our study was the first to show IRF3 phosphorylation in T cells upon cGAMP stimulation.



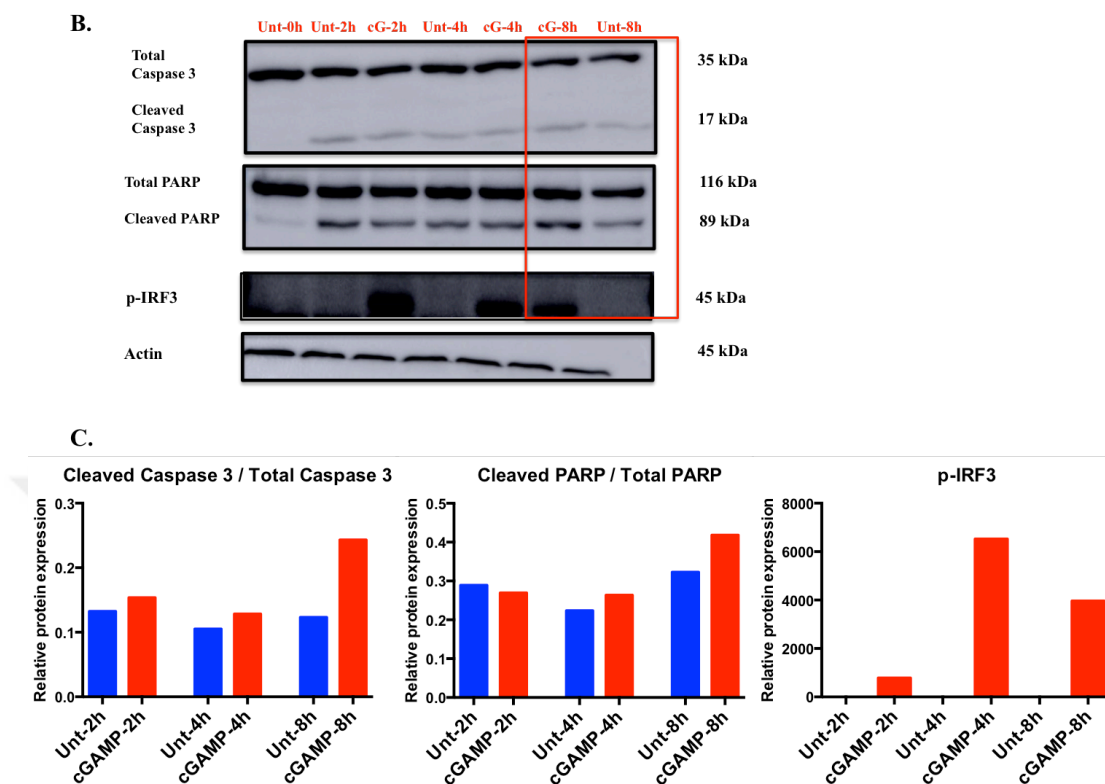


Figure 3.7: cGAMP has an apoptotic effect on PanT cells. (A) Activated T cells were stimulated with cGAMP (2.5 $\mu\text{g/ml}$) to check mRNA expression level of Caspase-3 and BAX at different time points. (B) Activated T cells were stimulated with cGAMP (2.5 $\mu\text{g/ml}$) and their protein expressions were analyzed at different time points. (C) Representative scheme of protein expression analysis after normalization. *** $p < 0.001$

3.2.2 cGAMP triggers IL-17 secretion from Pan T cells

We demonstrated that cGAMP promoted signaling pathways related to apoptosis. Hence, we hypothesized that it could be other functional effects on T cells related with immune response. To check our hypothesis, we decided to perform general screening for the cytokines that are highly secreted by both CD4^+ and CD8^+ T cells. Again, Pan T cells were stimulated with cGAMP for 3 days and ELISA was performed from cell supernatants. According to literature, we could have expected to see an increase in IL-4 secretion since TBK-1 also phosphorylates STAT-6, which triggers Th2 differentiation [105]. However, the results showed that the OD values of $\text{IFN}\gamma$, IL-10 and IL-4 did not

show any change in response to cGAMP. Surprisingly, cGAMP treatment led an increase in the level of IL-17 (Figure 3.8).

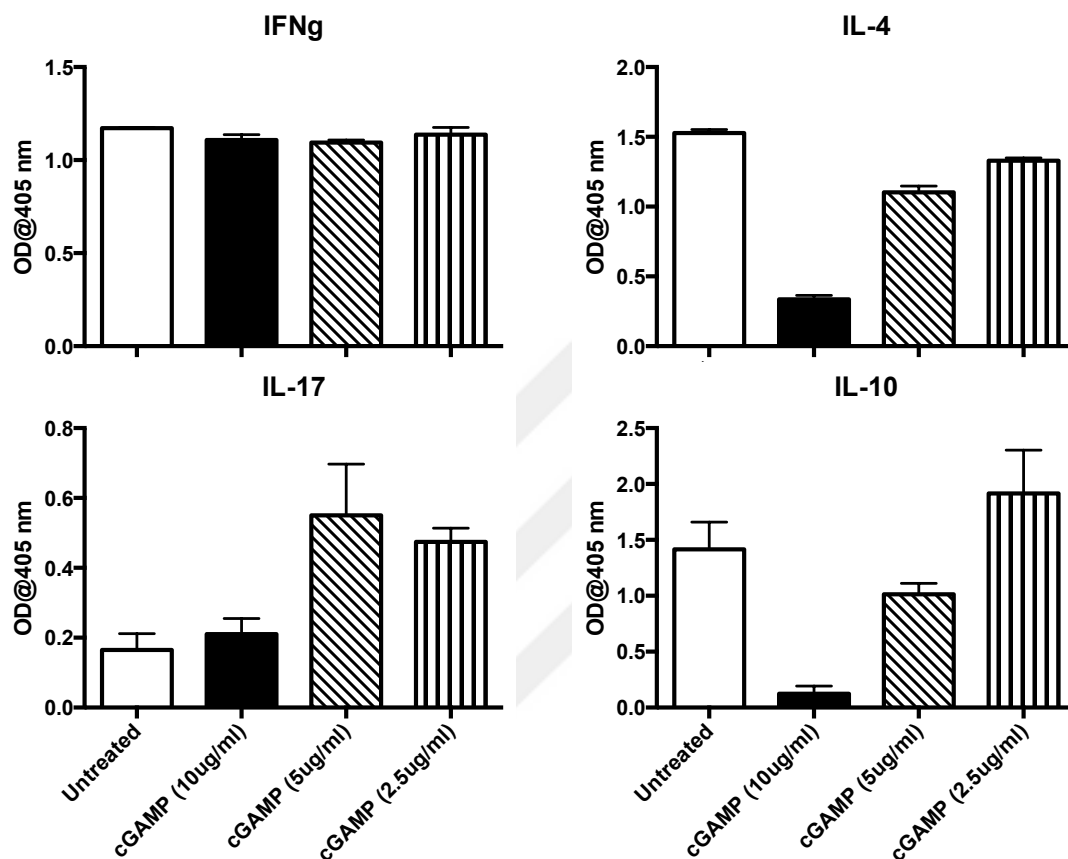


Figure 3.8: Screening for functional analysis of cGAMP on Pan T cells. *Pan T cells isolated from murine spleen were stimulated with cGAMP at different doses for 3 days and ELISA was performed from their supernatants.*

Hereafter, we focused on IL-17 secretion from Pan T cells upon cGAMP treatment. To confirm the secretion of IL-17, Pan T cells were incubated with cGAMP for 3 days. Each day, IL-17 concentration was measured. The concentration differences of IL-17 between untreated and treated samples revealed that at day 2 and 2.5 μ g/ml dose of cGAMP outsourced IL-17 secretion compared to untreated cells. Again, that was an unprecedented finding when existing literature data is considered.

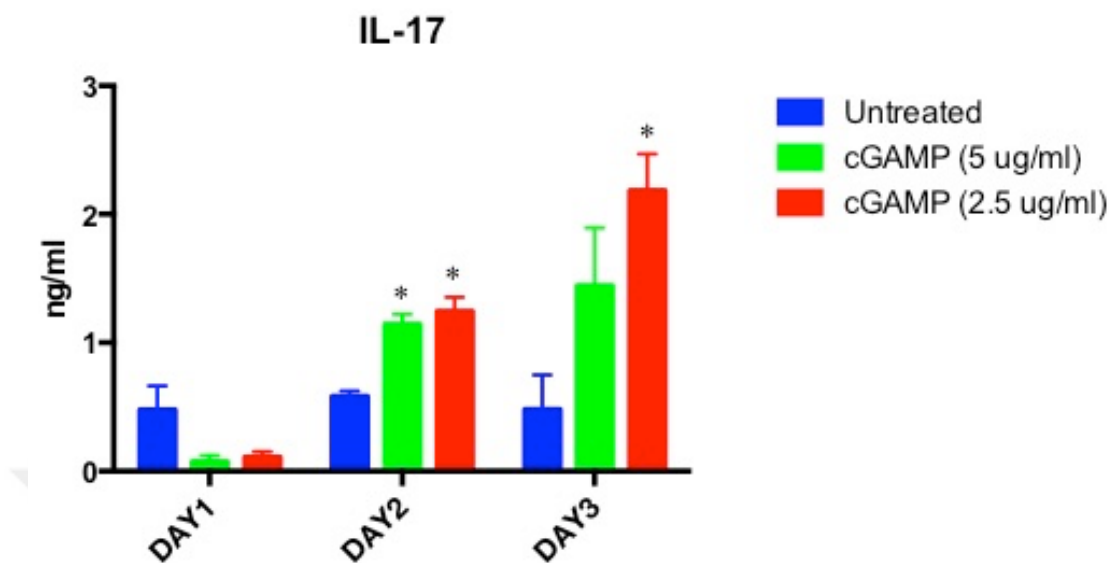


Figure 3.9 cGAMP triggers IL-17 secretion from Pan T cells. *T cells were stimulated with cGAMP at doses 5 and 2.5 $\mu\text{g/ml}$ for 3 days and IL-17 secretion was determined daily from supernatants by ELISA. * $p < 0.05$*

