Anti-cancer Immunologic Function of Recombinant Interleukin-18

Jirakrit Saetang

A Thesis Summited in Partial Fulfillment of the Requirements for the
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Prince of Songkla University
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ชื่อวิทยานิพนธ์ การทำงานของรีคอมบิแนนท์อินเตอร์ลิวคิน-18 ในการเสริมภูมิคุ้มกันต้านมะเร็ง

ผู้เขียน นายจิรกฤต แซ่ตั้ง

สาขาวิชา ชีวเวชศาสตร์

ปีการศึกษา 2560

บทคัดย่อ

Interleukin-18 (IL-18) เป็นไซโตไคน์ (cytokine) ชนิดหนึ่งที่มีการรายงานถึงฤทธิ์ในการต้านมะเร็งผ่านการกระตุ้นภูมิคุ้มกันต้านมะเร็งซึ่งได้มีการศึกษาการนำ IL-18 มาใช้ต้านมะเร็งในสัตว์ทดลอง และมีรายงานถึงการนำไปใช้กับผู้ป่วยมะเร็งได้อย่างปลอดภัย ในงานวิจัยชิ้นนี้ได้ทำการเปลี่ยนกรดมะนิโนของ IL-18 เพื่อเพิ่มประสิทธิภาพของโปรตีน โดยทำการเปลี่ยนในรูปแบบ E6K T63A และ E6K+T63A และเมื่อทดสอบความสามารถของ IL-18 แต่ละชนิดในการกระตุ้นให้เซลล์ NK-92MI สร้าง interferon-γ พบว่าโปรตีนดัดแปลง E6K+T63A มีความสามารถในการกระตุ้น IFN-γ มากกว่าโปรตีนตัวปกติ โปรตีนดัดแปลง T63A และ โปรตีนดัดแปลง E6K ประมาณ 16 4.2 และ 1.7 เท่า ตามลำดับ และจากการทดสอบฤทธิ์ต้านมะเร็งในสัตว์ทดลองพบว่า E6K+T63A มีความสามารถในการต้านการเจริญของก้อนมะเร็งมากที่สุด ตามด้วย E6K ซึ่งเมื่อนำก้อนมะเร็งมาศึกษาชนิดของเม็ดเลือดขาวภายในบริเวณก้อนด้วยวิธี flow cytometry พบว่า ในก้อนมะเร็งของหนูที่ได้รับโปรตีน IL-18 ชนิด E6K+T63A มีปริมาณของ helper T cell type 1 (Th1) และ cytotoxic T lymphocyte (CTL) ในปริมาณที่มากกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ ผลจากการศึกษาโครงสร้างของ IL-18 ที่ดัดแปลงกรดอะมิโนด้วยวิธี MD simulation พบว่า การดัดแปลง IL-18 ในรูปแบบ E6K+T63A ทำให้โปรตีนเกิดการเปลี่ยนแปลงโครงสร้างในบริเวณที่กรดอะมิโนตัวสำคัญในการเกิดอันตรกิริยากับตัวรับ ทำให้โปรตีนมีความสามารถในการจับกับตัวรับที่ดีขึ้น นอกจากนี้ ถึงแม้โครงสร้างของโปรตีนจะเปลี่ยนแปลงไป แต่ผล RMSD และ RMSF แสดงให้เห็นว่า โครงสร้างโดยรวมของโปรตีนยังคงใกล้เคียงกับโปรตีนต้นแบบ จึงยังสามารถเข้าจับกับตัวรับได้ตามปกติ ซึ่งโดยสรุปแล้ว โปรตีน IL-18 ชนิด E6K+T63A เป็นโปรตีนดัดแปลงที่เกิดขึ้นใหม่และมีประสิทธิภาพสูงกว่าปกติ ซึ่งในสัตว์ทดลองพบว่าโปรตีนชนิดนี้มีความสามารถในการชะลอการเจริญของเซลล์มะเร็งและกระตุ้นระบบภูมิคุ้มกันต้านมะเร็งได้ จึงทำให้โปรตีนชนิดนี้เป็นโปรตีนในระบบภูมิคุ้มกันชนิดหนึ่งที่อาจนำไปพัฒนาเป็นยาในการรักษามะเร็งได้ในอนาคต

คำสำคัญ: IL-18, ภูมิคุ้มกันต้านมะเร็ง, E6K+T63A, MD simulation

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Author Mr. Jirakrit Saetang

Major Program Biomedical Sciences

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ABSTRACT

Interleukin-18 (IL-18) is a cytokine species that can be used as a tumor suppressor protein in animal model and this protein can be applied to patients safely. In this study, we have developed the engineered types of human IL-18 including E6K T63A and E6K+T63A based on protein-receptor interaction. When the testing of the ability of each engineered IL-18 in interferon (IFN)-γ induction was performed, the result showed the higher activity of E6K+T63A IL-18 than native, T63A and E6K IL-18 around 16, 4.2 and 1.7 times, respectively. Moreover, results from the animal study demonstrated that mice treated with E6K+T63A IL-18 exhibited the lowest tumor volume, and expanded the survival period of this group of mice. This may be mediated by the recruitment of Th1 and cytotoxic T lymphocyte (CTL) to the tumor mass effected by our engineered IL-18 since flow cytometry revealed the higher proportion of Th1 and CTL cells in the tumor region when compared to the control group. Molecular dynamic simulation was also used to investigate the role of inter- and intra-molecular alterations of the protein after mutations were performed, and it has been revealed that the mutation affected the key residues containing region of IL-18, leading to the effective interaction between our engineered IL-18 and its receptors. Although these structural changes occurred with the higher activity, root mean square deviation and root mean square fluctuation demonstrated that the overall structure still be the same. This all confirms the performance of our engineered type of IL-18 to be a candidate as a cancer therapeutic agent.

KEYWORDS: IL-18, Anti-cancer immunity, E6K+T63A, MD simulation

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LIST OF ABBREVATIONS AND SYMBOLS

A, Ala	=	Alanine
ADCC	=	Antibody-dependent cellular cytotoxicity
AOX	=	Alcohol oxidase
ATCC	=	American Type Culture Collection
BCL	=	B cell leukemia
BMGY	=	Buffered Glycerol-complex Medium
BMMY	=	Buffered Methanol-complex Medium
BSA	=	Bovine serum albumin
C, Cys	=	Cysteine
CARs	=	Chimeric antigen receptors
CD	=	Cluster of differentiation
CTL	=	Cytotoxic T lymphocyte
CTLA4	=	Cytotoxic T-lymphocyte associated antigen 4
D, Asp	=	Aspartic acid
DC	=	Dendritic cell
DNA	=	Deoxyribonucleic acid
E, Glu	=	Glutamic acid
E. coli	=	*Escherichia coli*
EGFR	=	Epidermal growth factor receptor
ESC	=	Embryonic stem cell
F, Phe	=	Phenylalanine
g	=	Gram
G, Gly	=	Glycine
GM-CSF	=	Granulocyte macrophage colony-stimulating factor
h	=	Hour
H, His	=	Histidine
HER	=	Human epidermal growth factor receptor

HRP	=	Horseradish peroxidase
I, Ile	=	Isoleucine
IFN	=	Interferon
IL	=	Interleukin
IL-18BP	=	IL-18 binding protein
k	=	Kilo
K, Lys	=	Lysine
kDa	=	Kilodalton
L	=	Littre
L, Leu	=	Leucine
m	=	Milli
M	=	Molar
M, Met	=	Methionine
mAb	=	Monoclonal antibody
MD	=	Molecular dynamics
MHC	=	Major histocompatibility complex
n	=	Nano
N, Asn	=	Asparagine
NaCl	=	Sodium chloride
NFκB	=	Nuclear factor κB
NK cell	=	Natural killer cell
NMR	=	Nuclear magnetic resonance
OD	=	Optical density
ORF	=	Open reading frame
p	=	Pico
P, Pro	=	Proline
P. pastoris	=	*Pichia pastoris*
PBS	=	Phosphate-buffered saline
PCR	=	Polymerase chain reaction
PDB	=	Protein Data Bank
PVDF	=	Polyvinylidene difluoride
Q, Gln	=	Glutamine

R, Arg	=	Arginine
RCSB	=	Research Collaboratory for Structural Bioinformatics
RMSD	=	Root-mean-square deviation
RMSF	=	Root mean square fluctuation
RNA	=	Ribonucleic acid
Rpm	=	Revolutions per minute
S, Ser	=	Serine
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNPs	=	Single nucleotide polymorphisms
T, Thr	=	Threonine
TBST	=	Tris-buffered saline with Tween 20
TCR	=	Toll-like receptor
TGF	=	Transforming growth factor
Th	=	Helper T cell
TILs	=	Tumor-infiltrating lymphocytes
TNF	=	Tumor necrosis factor
V, Val	=	Valine
VEGF	=	Vascular endothelial growth factor
VMD	=	Visual Molecular Dynamics
W, Trp	=	Tryptophan
Y, Tyr	=	Tyrosine
YPD	=	Yeast extract- peptone-dextrose
α	=	Alpha
β	=	Beta
γ	=	Gamma
μ	=	Micro

CHAPTER 1

INTRODUCTION

1.1 Background and rational

As cancer is currently one of the most prevalence public health problems worldwide, effective cancer control and therapy take priority over other health issues. Nowadays, there are several cancer screening tests which have been performed to detect some cancer risk status leading to the early diagnosis (1). Although this system has been widely used in many countries, the incidence of cancer still has been increasing because of some risk behaviors such as smoking, alcohol consumption, physical inactivity, or western diet intake are still existed (2). Cancer treatments currently consist of three major modalities, surgery, chemotherapy, and radiotherapy, which one or more treatment modalities may be used to provide the most effective treatment. Alternatively, immunotherapy, a type of treatment that uses the body's immune system to facilitate the killing of cancer cells, was recently more interested in both basic and clinical research. There are several types of immunotherapy, including monoclonal antibodies, cancer vaccines, and non-specific immunotherapies (3). In this study, we tried to develop a non-specific immunotherapy method that boosts the immune system in a general way by using IL-18 as activator.

IL-18 is a cytokine species in IL-1 family that can be generated by many cell types including intestinal epithelial cells, macrophages, dendritic cells or Kupffer cells (4–7). IL-18 is recognized as a protein that drive the polarization of $CD4^+$ T cells toward the helper T cell type 1 (Th1) phenotype, when co-stimulate with IL-12 or IL-15, mediating cellular immune response, which is characterized by the production of IFN-γ (6,8,9). Several researches reported that IL-18 has anti-cancer properties through the stimulation of natural killer cells (NK cells), cytotoxic T lymphocytes, inhibiting the growth and spread of cancer cells and increasing the expression of Fas-ligand in immune cells for Fas expression cancer cell killing (10–14).

Moreover, there are several successful studies in which IL-18 was used as a tumor suppressor protein in animal model and this protein can be applied to patients safely (15–18).Tumor regression activity of IL-18 is achieved by administrating the cytokine with intraperitoneal (19–21), intravenous (19), intratumoral (22) and peritumoral (23) routes. This anti-tumor functions of IL-18 are mainly mediated through IFN-γ (24) which generally know that IL-18 is an effective activator of IFN-γ production by T cells, NK cells and macrophages and this function can be synergistic in with IL-12 (25). Tse et al. showed that neutralizing antibody against IFN-γ completely eliminated IL-18-mediated anti-tumor effects (24). Consistently, Nagai et al. also demonstrated that IFN-γ played a role for the tumor inhibitory effects of IL-18 using a gene-transfer approach (26).

In this study, we have been successful to improve the activity of IL-18 by site-directed mutagenesis and produced it in yeast, *Pichia pastoris*. The reports demonstrated that there are three sites of IL-18 that play an important role on its receptor binding: site I and II are specific to IL-18Rα and site III to IL-18Rβ (27). Arg13, Asp17, Met33, Asp35 and Asp132 are included in site I, Lys4, Leu5, Lys8, Arg58, Met60 and Arg104 are in site II, and Lys79, Lys84 and Asp98 are involved in site III (27). It is not only residues mentioned above; Glu6, Lys53 and Thr63 are also reported to be important in bioactivity and binding mode of IL-18 (28,29). To increase the activity, engineering of IL-18 by altering binding residues were performed. Since Met33 and Met60 in site I and II are surrounded by other charged amino acids such as Asp, Arg and Lys, a methionine was replaced by a glutamine (Q) to increase the site polarity, based on an implication that these site I and II require dipole/electrostatic force to facilitate IL-18 binding. Moreover, according to the report that a substitution of E6 with an alanine and a lysine can enhance IL-18 activity (28,29), E6K was also taken in this study as a comparative standard. Thr63 is another interesting residue, reported for increased activity when it was alanine-substituted. By testing with NK cell line for inducing IFN-γ, we found that the stimulation property of E6K+T63A IL-18 mutant was higher than wild-type protein about 15-20 times which is the highest activity that ever reported before. Moreover, we also tested the anti-cancer activity of this engineered IL-18 compared to wild-type by using tumor animal model that is a common model for evaluating the immunotherapy efficiency.

1.2 Review of literatures

1.2.1 Cancer immunobiology

Cancer immunobiology is a scientific topic which has been studied for a long time. It has been firstly mentioned in the cancer immunosurveillance hypothesis describe that cancer cells can be destroyed by the immune system before they develop into a tumor (30–32). Moreover, several experiments have shown that there are the other molecules that play a crucial role in anti-cancer immune response, such as IFN-α components (33–35), TNF-related apoptosis-inducing ligand (TRAIL) (36), IL-12 (37) or tumor necrosis factor-α (TNF-α) (35). This knowledge has led to the cancer immunoediting hypothesis, used to describe the dual opposing functions of immunity, cancer destruction and cancer promotion, by the immune system, which run into three phases, elimination, equilibrium, and escape (Fig. 1) (30,32,38).

1.2.1.1 Elimination phase

One evidence which shown the important function of immune system that protect the body from cancer is the analysis of 122,993 acquired immunodeficiency syndrome (AIDS) patients, whom have increased incidence of non-AIDS associated cancer, for instance, cancer of tongue, lung, skin or multiple myeloma (39). Moreover, drastic primary and secondary immunodeficiencies have been shown to have a relationship with high risk of cancers (40).