To confirm our ELISA results, the expression level of IL-17 and its transcription factor, RORc were monitored. cGAMP treatment enhanced the expression level of IL-17 and RORc (Figure 3.10.A). Previous studies showed that IL-17 expression was significantly associated with STAT3 phosphorylation [98]. To assess whether IL-17 secretion was linked with STAT3 phosphorylation, we investigated phosphorylation status of STAT3. Consistent with previous findings and literature data, cGAMP treatment indeed induced phosphorylation of STAT3 (Figure 3.10.B). All these findings prompted us to identify the source of IL-17. It is a known fact that the primary source of IL-17 comes from Th17 population among Pan T cells. To differentiate whether IL-17 secretion was related with Th17 subpopulation, the expression levels of Th17 related genes including RORa, SOCS1 and RORc together with intracellular IL-17 were measured. The qPCR results indicated that the expression of all Th17 related genes increased.

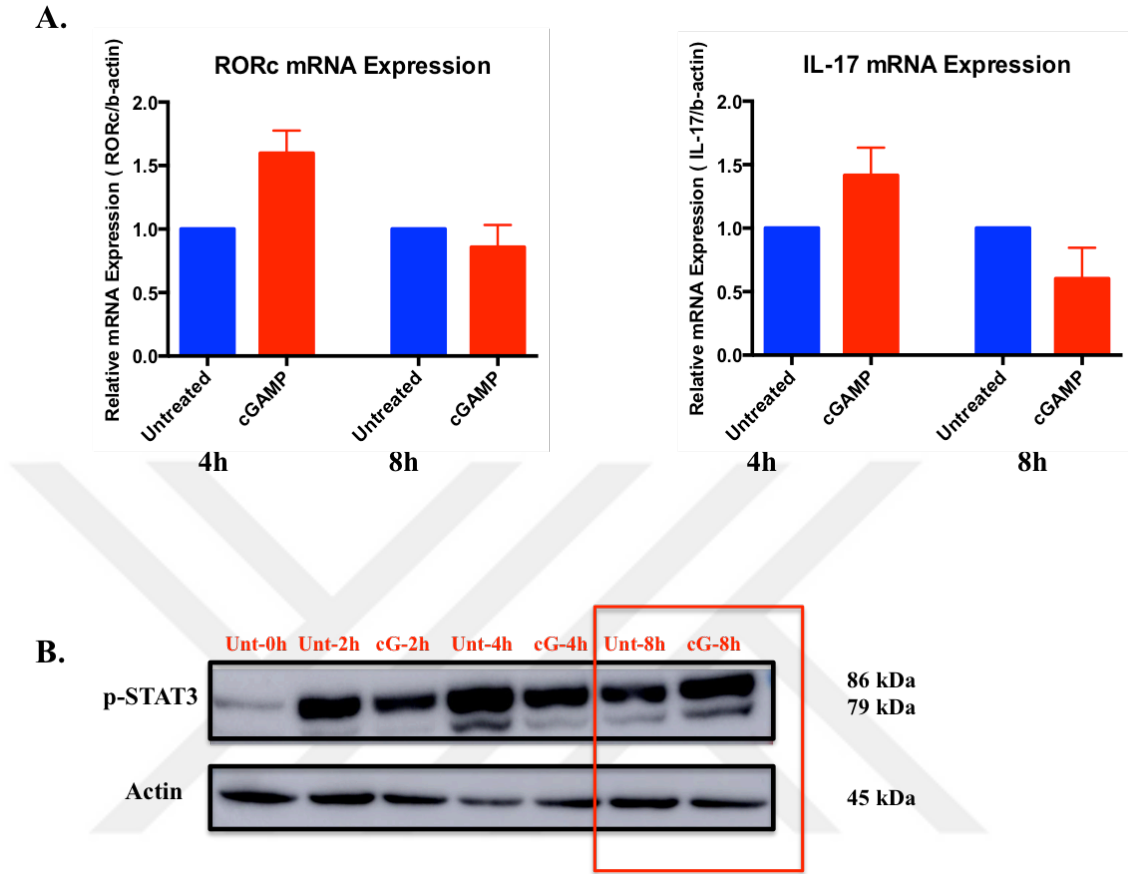


Figure 3.10: The expression of transcription factors related with IL-17 increases in the presence of cGAMP. (A) Isolated Pan T cells were treated with cGAMP (2.5 $\mu\text{g/ml}$) for 4 and 8 hours. Then, gene expression levels for RORc and IL-17 were detected by qPCR. (B) Isolated Pan T cells were treated with cGAMP (2.5 $\mu\text{g/ml}$) for 2, 4 and 8 hours. Phospho STAT3 protein levels were analyzed by western blot.

To determine the expression level of Th17 related genes did not state that only CD4^+ T cells were responsible from IL-17 secretion. We observed that both CD4^+ and CD8^+ T cells significantly enhanced IL-17 secretion upon cGAMP treatment (Figure 3.12). The message transcripts peaked by 36h of incubation with cGAMP and sustained by 48h according to our findings (Figure 3.11 and 3.12). To date there was no report describing the generation of IL17^+ T-cells upon cGAMP treatment. When taken together, our findings strongly suggested that cGAMP treatment of Pan T-cells induces IL17 from both CD4^+ and CD8^+ T-cells subpopulations.

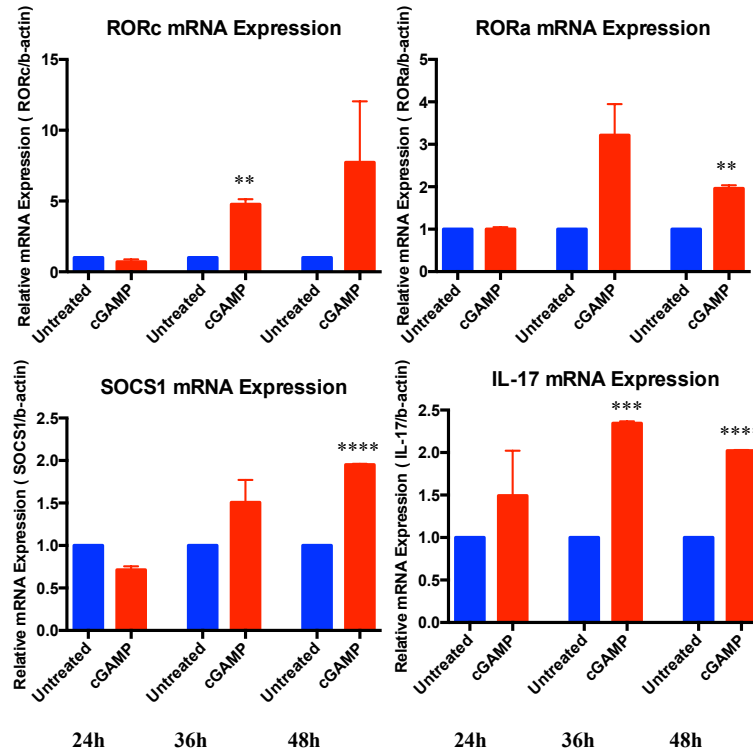


Figure 3.11: cGAMP induces expression of Th17 related genes in Pan T cells. *Isolated Pan T cells were stimulated with cGAMP (2.5 μ g/ml). Th17 related gene expression analyses were done at different time points. . **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$*

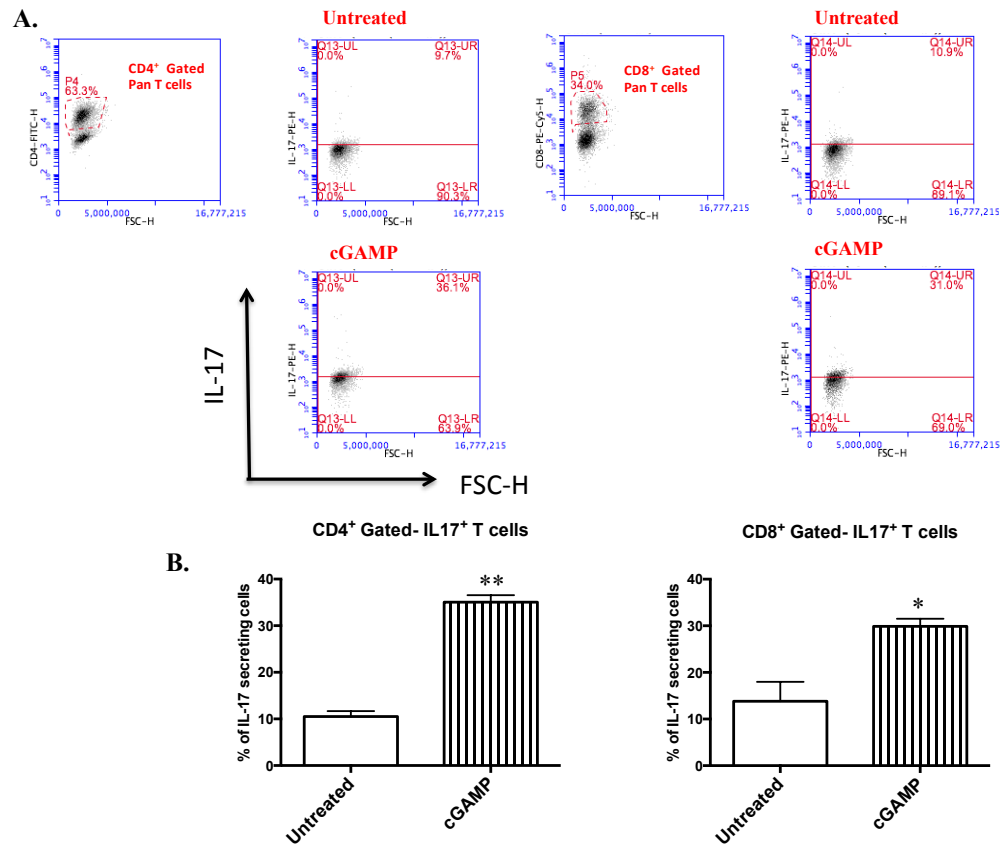


Figure 3.12: IL-17 secretion is from both CD4⁺ and CD8⁺ T cells in the presence of cGAMP.