The next several deep studies then illustrated that lymphocytes are the main cells of the immune system, which play an important role in cancer clearance. Abnormal lymphocyte mice created by deleting recombination activating gene-2 (RAG-2) show more severity of tumorigenesis than normal mice when induced by methylcholanthrene (MCA). Interestingly, when tumor from RAG-2 depleted mice was transplanted onto normal mice, this cancer cells can be destroyed by 40%. In contrast, tumors taken from normal mice had rapid growth property when transplanted into the same type mice or RAG-2 depleted mice indicating that tumor taken from normal mice has lower immunogenicity than RAG-2 depleted mice and this evidence

became the basis of immunoediting hypothesis (Fig. 2) (41). Some of lymphocytes and their mechanisms including T helper type 1 (Th1) cells, $CD8^+$ T cells, NK cells and NKT cells were widely reported in anti-tumor roles.It is thought that not only lymphocytes but non-lymphoid cell, such as type 1 macrophage (M1), type 1 neutrophil (N1), eosinophil and DCs, also have anti-tumorigenic roles. M1 macrophage can be recruited to the tumor microenvironment and secrete cytokines which help Th1 cell polarization, enhance B cell proliferation, activate DCs and $CD8^+$ T cells (42). N1 neutrophils, characterized by high expression levels of Fas, inflammatory cytokines and chemokines, exhibit strong cytotoxicity against tumor cells (43).

Molecules such as type I and type II IFN were also reported in anti-tumor immune response. Type I IFN (IFN-α/β) is required for helping the cross-presentation of tumor antigens from $CD8\alpha^+/CD103^+$ DCs to $CD8^+$ T cells and type II IFN, IFN- γ, which is made by cells in the immune system, such as NK cells, NKT cells, $CD4^+$ Th1 cells or $CD8^+$ T cells also play a vital role in the anti-cancer immune response (30,32,44–47). Perforin (pfp) is another molecule that is important in anti-tumor immunity by NK cells and tumor-specific $CD8^+$ T cells. In the experiment with MCA induced cancer model, perforin deficient mice have the more cancer severity than normal mice about 2-3 times (35,51,52). In addition to perforin, NKG2D, an activating- NK cell receptor, is another best characterized molecule contributing to NK cell-related cancer immonosurveillance. Up-regulation of NKG2D ligands such as major histocompatibility complex (MHC) class I chain-related A/B (MIC-A/B) or UL16 binding protein (ULBP) 1–5 molecules are involved in cellular stress such as DNA-damage or cell-cycle progression, which is usually found in tumor cells. Moreover, cytokine immunotherapy using IL-2 or IL-12 is likely to be effective via the NKG2D- and perforin-mediated mechanism (55). Another study by Cretney and colleagues showed that mice lacking TRAIL, a death receptor ligand, are more susceptible to experimental and spontaneous tumor metastasis, showing the anti-tumorigenic role of this molecule (56).

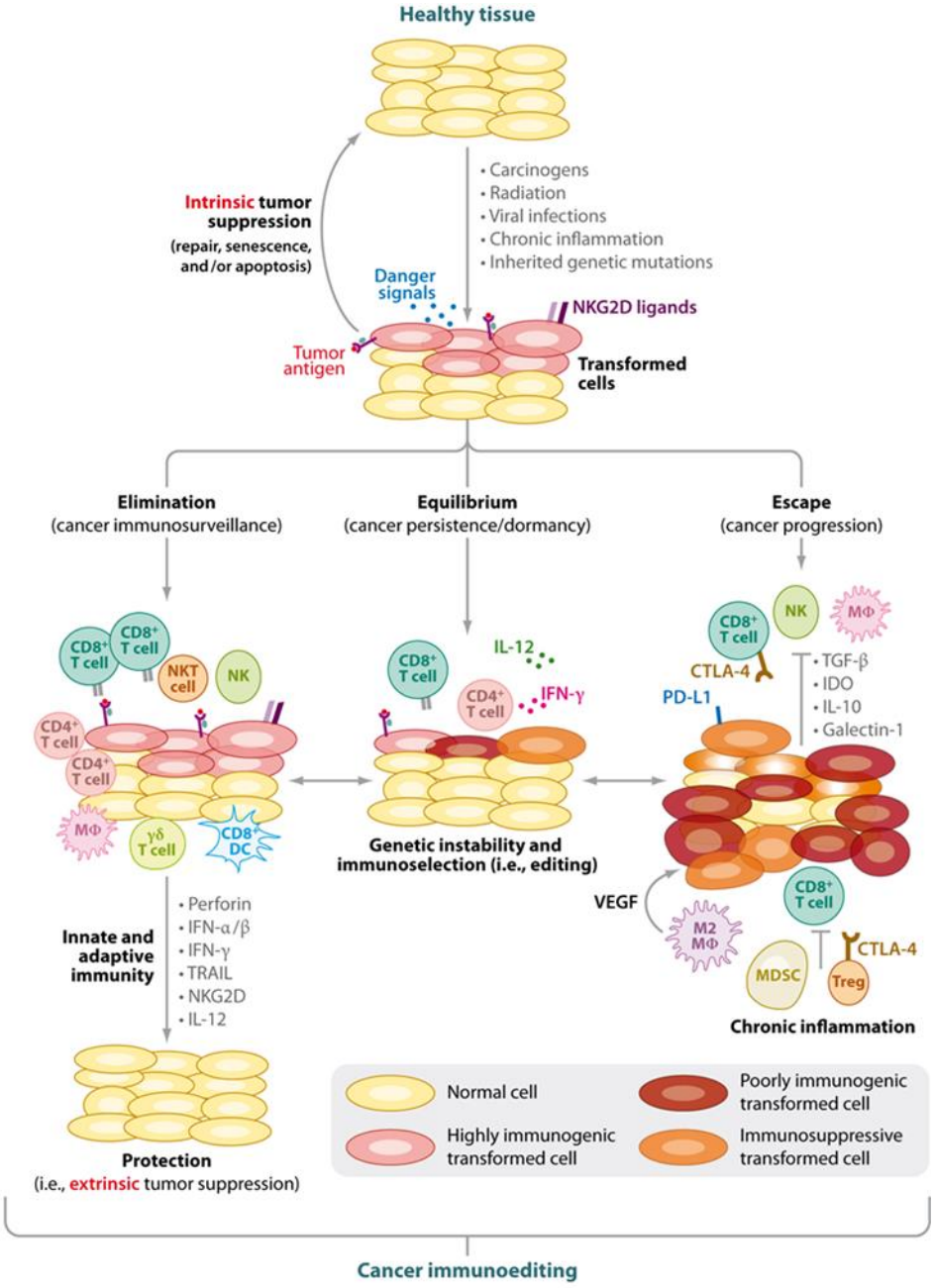


Figure 1. Three phases of cancer immunoediting hypothesis: elimination, equilibrium, and escape (32).

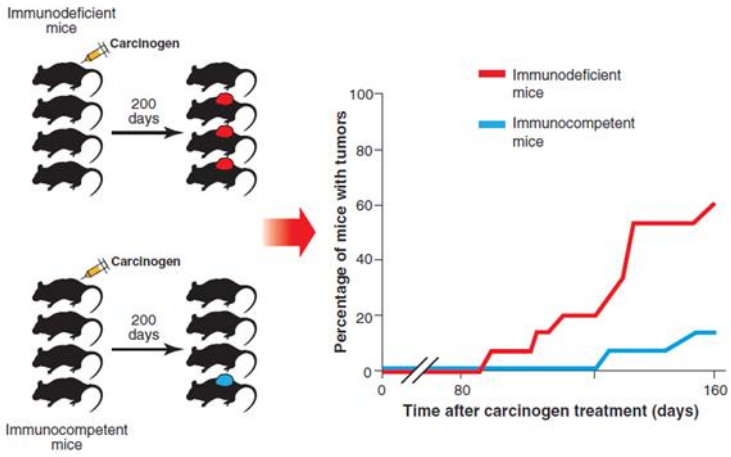


Figure 2. The important role of immune status in tumor susceptibility (42,46).

Taken together, although all data have been demonstrated the effective immunity against tumor in almost cases, cancer patients still died from the disease. This is likely because the cancer immunosurveillance resulting in the pressure selecting the low immunogenic tumor cell to gain the ability to persist in equilibrium phase.

1.2.1.2 Equilibrium phase

Although almost cancer cells can be destroyed by immune cells, some of them can be changed in their immunogenicity and adapt themselves to the low immunogenic stage, which allow cancer cells to increase their number and alter the environment around them to be suitable for growth and then spread out to other organs. During this phase, the equilibrium interaction between tumor clearance by immune cells and tumor cells growth by changing their immunogenicity is the 2nd phase according to immunoediting hypothesis, which leads to the tumor dormancy stage (30–32). This stage may continue for a long time causing the selection of low immunogenic cancer cells, which can evade the immune system making tumor overgrowth finally.

The evidences of equilibrium phase were from both clinical and experimental studies. The events of relapse which are seen in many cancer patients after remission for one to two decades indicating that some tumor cells still remained in the body

despite the treatment completely performed (48). In addition, six recurrence cases of non-small cell lung carcinoma between 7 and 14 years after getting remission were noted, and all were related with immunosuppressive treatments (49). Another study also illustrated that when tumor free-like kidneys from a donor, patient who was cured from skin cancer for a period of 16 years, were transplanted into 2 recipients, both of them had a metastatic melanoma lesion without primary lesion, indicating that immune suppression by immunosuppressive drugs result in rapid growth of cancer cells in transplanted organ while cannot be grown in the donor body (50).

In animal model, the preliminary studies of tumor dormancy used the BALB/c B cell leukemia/lymphoma 1 (BCL1) model, which showed that 70% of mice vaccinated with BCL1 idiotype vaccine did not develop splenomegaly after BCL1 challenge for more than 60 days while control mice appeared this character in 25-30 days (48,51). Interestingly, these mice can still be found to have BCL1 cells in their circulating system, and the tumor was relapsed in the period of more than 610 days (52). Importantly, in BCL1 Ig-immunized mice, $CD8^+$ T cells or IFN-γ depletion decreased the period and incidence of dormancy, indicating that these factors are associated ~~in~~ with maintaining the dormancy state (48). In a skin cancer model, when mice lacking E3 ligase Casitas B-lineage lymphoma b (Cbl-b), a $CD8^+$ T cell inhibition protein, were induced to develop squamous cell carcinoma by ultraviolet B (UVB) radiation, it did not show any apparent tumor lesions in 400 days while wide type mice had serious tumor lesions. However, 10 days after injection of mAbs against $CD8^+$ T cells, 50% of these mice displayed cancer lesions while no lesion~~s~~ could be detected in the control group (53). In addition, there is another report, showing that cancer cells can be destroyed by $CD8^+$ T cells through IFN- γ (46).

1.2.1.3 Escape phase

Because of heterogeneous population of tumor cells, there are some cancer cells that can survive and adapt themselves to suppress or invade immune system although almost can be killed in elimination phase. A long studied mechanism is decreasing antigen presentation to T cells, including down-regulation of the main component needed for antigen presentation or reducing the expression of MHC class I

molecule (30,32,54). Another mechanism is IFN- γ resistance by epigenetic changes or somatic mutations of its responding component genes, such as IFNGR1, IFNGR2, JAK1, JAK2 and STAT1, leading to the inhibition of MHC class I expression and antigen presentation (30,32,54,55).

Immunosuppressive cytokines secreted by tumor cells in tumor microenvironment have been long recognized as one of the strategies to evade the immune system. Moreover, there is a group of cells called myeloid-derived suppressor cells (MDSCs), which play an important role in suppressing anti-tumor immunity. This group of cells contains various types of myeloid cells, such as myeloid progenitor cells, immature macrophage, granulocyte and DCs, which can secrete some immunosuppressive cytokines (TGF-β, IL-10), inhibit T cell activation by inducing T cell receptor nitrosylation, attract regulatory T cells into a tumor lesion, suppress IFN-γ production in DC-induced $CD8^+$ T cells or interrupt NK cell and NKT cell activation (32,38,54).

Expressing immune-inhibitory ligands in the tumor cell itself is also another way to inhibit immune cell activation through cell-to-cell contact. B7-H1 (PD-L1), HLA-G and HLA-E can interact to the receptor on the surface of T cells, leading to T cell apoptosis, antigen presenting cell anergy, resistance to NK cell killing and decreasing the cytotoxicity of T cells (32,56,57). Besides, indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the degradation of tryptophan by kynurenine pathway, has been studied in its role in supporting tumor growth (54,58).

Although the knowledge about immunoediting hypothesis is now better understood, gaps in this area still persisted. Knowledges in the interaction among signaling molecules, the understanding about the suppress on mechanism by a tumor cell itself or the relationship between immune cells in tumor microenvironment are the important things, which help to improve the cancer therapeutic methods.

1.2.2 Cancer immunotherapy

In addition to standard therapeutic methods, several other techniques have been studied with an aim to cure cancer patients. The most interesting method is immunotherapy, which is based on the stimulating cancer patient's immune system to attack the malignant tumor cells. This can be achieved by using monoclonal antibodies (mAbs), cancer vaccines, and non-specific immunotherapies (3).

1.2.2.1 Monoclonal antibodies (mAbs)

The use of mAbs is one way of immunotherapy to fight cancer. Researchers have learned how to design antibodies that specifically target a certain antigen in cancer cells. Because of this reason, this method is sometimes called targeted therapies. Cancer killing mechanisms of mAbs can be divided into three types (Fig. 3) (59).

1.2.2.1.1 Immune-mediated cell killing: This mechanism is mediated by triggering the immune system to attack and kill cancer cells. This includes complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) and regulation of T cell function (59). Moreover, to improve better immune activation, there are some efforts that aim to modify the Fc domain of mAbs resulting in higher affinity of Fc domains and enhancement of ADCC. Fucosylation modification, have also become another strategy for new antibodies that are being introduced into clinical used (59,60). Moreover, turning-off the inhibitory mechanism of immune cells is another strategy to achieve therapeutic effect of mAbs. For instance, ipilimumabis designed to seek out and lock onto cytotoxic T-lymphocyte associated antigen 4 (CTLA4), molecule that is normally found in the downregulated mechanism of the activated T cell, to boost a longer anti-tumor respond of T cells (61–63). In addition, to enhance the efficacy of this cancer drug, some modification such as glycosylation site improvement, amino acid change or adding the tumor toxic molecules have been performed (64–67).