(A) Pan T cells were stimulated with cGAMP for 3 days. After 3 days, cells were stimulated with Brefeldin A, PMA and Ionomycin for 5 h and then, stained with anti-CD4, CD8 and IL-17. (B) Percentage of IL-17 secreted CD4⁺ and CD8⁺ T cells. . ** $p < 0.01$, * $p < 0.05$

Interestingly and repeatedly during flow cytometry analyses, we unexpectedly observed that CD4⁺/ CD8⁺ ratio of Pan T-cell population drastically increased upon cGAMP treatment. This rise started at day 2 and persisted at day 3 (Figure 3.13).

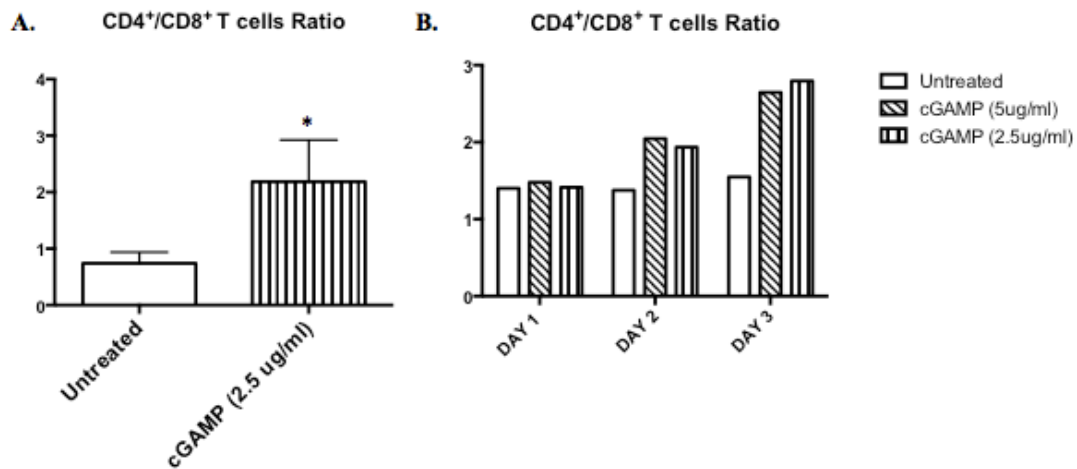


Figure 3.13: cGAMP increases CD4⁺/CD8⁺ ratio of Pan T cells. (A) Pan T cells were stimulated with cGAMP for 3 days. At the end of the 3 days, cells were stained with anti-CD4 and anti-CD8 antibodies to detect the ratio of CD4⁺ vs CD8⁺ T cells by flow cytometry (B) Time course change in the ratio of CD4⁺ and CD8⁺ T cells. * $p < 0.05$

This relative increase in the CD4⁺/CD8⁺ ratio was primarily due to the reduction of CD8⁺ T cell number rather than the increase in CD4⁺ T cells. From all these findings regarding the functional assays, we reached a conclusion that CD4⁺ T cells, especially Th17⁺ sub-population survive while CD8⁺ T cells were dying in the presence of cGAMP. To test this possibility, subsequent assays were performed only with CD4⁺ T-cells.

3.3 Efforts to understand the effect of cGAMP on CD4⁺ T cells

Since we could not differentiate which type of T cells internalized cGAMP in previous experiments, internalization studies were conducted for CD4⁺ T cells. First, CD4⁺ T cells were magnetically isolated by CD4⁺ MACS isolation kit and their purity was measured (Figure 3.14). The yield of purity after MACS separation was found to be over 94.7±2.62%.

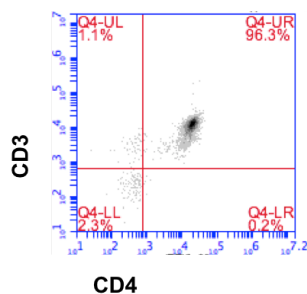


Figure 3.14: Isolation efficiency of CD4⁺ T cells. CD4⁺ T cells isolated from murine spleen were stained with CD3/CD4 to check efficiency of the isolation and the purity of cells. A representative plot following purification is presented

Internalization kinetics for a period of 4h was then investigated with these CD4⁺ T-cells. The results showed that FAM⁺ CD4⁺ T cells could easily bind and internalize cGAMP (Figure 3.15). As early as 30 mins of post-incubation with cGAMP, over 90% of T-cells were positive for cGAMP, of these, over 80% internalized cGAMP (Figure 3.15 A and B).

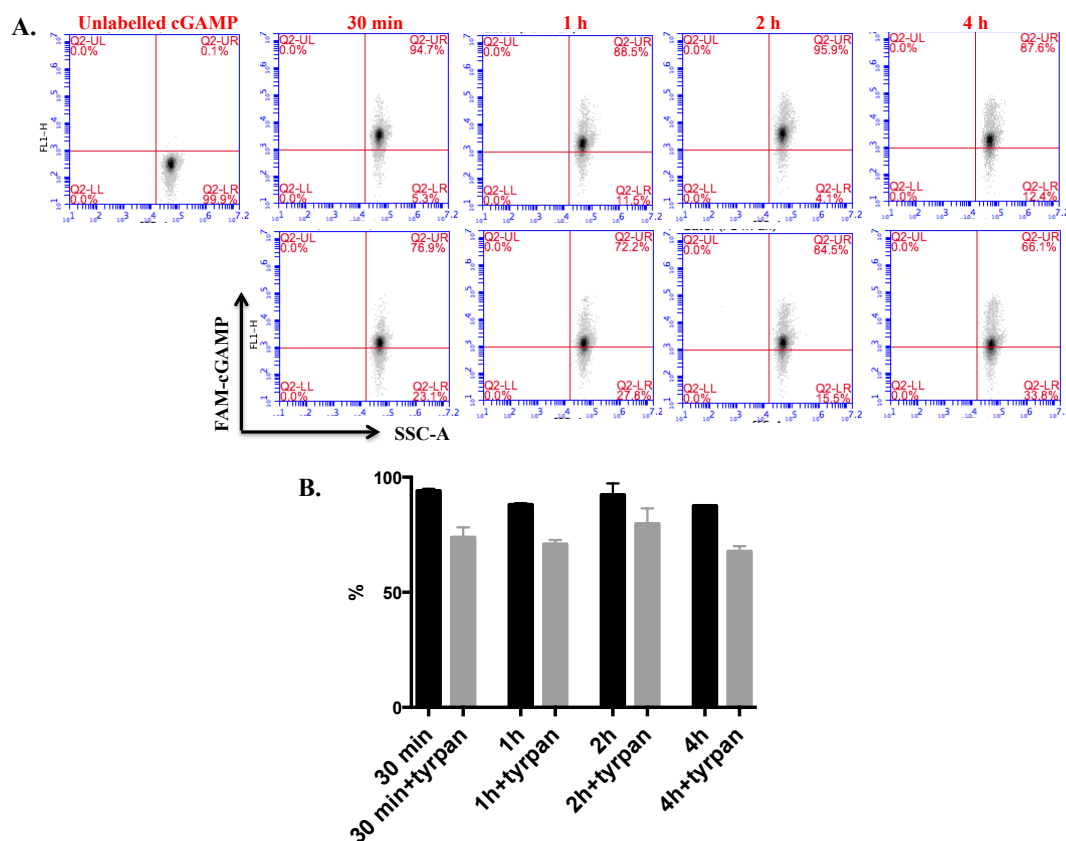
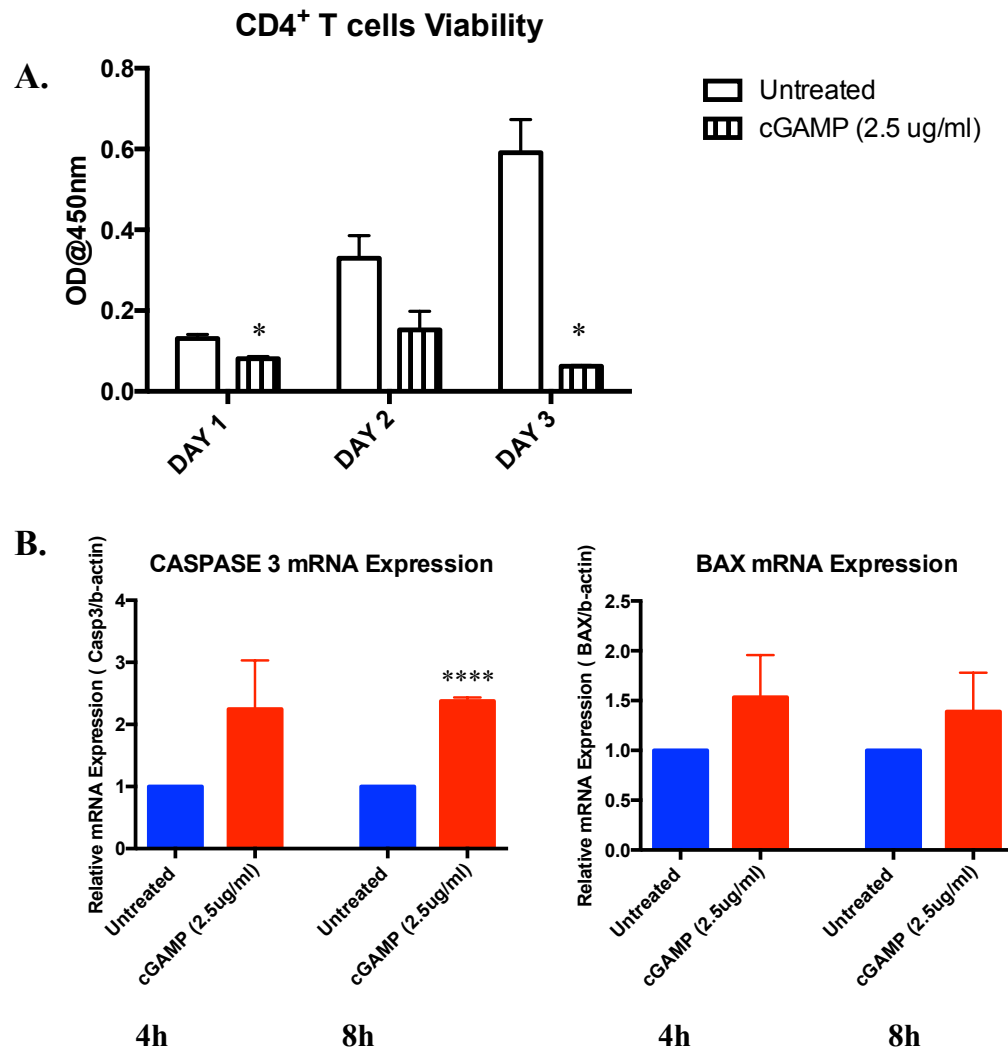


Figure 3.15: Activated CD4⁺ T cells internalize cGAMP. (A) CD4⁺ T cells were stimulated with cGAMP (2.5 μ g/ml) or FAM labeled cGAMP (2.5 μ g/ml) and their fluorescence signals were measured at different time points. (B) Percentage of binding, uptake and internalization of cGAMP. Black bars show binding, uptake and internalization efficiency. On the other hand, gray bars show only internalization efficiency.

3.3.1 cGAMP has a cytotoxic effect on CD4⁺ T cells

Next, we attempted to check whether cGAMP triggered a detrimental effect on CD4⁺ T cells viability. We did not anticipate that a significant degree of death would happen, since previous finding suggested that more CD8⁺ T cells undergo apoptosis with cGAMP than CD4⁺ T-cells. Contrary to our expectations our results pointed out that, the viability of cGAMP stimulated CD4⁺ T cells also goes down (Figure 3.16.A). In addition to that, the mRNA levels of Caspase 3 and Bax increased with cGAMP (Figure 3.16.B). As we

experienced in Pan T stimulations, the protein levels of cleaved caspase-3 and cleaved PARP augmented upon cGAMP treatment in CD4⁺ T cells. Moreover, IRF3 phosphorylation in CD4⁺ T cells was elicited in the presence of cGAMP starting with 2h of stimulation and persisted by the end of 8h of ligand treatment (Figure 3.16.C).



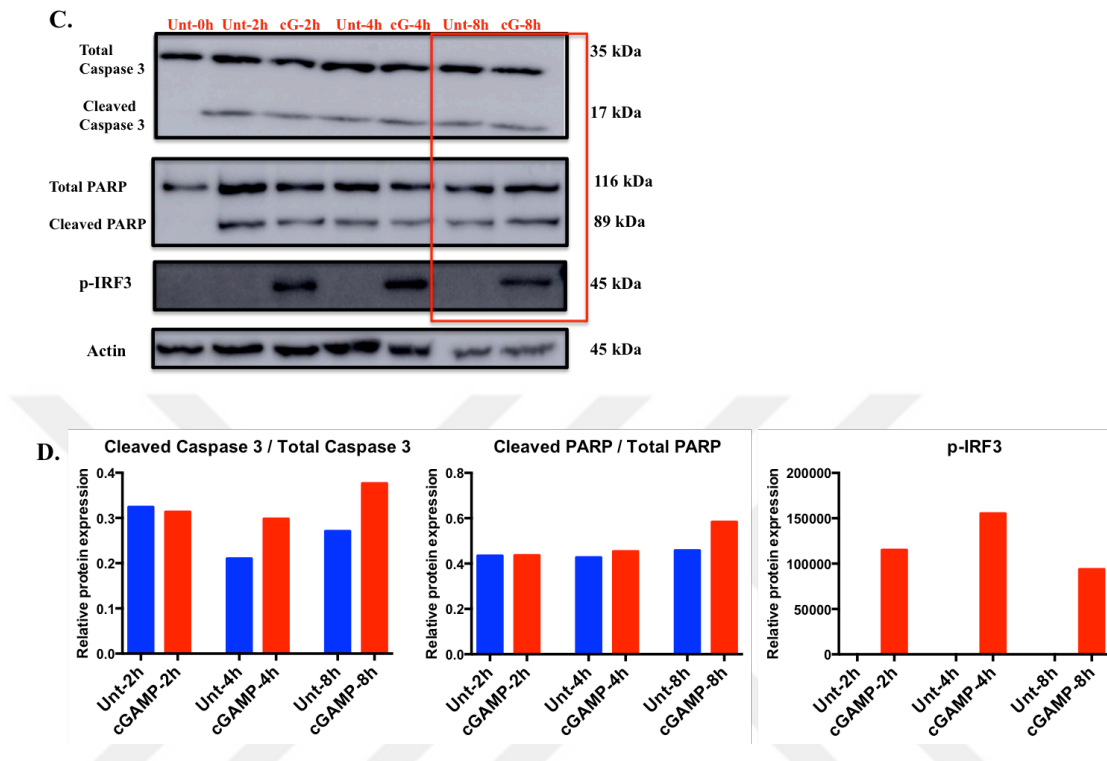


Figure 3.16: cGAMP induces apoptosis of CD4⁺ T cells. (A) CD4⁺ T cells isolated from murine spleen were treated with cGAMP and their viabilities were measured for 3 days by cell counting kit-8. (B) RNA isolation was performed from CD4⁺ T cells exposed to cGAMP for 4 and 8 hours. Then, gene expression levels for Caspase-3 and BAX were determined by qPCR. (C) Proteins were isolated from CD4⁺ T cells exposed to cGAMP for 2,4 and 8 hours. Apoptosis related proteins were determined by western blot. (D) Representative graph for protein expressions after normalization. . **** $p < 0.0001$

3.3.2 cGAMP did not trigger IL-17 secretion from CD4⁺ T cells

In the light of the findings about Pan T cells, we hypothesized that cGAMP treatment enhanced the differentiation and proliferation of Th17⁺ cells. First, we screened helper T cells related cytokines as we did for Pan T cells. We did not observe any increase in IL-17 secretion together with other cytokines in the presence of cGAMP (Figure 3.17).

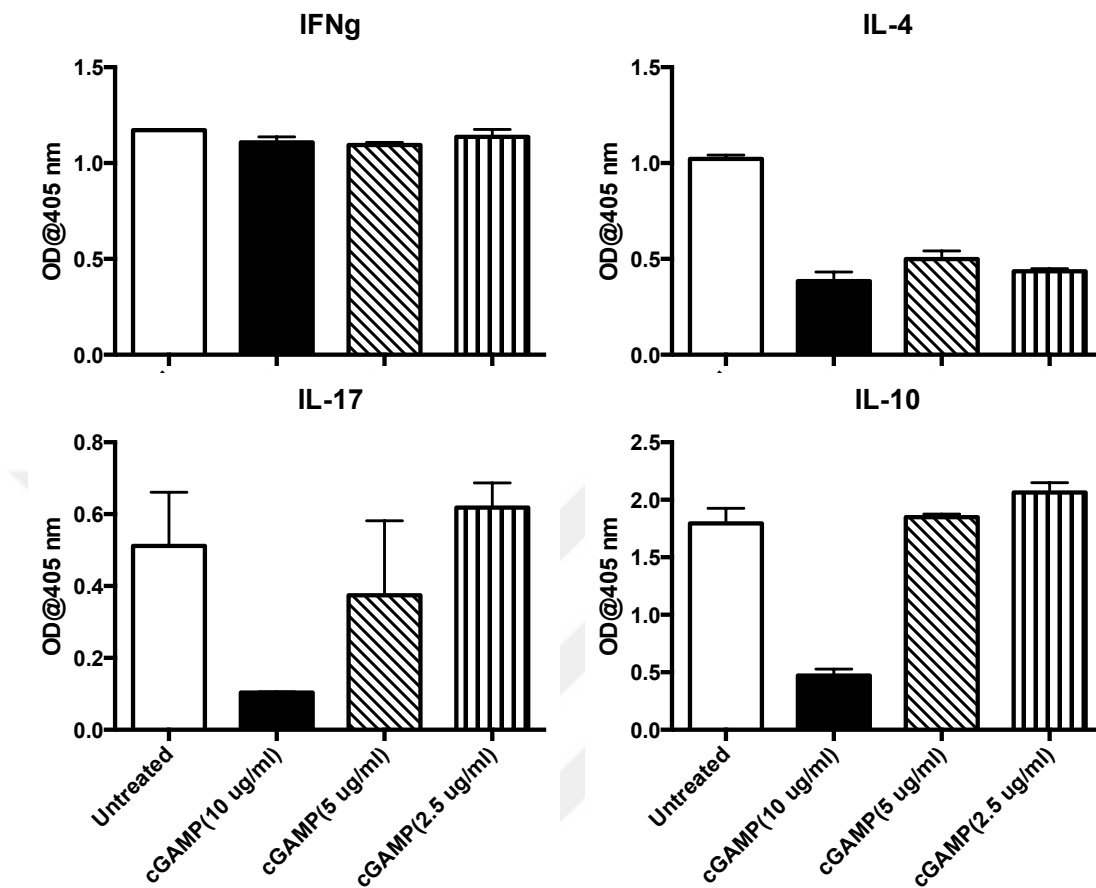


Figure 3.17: Screening for functional analysis of cGAMP on CD4⁺ T cells. CD4⁺ T cells isolated from murine spleen were stimulated with cGAMP at different doses for 3 days and ELISA was performed from their supernatants