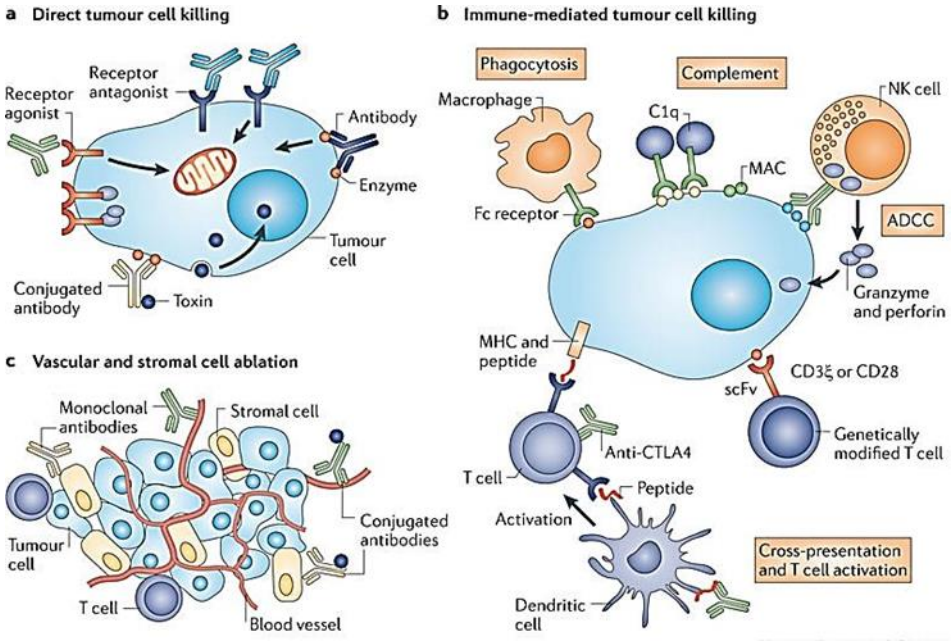


Figure 3. Three mechanisms of mAbs for treating cancer (76).

1.2.2.1.2 Specific effects on tumor microenvironment: In addition to mediating the immune cell activation and cancer killing, antibodies can activate or antagonize immunological pathways that are important in cancer immune surveillance. For example, many solid tumors generate vascular endothelial growth factors (VEGFs), which stimulate angiogenesis when bind to its receptor on the vascular endothelium. Bevacizumab is a mAb that neutralizes this cytokine resulting in inhibiting the binding to its receptors and approving for the treatment of breast, colorectal and non-small-cell lung cancer in combination with cytotoxic chemotherapy (59,60). Transforming growth factor-β (TGF-β), a cytokine which can inhibit T cell activation, differentiation, and proliferation, is another target for therapy. Fresolimumab (GC1008) is a TGF-β-specific antibody which neutralizes all three forms of TGF-β. The phase I study demonstrated the preliminary evidence of antitumor activity in patients with advanced malignant melanoma or renal cell carcinoma (60,68).

1.2.2.1.3 Direct action to tumor cells: Many tumors express high level of growth factor receptors which can be targets of therapeutic antibodies. Cetuximab, for example, is a recombinant chimeric mAb which targets epidermal growth factor receptor (EGFR) leading to the inhibition of EGFR-mediated signal transduction (60). This results in cell growth inhibition and leads to apoptosis (3). Some types of antibody are modified to joint with toxic substances which aim to deliver drugs, toxins, or radioactive isotopes directly to the cancer cell. Ibritumomab and tositumomabradioconjugate are radioisotope-conjugated mAbs that serve as delivery systems of radioactive isotope to the cancer cells and are indicated for the B-cell NHL treatment (3).

Gemtuzumabozogamicin for CD33-positive AML and brentuximabvedotin for CD30-positive Hodgkin's lymphoma are chemolabeled antibodies which have been approved by the Food and Drug Administration (FDA) (59). These have provided the first proof-in-principle for antibodies selectively deliver drug to cancer cells. Moreover, vivatuxin, which is an ^{131}I-labelled chimeric mAb against intracellular DNA-associated antigens, is approved by the Chinese drug regulator for the treatment of lung cancer (69).

2.2.2 Cancer vaccine

Vaccine development is an alternative method for treating cancers. The vaccine has evolved into two types: 1) prophylactic or preventative vaccines which are against cancers caused by viruses such as HBV (hepatitis B virus), or human papillomavirus, and 2) therapeutic vaccines for the treatment of developed tumors (70). In the past few years, development of vaccines to treat or prevent cancer with the aim to stimulate $CD4^+$ T cells, $CD8^+$ T cells and the stimulation of IFN-γ have been very successful. All these factors have been reported to show the relationship with improved survival rate in cancer patients (70,71). With scientific advances in molecular biology and cell culture technology, adoptive cell transfer has been consequently studied and developed to be another way to treat cancer as cancer vaccine (64,70).

The first successful experiment leading to a phase III randomized cancer vaccine trial is sipuleucel, a DCs-based vaccine, for prostate cancer. DCs, a professional antigen presenting cell, are separated from patient and then activated in vitro with fusion protein, consisting of prostate cancer-specific antigen, prostatic acid phosphatase, and DC activator, granulocyte macrophage colony-stimulating factor (GM-CSF) (72,73). Antigen-specific activated DCs are next returned from the laboratory into the patient, resulting in the activation of anti-tumor immune response and increasing the overall survival about 4 months (72,73).

In addition to DCs-based vaccine, adoptive T cell transfers therapeutic cancer vaccines is another method for cancer treatment. This method starts by selecting T cells with specificity for tumor antigen, grow it in the laboratory or modify a T cell receptor to be a chimeric antigen receptors (CARs) before sending back to the same patient (74). A major source of $CD4^+$ and $CD8^+$ T cells is tumor-infiltrating lymphocytes (TILs), which is highly specific to, but cannot destroy cancer cells because of the surrounding immunosuppressive scenario in the tumor microenvironment (32,54,64). It was found that when the TILs from melanoma patients with metastatic stage were grown in vitro and taken back into patients, 50% had objective responses (75). But when $CD4^+$ and $CD8^+$ T cells, which are specific to only one type of antigen, were used in the same way, effectiveness of the therapy reduced probably because of the heterogeneity of cancer cells (76,77). Even the development of a more effective way of this protocol has been improved by engineering TCRs, the result is not as good as expected due to MHC restriction as well as side effects caused by destruction of normal tissue (64,70,78). For this reason, CARs need to play more roles in adoptive cell transfer.

CARs are engineered ligand-binding domain or ectodomain, which specifically binds to the antigen, linked to the TCR constant domain or endodomain through a transmembrane domain for inducing signaling cascade (64,76). Enhancing the T cell efficacy can be done by increasing the co-stimulatory signal activation via CD3z. In the second generation CARs, researchers add intracellular signaling domains such as CD28, 4-1BB or ICOS to the cytoplasmic tail of the CAR to improve tumoricidal activity of a T cell, including T cell proliferation enhancement, anti-tumorigenic cytokine induction, or longer cytotoxicity stage maintaining (79,80).

Moreover, the third generation CARs has recently been developed for stronger activation and longer survival period of T cells survival by adding 4-1BB orOX-40 (Fig. 4) (81–83). Although the development of the third generation has been performed, experiments using CD20-specific CD28/4-1BB/CD3z T cells in patients with lymphoma found that the response was still not satisfactied (84).

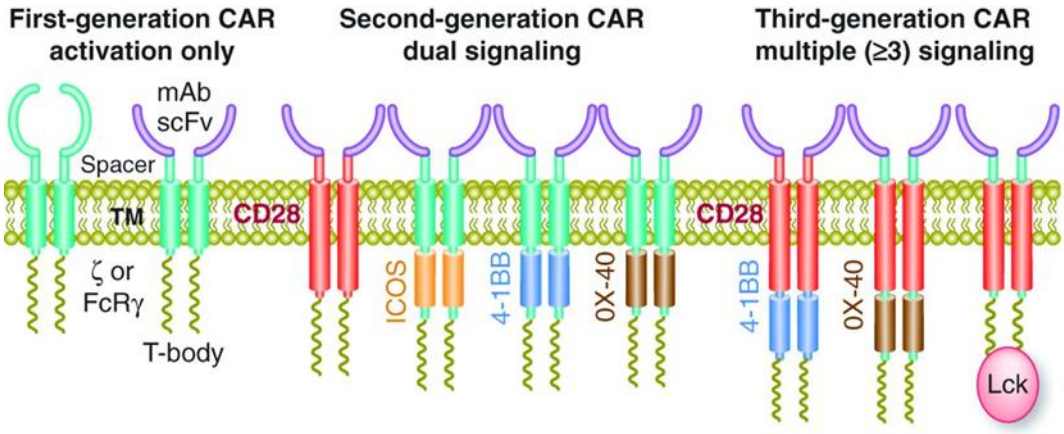


Figure 4. Three generations of CARs (78).