Then, IL-17 concentrations were measured 3 days after stimulation, there was no significant change between untreated and cGAMP treated CD4⁺ T cells (Figure 3.18).

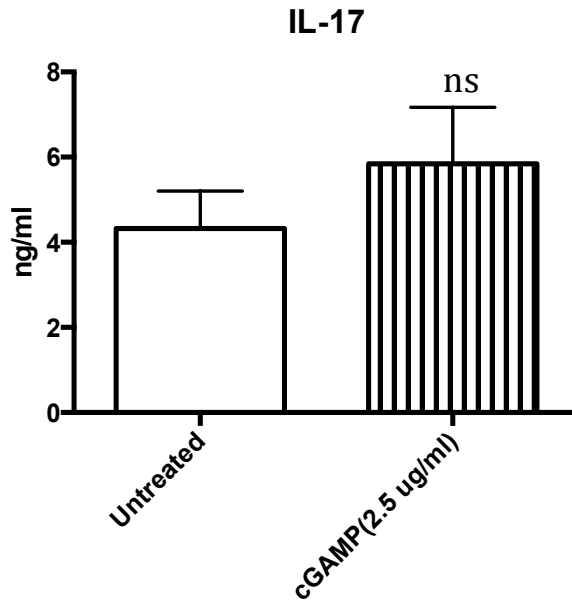
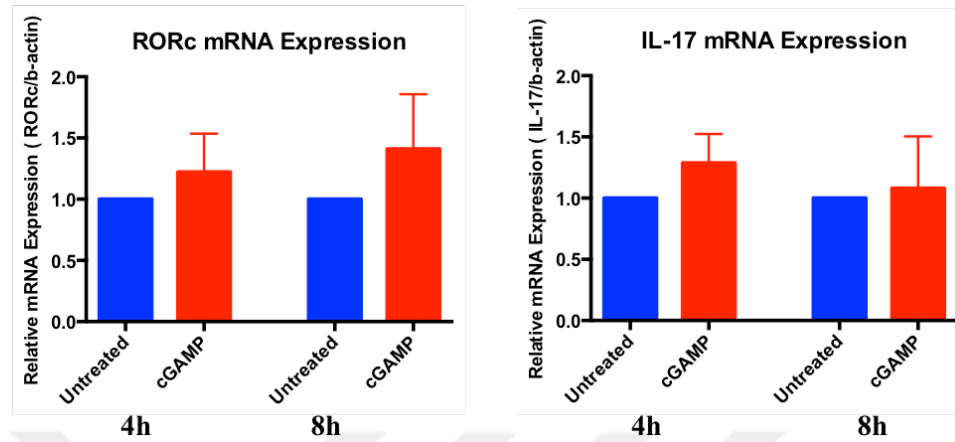


Figure 3.18: cGAMP fail to induce IL-17 secretion from CD4⁺ T cells. CD4⁺ T cells isolated from murine spleen were treated with cGAMP (2.5 µg/ml) for 3 days and IL-17 concentration was measured from supernatants.

Unexpectedly, neither gene expression levels of RORc nor IL-17 and western blot of phospho STAT3 increased by cGAMP (Figure 3.19). Lastly, intracellular IL-17 levels were analyzed. Similarly, cGAMP did not significantly induce IL-17 secretion from CD4⁺ T cells (Figure 3.20). Although there was a substantial increase in IL17 production in response to cGAMP treatment, this change was not significant between treatment groups (Figure 3.20). As a conclusion, our hypothesis about CD4⁺T cells in response to cGAMP treatment was not verified in these sets of experiments and we abandoned this set of work.

A.



B.

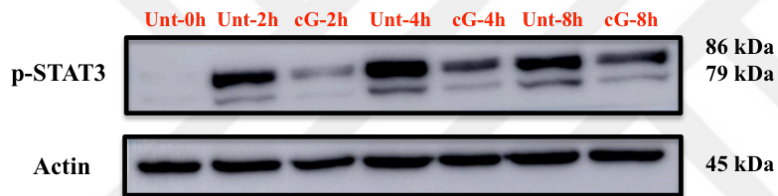


Figure 3.19: cGAMP treatment fail to elicit expression levels of IL-17 and IL-17 related transcription factors in CD4⁺ T cells. (A) Isolated CD4⁺ T cells were treated with cGAMP (2.5 µg/ml) for 4 and 8 hours. Then, gene expression levels for RORc and IL-17 were detected by qPCR. (B) Isolated CD4⁺ T cells were treated with cGAMP (2.5 µg/ml) for 2,4 and 8 hours. Phospho STAT3 protein levels were analyzed by western blots.

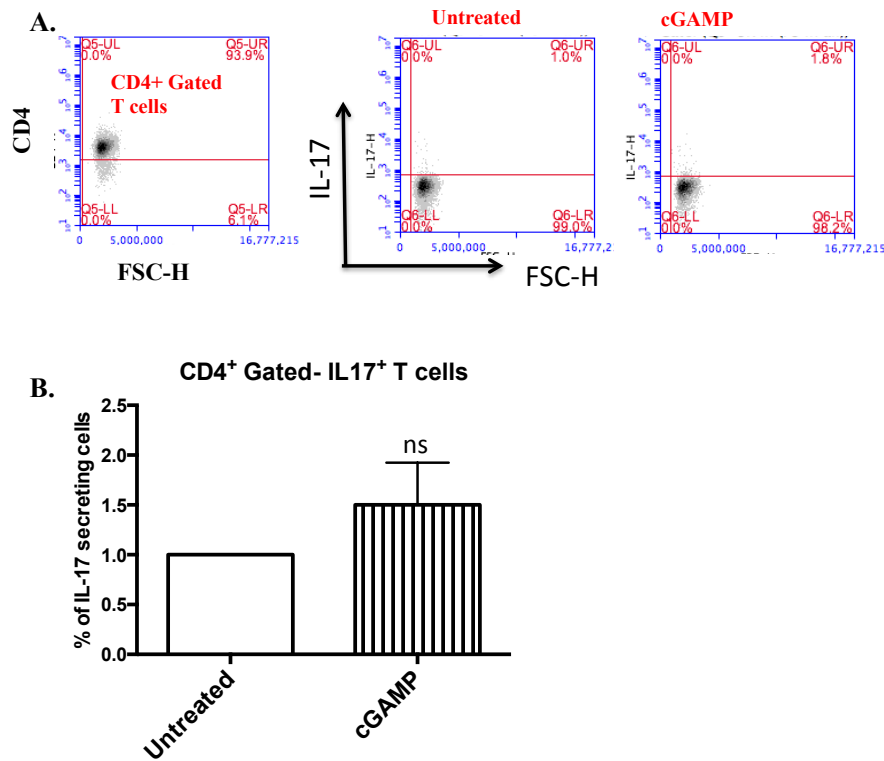


Figure 3.20: IL-17 secretion in CD4⁺ T cells by cGAMP. (A) CD4⁺ cells were stimulated with cGAMP for 3 days. After 3 days, cells were stimulated with Brefeldin A, PMA and Ionomycin for 5 h and then, stained with anti-CD4, and IL-17. (B) Percentage of IL-17 secreted CD4⁺ T cells.

3.4 Alternative approaches to decrease cytotoxic effect of cGAMP while maintaining IL-17 secretion

The next step was to find an alternative way that would reduce the apoptotic effect of standalone cGAMP administration while maintaining IL-17 secretion. Since cGAMP led to phosphorylation of IRF3, we postulated that there was a link between phosphorylation of IRF3 and IL-17 secretion through STAT3. Therefore, we sought to utilize another interferon inducer, namely R848, which is a TLR7/8 ligand and promotes IRF3 phosphorylation. We once again purified Pan T-cells and stimulated them with cGAMP

(2.5 $\mu\text{g/ml}$) in the presence and absence of R848. We observed that R848 inclusion diminished the detrimental effect of cGAMP at day 3 and 4 (Figure 3.21).