Currently, various tumor antigens have been found and molecularly characterized, some of them can be cloned, produced in vitro, and directly vaccinated to a patient. Moreover, some cytokines or mAb are additionally included in the vaccine, and some mix various peptide antigens together in one (85). For an example, Walter and colleagues used a new technique to find the renal cell cancer antigens, which are the clinically-relevant, naturally-presented HLA-associated peptides. Ten molecules, which were specific to the most common type of Caucasian HLA, HLA-A*-02, and HLA-DR, or MHC class II, were produced under the IMA901 name, which provides the better outcome when use as an adjuvant to the cyclophosphamide (CTX) (86,87).

2.2.3 Non-specific immunotherapies

Non-specific immunotherapies do not target cancer cells specifically. They stimulate or switch the immune circumstance in tumor microenvironment in a more general way to be against cancer cells. This switching is generally from immunosuppression to anti-tumor progression by using some cytokines and chemokines.

In 1992, high-dose IL-2 (IL-2) was the first immunotherapy approved by the FDA to be used as a primary or stand-alone cancer treatment. IL-2 stimulates the proliferation of cytotoxic $CD8^+$ T-cells and NK cells, both of which promote tumor regression. The evidence was a proof-of-concept for potent activity of IL-2-activated killer cells (termed lymphokine-activated killer, or LAK cells) against established malignancy in animal models. Today, high-dose IL-2 may also be used for metastatic melanoma.

IFN therapy is used as a treatment for many cancers in combination with chemotherapy and radiation. IFN-α, a type I IFN, is the only currently approved adjuvant therapy for patients with high-risk stage II or stage III melanoma (88). Moreover, IFN-α is approved for the treatment of some hematologic malignancies and AIDS-related Kaposi's sarcoma (88). IFN-β is another type I IFN, which is more effective than IFN-α in stimulating anti-proliferative effects in cancer models (89). However, this IFN has been limited in the clinical use because of low bioavailability and sustained side effects (88). IFN-γ is cytotoxic to some malignant cells and has modest anti-angiogenic activity (88,90). It also shows to be involved in anti-proliferative and pro-apoptotic effects against cancer cells (91,92).

IL-15 has indicated significant therapeutic response in many pre-clinical mouse models of cancer. These therapeutic effects are mediated through the activation of effector CTL in an antigen-independent manner and may be also important for the persistence of memory CD8+ T cells (93,94). IL-15 has just entered phase I studies for both *in vivo* and in *ex vivo* in adoptive cell therapies (88). In addition to IL-15, IL-12 has been reported to have anti-tumor effects in models of melanoma, colonic carcinoma, mammary carcinoma and sarcoma mediated by anti-angiogenic effect through IFNs induction, particularly IFN-γ and IP-10, and has then been studied as a

therapeutic molecule for cancer in preclinical and phase I clinical trials (95–98). IL-18 is another molecule which is originally identified as an IFN-γ inducing factor. It induces IFN-γ expression in NK and $CD8^+$ T cells and increases their cytotoxicity (6,8,9). Moreover, it can stimulate natural killer cells (NK cells), cytotoxic T lymphocytes, inhibit the growth and spread of cancer cells and also increase the expression of Fas-ligand in immune cells for Fas expression cancer cell killing (10–14). A phase I study of IL-18 reported the safety and efficacy in terms of higher levels of IFN-γ and GM-CSF in patients after IL-18 injection (16–18).

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a highly pleiotropic cytokine which is approved by the FDA for neutrophil recovery and reducing the chance of infections after chemotherapy in acute myelogenous leukemia patients (88). The efficacy of GM-CSF in therapeutic responses has been shown in murine melanoma model in which transgenic expression of GM-CSF led to protection from tumor challenge in more than 90% of the treated animals (99). GM-CSF has also been shown to have antitumor effects in melanoma when injected directly into metastatic lesions (100). Moreover, GM-CSF may provide a more regulatory role in the induction of DC-mediated T cell immune response through complex interactions with milk fat globule-8 (MFG-8) (101).

In addition to therapeutic cytokines mentioned above, other molecules, for instance, CXCL10 and XCL1, chemoattractive cytokines for $CD8^+$ T cells, NK cells and monocytes, have also been involved in adoptive transfer therapy. Moreover, CCL5, CCL2, CCL21 and CCL16 can be used to recruit DCs, macrophage and T cells to tumor microenvironment in combining with IL-12, cytosine-phosphorothioate-quanine (CpG), Toll-like receptor 3 (TLR3) activator or αIL-10 mAb (62).

These proteins have been increasingly used as common therapeutic drugs. As recombinant DNA technology has been developed, it allows therapeutic proteins to be produced on a large scale. However, in the fact of pharmaceutical view, proteins are not an ideal drugs because of high tendency to aggregate, chemical instability in some cases and the complex production, storage, and administration (102). Hence, the redesign of proteins to increase their clinical potential has to be considered as the next major step in the evolution of therapeutic protein. Controlled modifications of specific biochemical and biophysical properties of proteins can influence their mechanism of

action, stability, side effects, efficacy, pharmacokinetics, ease of production and storage, and susceptibility to degradation (102).

High-dose IL-2 has been approved for metastatic melanoma. Its stability is also enhanced by point mutagenesis of free cysteines to serines preventing undesired disulfide bridges which may be lead to protein aggregation (103). In addition, to enhanced its binding affinity, variants of IL-2 with greater than 100-fold increases in affinity has been developed by using yeast display and directed evolution approaches (104). This mutant effectively stimulate T cell proliferation for up to 6 days following a 30-minute incubation with the protein (104). Recently, an IL-2 variant displaying ~250-fold increased affinity to IL-2Rβ was developed through in vitro evolution using yeast display (105). Interestingly, most of the 5 mutations resided in the core of IL-2 resulted in a conformational change similar to the one induced after IL-2Rα binding. This mutant, also known as super-2, displayed increased ability to stimulate cells displaying IL-2Rβγ, when compared to unmodified IL-2. Accordingly, super-2 mediated more potent expansion of $CD8^+$ T-cells and NK cells, which is correlated with increased anti-tumor activity in mice. By shifting the balance of IL-2 stimulation towards cells displaying IL-2Rβγ, super-2 is predicted to mediate tumor regression with a reduced incidence of treatment-associated adverse effects (105).

G-CSF is another cytokine which may be used during treatment with chemotherapy to shorten the duration of leukopenia. To enhance its stability, this protein has been engineered from a low α-helical propensity amino acid such as proline or glycine within α-helixs to a high α-helical propensity amino acid such as alanine to increase its chemical resistance (103,106). Moreover, a computational approach has also been included to develope a defined three-dimensional structure with 10 core amino acid substitutions that has decreased aggregation and increased thermal stability (107). Moreover, rationally designed G-CSF variants with reduced affinity for G-CSF receptor at endosomal pH (~ 5–6), but has unaffected affinity at physiological pH, showed increased cytokine recycling correlated with prolonged half-life and enhanced potency in vitro (108). This pH- specific reduction in affinity was achieved by mutating a number of residues involved in receptor binding to histidine, a process known as “histidine switching”. Since histidine (p*K*a = 6.5) has a net positive charge at endosomal pH, but is neutral at physiological pH, these

substitutions exclusively targeted disruption of receptor binding in endosomes, thereby promoting G-CSF recycling (108).