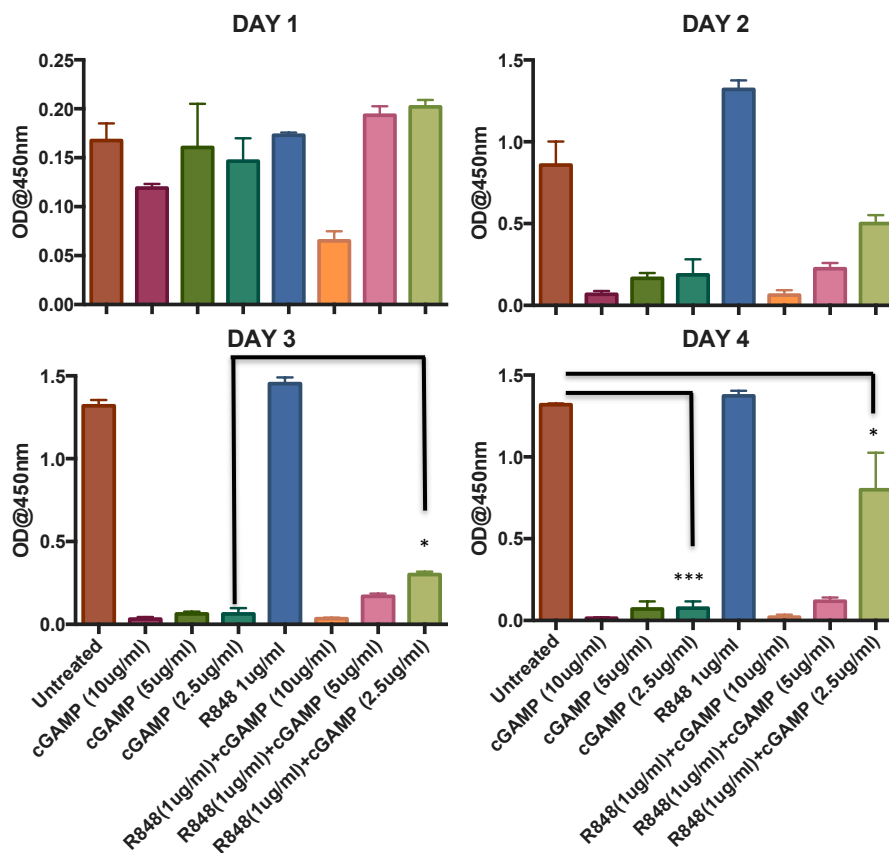


Figure 3.21: TLR 7/8 ligand, R848 diminishes the cytotoxic effect of cGAMP in Pan T cells. Pan T cells isolated from murine spleen were treated with cGAMP at different doses alone or together with R848 for 4 days. Their cell viabilities were measured for each day. . *** $p < 0.001$, * $p < 0.05$

Strikingly, R848 maintained IL-17 secretion from Pan T cells (Figure 3.22). However, although IL-17 secretion was continued, the expression levels of Th17 related genes except IL-17 itself decreased, unexpectedly (Figure 3.23). Maybe, R848 could have an impact on CD8⁺ T cells.

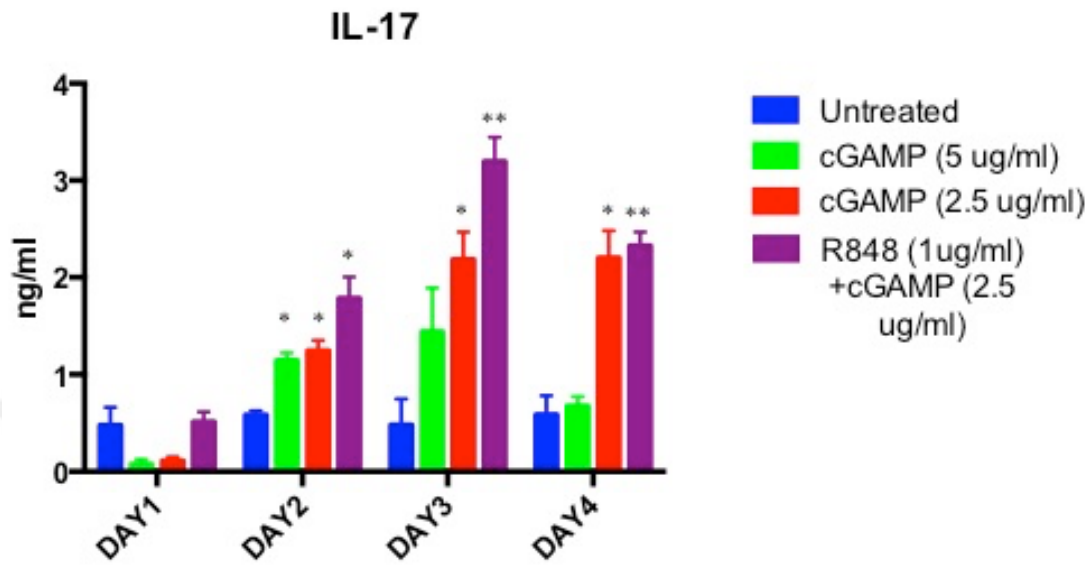


Figure 3.22: TLR 7/8 ligand, R848 improves IL-17 secretion from Pan T cells in the presence of cGAMP. *Pan T cells isolated from murine spleen were treated with cGAMP alone or together with R848 for 4 days. IL-17 secretions were determined for each day by ELISA. . ** $p < 0.01$, * $p < 0.05$*

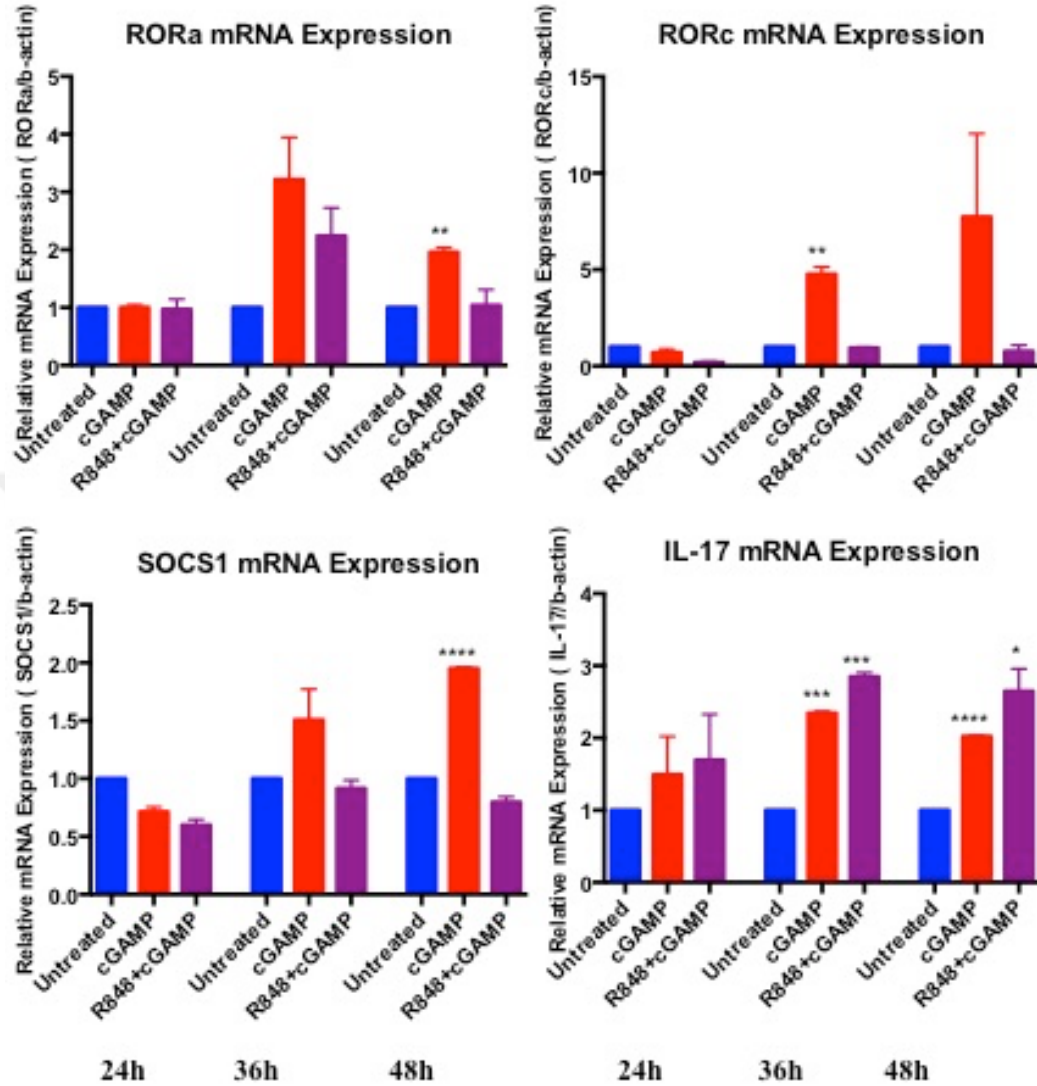
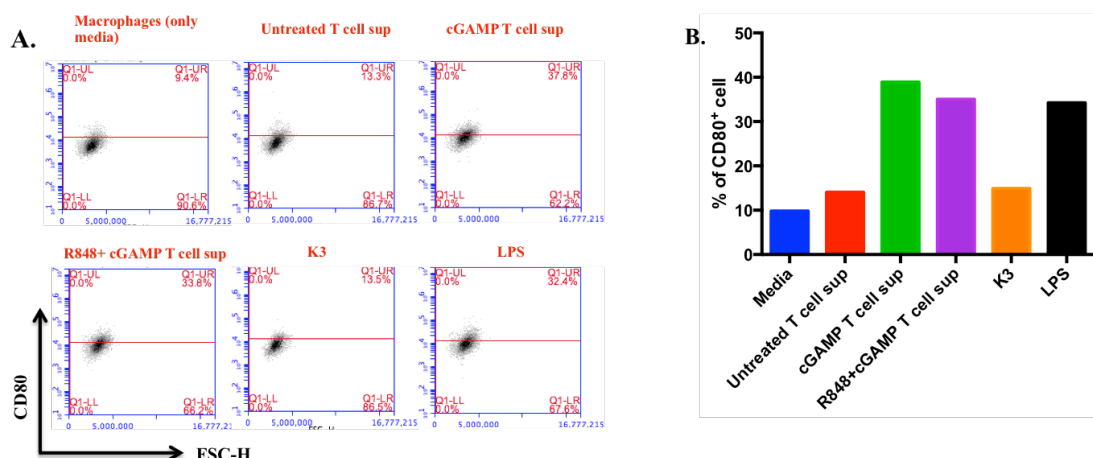