1.2.3 Tumor animal model

Animal tumor models are important tools for preclinical tumor studies because they provide an *in vivo* environment that cannot be reproduced *in vitro*. These models can be used to investigate the factors included in malignant transformation, invasion and metastasis, and to test response to therapy. Currently, several techniques have been established and applied for animal tumor model. Selecting the appropriate set of techniques is a crucial step in generating mouse models of cancer.

1.2.3.1 Transplanted tumor model

1.2.3.1.1 Syngeneic model (allograft model)

Syngeneic models consist of tumor tissues derived from the same genetic background as a given mouse strain.This model offer several benefits as they are available with hosts at a low cost, reproducible and grow in immunocompetent hosts which retain the tumor-stromal interactions (109,110). Moreover, a wide variety of tumor types can be performed by this model which have a long history of use and therefore a strong baseline of drug response data as well as the studies with this model are easily conducted with simplicity of statistical analysis required for data validation (109,110)**.** Therefore, this model has been applied for a variety of studies especially immunotherapeutic study.

It is not only the preclinical cancer investigation that uses syngeneic models in the experiment; some kinds of transplantation experiment also applied this model for studies. To illustrate, Dare et al. performed murine syngeneic heart transplant model to assessed the efficacy of the mitochondria-targeted anti-oxidant MitoQ in reducing post-transplant ischemia-reperfusion injury (111). Although syngeneic transplantable tumors have long been employed in tumor immunology investigations; they have several properties that lack in human diseases and do not closely mimic those

characteristics. Consequently, this makes them suboptimal for predicting immunotherapy efficacy in patients (112). First of all, the tumor cells are rodent. In the present days, specific molecules have been used as targets in modern medicines. So, the identity between the mouse and human attributes of target biomolecules can be a serious limitation for syngeneic models (112,113). Moreover, since most transplantable tumor cell lines were obtained many years ago and syngeneic mouse strains now may no longer be completely syngeneic with these cell lines. Therefore, many transplantable tumors may be partly compatible with their syngeneic host (112). Finally, transplantable tumors tend to be aggressive and grow very rapidly following inoculation, while human tumors generally develop more slowly through a step of damage DNA accumulation leading to the transformation from pre-malignant to malignant pathologies. Hence, the immunopathologic scenario of patients is slowly progress in tumors, whereas in experimental animals the immune system with transplanted tumors is suddenly exposed. These dynamic differences may result in the variation of immunological outcomes, such as tolerance versus activation (112,114).

1.2.3.1.2 Xenograft model

Another transplantation model is called a xenograft, which involves actual human cancer cells or solid tumors which are transplanted into a special host in different species, usually a mouse. The mouse is immunosuppressive, such as in genetically manipulated athymic mice (*nu/nu*), or in severe combined immunodeficiency mice, which the immune components are defected in both T cells and B cells. This resulting in the event that foreign cells will not be rejected by the host when a transplantation is performed. These transplants may be orthotopic, meaning that the tumor is placed in the site it would be expected to arise naturally in the host: human liver tumor cells placed in the mouse liver. Alternatively, they may be subcutaneous, or placed just beneath the host skin. An advantage of xenografting is that malignant cells are human which carries human genetic materials. Therefore, it may be more representative of the properties and mutations and consequently express the human homolog of the target biomolecules (115).

As a xenogeneic model has several benefits as descried, many investigations have used this model for a cancer study and anti-cancer drug development in both solid tumors and hematologic malignancies.However, this provides the nonhuman microenvironment around the tumor that altered interaction patterns between the human tumor and the extracellular matrix of the murine host. To solve this problem, nude mouse models of patient-derived cancer xenograft have been established for minimizing these differences when patient tumor mass are directly transplanted into mice, whereby the key characteristic of xenograft closely resembles the histopathology of the patient tumor (116,117). Taken together, this suggested that patient-derived xenograft platform is beneficial for preclinical investigation of novel therapeutic approaches.

However, these models are more costly to operate than syngeneic models and, most importantly, the microenvironment around the tumor is provided by the nonhuman host. Moreover, because mouse hosts are immunodeficient and susceptible to microbes, it is therefore necessarily to house the mice in a microbe-free environment. Therefore, it is important to note that although some anti-tumor agents show activity against human tumor xenografts, it may be unsuccessful to demonstrate anti-cancer activity in human clinical trials (118).

1.2.3.2 Autochthonous tumor models

In this model, a tumors arises or is induced in a rodent host grown outbred or inbred, or those from animals containing genetic mutations which are responsible to tumor susceptibility (114).

1.2.3.2.1 Carcinogen-induced models in immunocompetent rodents

Carcinogen-induced tumors of various solid and hematological cancers, usually in rats, are among the oldest models that have proven to be useful pre-clinical models in cancer biology. These models utilize the topical, i.p., or p.o. (per os; oral administration) of a diverse of polycyclic aromatic hydrocarbon carcinogens e.g., N-nitroso-N-methylurea (NMU), 7,12-dimethyl-benz(*a*)anthracene (DMBA),

azoxymethane (AOM), and tobacco-smoke associated carcinogens and nitrosamines including benzo(*a*)pyrene (BaP), diethyl-nitrosamine (DEN), 4-methylnitrosamino-3-pyridyl-1-butanone (NNK), and N-nitrosobis (2-oxopropyl) amine (BOP) in established protocols either alone or in combination with known tumor promoter agents, e.g., phorbol esters, to induce specific cancers in a variety of immune-competent mice and rodent strains (119). Chemical carcinogens cause tumor formation by interacting with some proteins or compounds, and disrupting the molecular pathways associated with cellular growth, proliferation, and death (120). This model also response to etiologically relevant environmental carcinogens and tumor-promoting agents. Moreover, exposure to such chemicals may stimulate the systemic effects that are difficult to replicate in genetically engineered mice (114). Indeed, these models are infrequently applied as cancer treatment models, since the tumors are internal; there is some variation; and because the hosts are rats, a larger animal (109).

1.2.3.2.2 Genetically engineered mouse models (GEMMs)

Transgenic mice, both germ-line and conditionally controlled, are invaluable tools for deeper understanding of complex biological mechanisms linked to the impaired regulation of specific both oncogenes and tumor suppressor genes in cancers. Such GEMMs provide excellent experimental systems increasingly used for preclinical testing of molecularly targeted therapies, as they depend on the specific gene abnormalities and intracellular signaling pathways engineered to drive the malignant process. These may be genetically engineered by exchange of the endogenous genetic element of a gene to more closely mimic human carcinogenesis in mice, called knock-in mice, or their genetic may be molecularly disrupted for suppressing its function, called knock-out mice.In these points, tumorigenic progress spontaneously and autochthonously in situ with the suitable tumor-stroma environment or organ of an immunocompetent host (121,122). Therefore, the therapeutic effect of drugs on the dynamics of the immune response mechanisms, the invasion and metastatic spread of primary tumors, and tumor neovascularization can

be better examined with GEMMs in fully immunocompetent host than in transplanted tumor models in an immunocompromised host (119).