Figure 3.23: TLR7/8 ligand, R848 decreases the expression of Th17 related genes while maintaining IL-17 expression. Isolated Pan T cells were stimulated with cGAMP (2.5 µg/ml) alone or together with R848 (1 µg/ml). Th17 related gene expression analyses were done at different time points. **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$

3.5 Effect of cGAMP treated Pan T cells on Macrophages

In the last part of this thesis, we planned to investigate whether IL-17 secreted from Pan T cell upon cGAMP stimulation could activate macrophages. If this activation could be established, it could uncover a novel function of cGAMP in adjuvant therapies. For that, RAW cells were stimulated with media from Pan T cell stimulated for 24 h. As positive controls, K3 (a TLR9 ligand) and LPS (a TLR4 ligand) were used. After 24h, the activation statuses of the RAW cells were monitored by flow cytometer. Media from T cells stimulated with cGAMP alone and together with R848 activated macrophages at the level comparable to direct LPS induction (Figure 3.24). Moreover, although we could not observe any increase in the percentage of MHCII⁺ macrophages, MFI value of MHCII positivity also increased in macrophages treated with media from cGAMP stimulated T cells compared with media alone and media from untreated T cell (Figure 3.24.C/D/E).



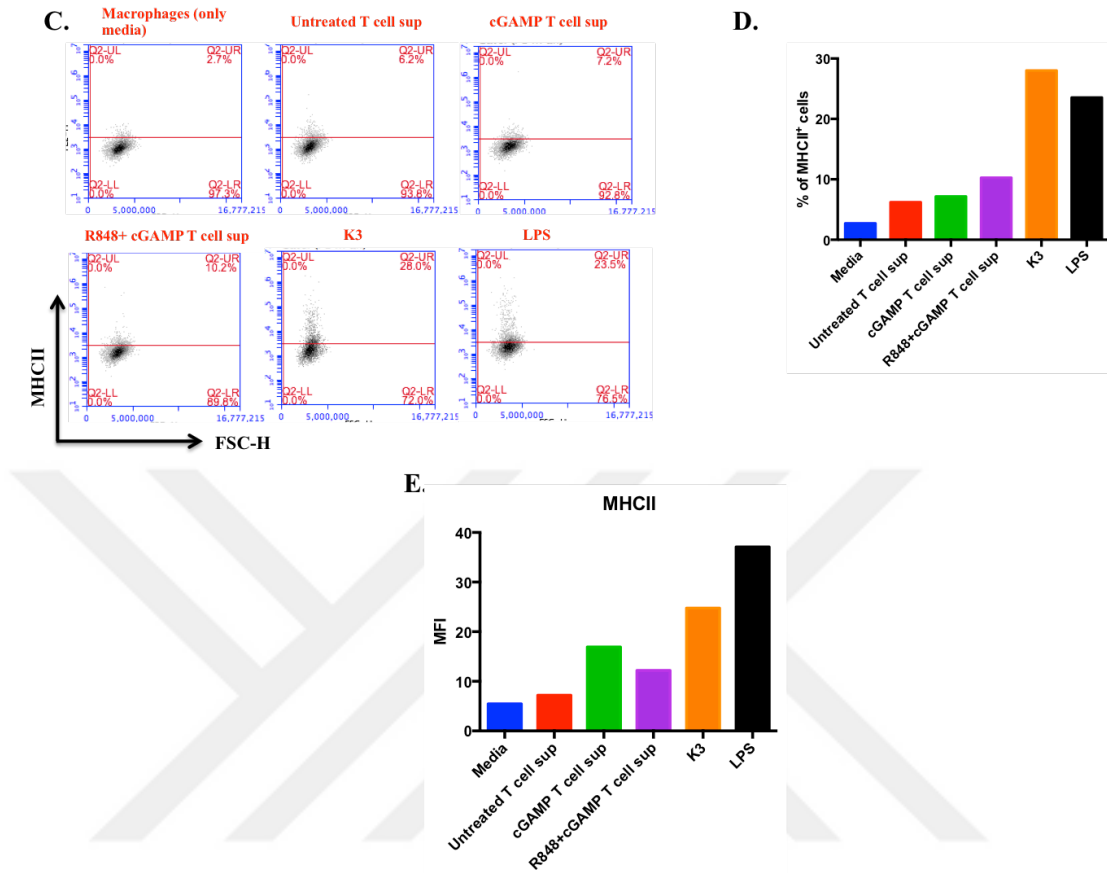


Figure 3.24: Condition media from cGAMP stimulated T cell activate macrophages. (A) RAW cells were stimulated with supernatants from Pan T cell stimulation and incubated for 24 hours. As a control, K3 and LPS were used. After 24 hours, Anti-CD80 staining was performed by flow cytometry. **(B)** Percentage of CD80⁺ RAW cells. **(C)** Dot plots showing MHCII⁺ population of macrophages upon conditioned media treatment for 24h. **(D)** Percentages of MHCII⁺ cells. **(E)** MFI ratios for MHCII staining.

These findings for the first time suggested that one of the STING ligands, cGAMP, not only activates T-cells to trans-differentiate to Th17 cells, but also contributes to macrophage activation. This observation alone is a novel finding in the cytosolic sensor STING mediated immune activation and could shape the breadth of application ranging from vaccine development to immunotherapeutic application. On the other hand, the cytotoxic potential of cGAMP usage on T-cells could be another finding to be considered

and might be important for the utilization of cGAMP or other cytosolic STING sensors in immune modulation.



Chapter 4

Discussion

Type I interferons (IFN- α/β) play a significant role not only in the protection against viral infectious but also in the progression of bacterial infectious, cancer and autoimmunity. Beside, they behave like a bridge between innate and adaptive immunity [99]. The activation of cytosolic receptors including TLRs, RLRs and cytosolic DNA sensors mostly result in the secretion of type I interferons by inducing IRF pathway. Secretion of type I interferons enhances the activation of macrophages, B cell switching mechanism and the differentiation of helper T cells into Th1 [100]. STING is the essential mediator for the recognition of cytosolic dsDNA, which triggers the production of type I interferon via TBK1-IRF3 pathway. STING can recognize dsDNA directly or sense the cyclic dinucleotides, which are secreted by pathogens. Beside, cytosolic ATP and GTP are converted into cGAMP upon DNA binding to cGAS enzyme and cGAMP interacts with STING and triggers the production of type I interferons, mostly IFN- β [17,18]. Since it has been shown that type I interferons are the key modulators to fight against bacterial, viral infectious and malignancies, STING activating cyclic di-nucleotides such as cGAMP are thought to be effective candidates for vaccine adjuvants. Although they have an enormous potential as vaccine adjuvants, a great deal of research is needed in terms of immune response and cytotoxicity to initiate human clinical trials [101]. Nevertheless, studies performed so far have focused the effect and the function of cGAMP in innate immune system. Its role in adaptive immune system still remains unclear. Hence, we

aimed to investigate the effect and the function of cGAMP in T lymphocytes to contribute the researches involving the vaccine adjuvant potency of STING activating dinucleotides.

Before investigation of the role of cGAMP, the culture conditions for primary T cells were optimized since proliferation of cells is a vital part to initiate an appropriate immune response. Magnetically isolated Pan T cells were stimulated with different antibodies and chemicals for their proliferation and activation. We observed that anti-CD3/CD28 is the most potent proliferative and activator condition compared with others (Figure 3.2). Trickett and her colleagues also support this observation by showing that anti-CD3/CD28 stimulation is more close to physiological situation for the in vitro proliferation and activation of T cells [94]. After that, anti-CD3/CD28 was used for the experiments involving T cells for their proliferation and activation unless indicated otherwise. Beside culture conditions, we had to be sure the expression of STING and the internalization of cGAMP by T cells before functional analysis. We saw that STING was expressed in T cells as well as macrophages did (Figure 3.3). Then, the internalization experiment showed that most of the cGAMP were internalized within 30 minutes (Figure 3.4). Yet, we could not see kinetic progression. It was most likely because of the half-life of STING. Upon cGAMP binding to STING, they are embedded into endosomes by moving out from endoplasmic reticulum and then degraded [102]. There are continuous activation and degradation cycles. That's why we could not observe kinetic internalization. After we showed that STING was expressed in T cells and could be activated by cGAMP which is easily internalized by T cells, our first strategy to understand the effect of cGAMP stimulation in T cells was to check T cell specific cytokines upon stimulation of Pan T cells with cGAMP and/or various TLR ligands. Surprisingly, when cGAMP mixed with TLR ligands, the secretion of cytokines including IL-4, IFN- γ and IL-17 dramatically reduced compared with T cells stimulated with TLR ligands (Figure 3.5). In addition to that, states of cells were checked on microscope after the stimulation. T cells stimulated with cGAMP and/or TLR ligands were dead or dying. To ensure that cGAMP stimulation caused an apoptosis in T cells, cell viability assay was performed by using different doses

of cGAMP. On the other hand, previous studies showed that cGAMP has no toxic effect on dendritic cells or macrophages. Our results also supported literature and showed that cGAMP is toxic only for T cells. Neither dendritic cells nor macrophages were affected from cGAMP stimulation (Figure 3.6). That was highly unexpected. Next, we wanted to clarify whether cGAMP causes an apoptosis in Pan T cells. Our results demonstrated that cGAMP treatment raised mRNA level of caspase-3 and Bax (Figure 3.7.A). Beside, cleaved caspase-3 and cleaved PARP, which are the fundamental apoptosis markers, increased in T cells stimulated with cGAMP. In addition to apoptosis, our study was the first to show IRF-3 phosphorylation in T cells upon cGAMP treatment (Figure 3.7.B). Although there is no study or data which relates cGAMP effect on apoptosis in T cells, Chattopadhyay et. al. demonstrated that there is a novel IRF3 activation pathway which induces apoptosis by interacting with pro-apoptotic protein Bax [103]. Beside, during this study, Tang and his colleagues showed that cGAMP caused an apoptosis in B cells via ER stress [104]. Hence, the effect of apoptosis in our study may be derived from those pathways. Observing apoptosis and IRF3 phosphorylation brought to our minds whether cGAMP had a functional effect such as differentiation on T cells. When helper T cell specific cytokines were scanned, IL-17 drastically increased with cGAMP stimulation (Figure 3.9). That was highly unforeseen because we had hypothesized that cGAMP might lead Th2 differentiation since it has been shown that TBK-1 phosphorylates STAT6 which is an important regulator for Th2 [105]. However; Zhang et. al., recently showed that TBK-1 is crucial to maintain continuous activation of STAT3 [106]. This study makes our work meaningful since STAT3 is an important regulator for IL-17 secretion and Th17 cells. We then checked the mRNA level of transcription factor of IL-17, ROR-c and IL-17 at early time points. As we expected, the expression of RORc and IL-17 increased in cGAMP stimulated Pan T cells (Figure 3.10.A). To understand whether those events were related with STAT3, phosphorylation status of STAT3 was examined. cGAMP stimulation raised phosphorylation of STAT3 (Figure 3.10.B). Since IL-17 mostly come from Th17 cells, the expression level of Th17 related genes including RORa and SOCS1 with RORc and IL-17 were examined at late time points. The mRNA levels of RORa and SOCS1 raised at 48h while RORc and IL-17 were increasing at early time points (Figure 3.11). Moreover, it was important to determine the source of IL-17 in

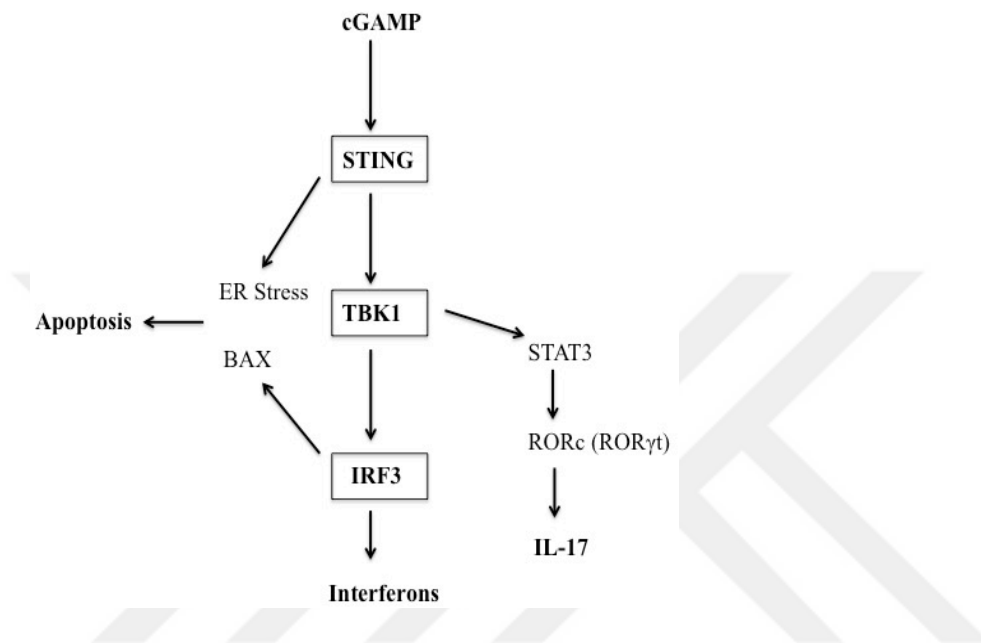
Pan T cell population. When intracellular IL-17 was stained, IL-17 secretion increased in both CD4⁺ and CD8⁺ T cells upon cGAMP stimulation (Figure 3.12). During flow cytometry analysis, CD4⁺/CD8⁺ T cell ratio abruptly increased (Figure 3.13). We thought that those results could be derived from the differentiation effect of cGAMP on CD4⁺ T cells. In other words, cGAMP firstly could affect and differentiate helper T cells into Th17 or enhance already exist Th17 cells. Secreted IL-17 then might trigger the secretion of IL-17 from CD8⁺ T cells. To test that, all experiments performed for Pan T cells were done for magnetically isolated CD4⁺ T cells. Although cGAMP stimulation caused an apoptosis in CD4⁺ T cells, we could not observe any IL-17 secretion from CD4⁺ T cells (Figure 3.16-18). Beside, the phosphorylation of STAT3 could not be enhanced upon cGAMP (Figure 3.19). To determine whether apoptosis masked the IL-17 secretion, intracellular IL-17 level was determined. Similarly, cGAMP did not increase IL-17 secretion from CD4⁺ T cells (Figure 3.20). Hereby, our hypothesis, which relates IL-17 secretion and CD4⁺ T cells failed. However, we noticed that $\gamma\delta$ T cells are also a significant source for IL-17. In fact, Hamada *et. al.* showed that in the presence of Listeria infection which produce cyclic di-nucleotides, $\gamma\delta$ T cells start to secrete huge amount of IL-17 [83]. Maybe that was the reason why we obtained huge amount of IL-17 secretion from Pan T cells but not from CD4⁺ T cells in the presence of cGAMP. On the other hand, several groups demonstrated that invariant natural killer cells (iNKT) can also secrete IL-17. Maybe, IL-17 secretion from Pan T cell stimulation may be derived from iNKT cells upon cGAMP stimulation. Since the response from Pan T cells were more close to physiologic situation, our second strategy was to reduce cytotoxic activity of cGAMP while maintaining IL-17 in Pan T cells. Since we related type I interferons and IL-17 secretion, we should have chosen TLR ligand, which induces, type I interferon. Since TLR3 ligand directly activates IRF-3, which may be related with apoptosis pathway, we decided to use TLR7/8 ligand, R848 that promotes proliferation of T cells [107]. As we expected, R848 reversed the death mechanism and promoted their survival while enhancing IL-17 secretion (Figure 3.21-22). Th17 related genes were also examined in mRNA level. Although R848 together with cGAMP increased the mRNA level of IL-17, Th17 related genes including RORa and SOCS1 dramatically decreased (Figure 3.23). This situation can also be related with R848+cGAMP effect on other

subtypes of Pan T cells including $\gamma\delta$ T cells and iNKTs. On the other hand, this data can contribute studies, which research the role of cGAMP as a vaccine adjuvant. Injection of cGAMP together with R848 can decrease toxicity of cGAMP and become more efficient vaccine adjuvant. Lastly, we wanted to determine the effect of IL-17, which was secreted from Pan T cells stimulated with cGAMP in innate immunity. RAW macrophages were stimulated with media from T cells, LPS and K3. According to our results, supernatant from T cells stimulated with cGAMP alone and together with R848, which are the most potent IL-17 inducers increased CD80 expression, which is the activation marker for macrophages (Figure 3.24.A). Also, another activation marker, MHCII was checked. Although there was no increase in the percentage of MHCII positive cells, MFI value of T cells stimulated with cGAMP raised compared with T cells stimulated with only anti-CD3/CD28 antibodies (Figure 3.24.B).

As a conclusion, our study is the first to understand the effect of cGAMP in T cells. Our findings not only show the toxic effect of cGAMP but also provide a strategy to reverse this toxic effect while maintaining its functional effect in terms of IL-17. Usage of cGAMP together with R848 would lead to design more efficient vaccine adjuvants. Beside, since IL-17 plays an important role for the elimination of extracellular bacteria and fungi in mucosal surfaces, our findings indicate that cGAMP would become an effective mucosal vaccine adjuvant against bacterial and fungal infectious.

The exact mechanism of apoptosis and IL-17 secretion are still unknown. Although we have some proposed mechanism, this mechanism should need to be proved. For the apoptosis effect of cGAMP, two independent signaling pathway including BAX-IRF3 dependent and ER stress related pathways might be further studied and identified. On the other hand, in the literature there is only a single study which related TBK1 and STAT3 during viral infectious. Hence, as another future perspective, the source of IL-17 and its signaling pathway might be further investigated. Moreover, other subtypes of Pan T cells including $\gamma\delta$ T cells and iNKTs can be separately isolated and analyzed in the presence of cGAMP to identify IL-17 source in detail. Lastly, we were unable to test the efficacy of

cGAMP treated T cells against bacterial or fungal infectious. In the future, its efficacy in in vivo against pathogens and long term efficiency can be tested.



Scheme 4.1: Proposed mechanism of the effect of cGAMP stimulation in T lymphocytes

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