Despite early models knocked out the genes in all tissues, GEMMs now allow for inducible, tissue-specific expression of oncogenes as well as conditional, tissue-specific deletion of tumor suppressors (123). Moreover, although transgenic mice may develop cancers in a variety of organs with an inherently regulated development closely resembling human carcinogenesis, the use of GEMMs has been limited not only as a result of their cost and limited availability but also because of asynchronous development of tumors in the transgenic host. These models often have low penetrance and can be heterogeneous with respect to tumor frequency, latency for tumor development, and growth properties. While most human xenografts are grown in inbred immunocompromised host (e.g., athymic nu/nu, and SCID mice), both syngeneic transplantation and transgenic GEMMs are developed in outbred murine strains.This different genetic backgrounds may cause tumor to be permissive or suppressive to varying degrees (123,124).This non-uniform genetic background of the host can result in the variety of outcomes in therapeutic efficacy of anti-tumor drug studies, including to differences in pharmacokinetic, metabolic, and tolerability profiles as well as to heterogeneity in the growth, latency, and progression of tumors (119).

1.2.4 Interleukin-18

IL-18 or IFN-γ inducing factor is a pro-inflammatory cytokine belonging to the IL-1 family, which can be generated by many cell types such as intestinal epithelial cell, macrophage, dendritic cell, Kupffer cell, and adipocyte (4–7,125). When cells are activated by IL-18 activator, this protein is first produced in 24 kDa pro-form and then is cleaved into 18 kDa mature form by caspase-1, which is stimulated when inflammasomes, an intracellular multiprotein, are activated (8,126,127). On the other hand, pro-IL-18 can be processed into its active form by other mechanisms, for instance, Fas ligand (FasL) stimulation leads to release of biologically active IL-18 in caspase-1-deficient mouse macrophages (128). Moreover,

other extracellular enzymes including protease 3 (PR-3), serine protease, elastase and cathepsin G can process this pro-form into the mature form (8,129).

1.2.4.1 IL-18 gene and stimulation

IL-18 gene is located on chromosome 11q22.2-q22.3 and is composed of six exons and five introns over 20.8 kb. It does not contain a TATA box, and the expression of the gene is regulated by at least two distinct promoters; one is located upstream of untranslated exon 1 (promoter 1) and the other upstream of exon 2 (promoter 2) (130). PU.1 is proposed as one of transcription factors of IL-18 based on its gene structure. However, other studies indicated that PU.1 acts only as a weak activator which requires other transcription factors to transcribe the gene (131). Other investigations show that a GC-rich region within the proximal promoteris also important in IL-18 expression (132). Moreover, nuclear factor (NF)-κB recognition sequences, found in the IL-18 promoter, is propose for its additional role in regulating *IL-18* gene expression (133).

IL-18 contains 504 polymorphic loci within a region including 3' and 5' near gene region. There are various functional polymorphisms in the promoter (134). Two single nucleotide polymorphisms (SNPs) at positions -607 A/C and -137 G/C in IL-18 promoter are proposed to influence the IL-18 production and activity(135,136). Because -137 position is a binding sites for histone 4 transcription factor 1 (H4TF-1) and -607 is cAMP responsive element binding protein (CREB) transcriptional binding site. These polymorphisms could alter the IL-18 promoter activity (136–138). Recent studies have suggested that IL-18 polymorphisms may be associated with the risk of allergic disease, some cancers, autoimmune disease, outcome of different infections such as hepatitis C and AIDS (134,135,138).

The IL-18 precursor is constitutively expressed in many cell types. This may be due to particular two locations of promoter as described previously. Both promoters are a TATA-less type, while most other cytokines use a TATA-type promoter (128). Interestingly, the promoter 2 activity is constitutive, whereas promoter 1 is up-regulated by activation, presumably leading to uncommon, constitutive expression of IL-18 (128). On the basis of these special features of IL-18

gene expression, the constitutive inactive precursor remains in the intracellular compartment. In order to induce biologically active mature IL-18 secretion, at least two components of activator are required. Several TLR agonists such as lipopolysaccharide, have been reported to induce IL-18 production, but the inactive precursors of IL-18 require cleavage into the active cytokines by an enzymatic process (8).

Oligomeric multi-protein complex called the inflammasomes are important regulators in maturation process of IL-18. Inflammasomes regulate the processing of pro-IL-18 by caspase-1 (8). The known caspase-1-activating inflammasomes are constituted from a PRRs of the nucleotide-binding oligomerization domain receptors (NLR) family such as NLR family pyrin domain (PYD) containing 1 (NLRP1), NLRP3, NLRP6, NLRP7, NLR family CARD domain-containing protein 4 (NLRC4), the DNA-sensing absent in melanoma 2 (AIM2) and retinoic acid-inducible gene *1* (RIG-1) receptors (139). The activation of inflammasome happens in response to pathogen or damage-associated molecular patterns (PAMPs or DAMPs) such as nucleic acid structures that are unique to microorganisms, bacterial secretion systems and their effector proteins, microbial cell wall components, ATP, nigericin, or aluminum hydroxide (8,139). Following cleavage by biologically active caspase-1, mature IL-18 is secreted from the cells and then bind to its receptor or IL-18 binding protein as negative regulation (140).

1.2.4.2 IL-18 signaling

Like IL-1, IL-18 provides its biological function by binding to a specific receptor on the surface of target cell, composed of two chains: IL-18 receptor α chain (IL-18Rα) (also known as IL-1Rrp1, IL-18R1 or IL-1R5) and IL-18 receptor β chain (IL-18Rβ) (also termed IL-18RacP, IL-18RII or IL-1R7) (Fig. 5). IL-18 bind directly to form a low-affinity complex with IL-18Rα, which recruit IL-18Rβ to form a high-affinity complex-inducing signaling pathways. The binding residues of IL-18 to it receptors were identified as three sites: site I and II are specific to IL-18Rα and site III to IL-18Rβ (27). Arg13, Asp17, Met33, Asp35 and Asp132 are included in site I, Lys4, Leu5, Lys8, Arg58, Met60 and Arg104 are in site II, and Lys79, Lys84 and

Asp98 are involved in site III (27). It is not only residues mentioned above; Glu6, Lys53 and Thr63 are also reported to be important in bioactivity and binding mode of IL-18 (28,29) (Table 1).

The binding of IL-18 to its receptor allow the recruitment of the intracellular adapter molecules, myeloid differentiation factor 88 (MyD88), through the Toll-like receptor/IL-1R (TIR) domain of MyD88, which associates with the TIR domain of Toll and interaction of cytoplasmic IL-18R to dock to the complex (141–143). This event allows dead domain of MyD88, IL-1 receptor associated kinase (IRAK) and IRAK-4 interaction with each other mediate the recruitment of IRAK, subsequently causing IRAK4-dependent IRAK phosphorylation (144). Activated IRAK migrates from the complex and translocates into the cytosol which it interacts with TNF receptor-associated factor 6 (TRAF6), an adapter molecule, resulting in its degradation by ubiquitylation and phosphorylation of transforming growth factor-β-activated kinase 1 (TAK1) (145). This leads to the nuclear translocation of the NF-κB and consequent modulation of gene expression (Fig. 5) (8,127).

Table 1. Possible binding site of IL-18 for IL-18R.

Binding site	**Amino acid positions**	**IL-18 receptors**
Site I	Arg13, Asp17, Met33 Asp35, Asp132	IL-18Rα
Site II	Lys4, Leu5, Lys8 Arg58, Met60, Arg104	IL-18Rα
Site III	Lys79, Lys84, Asp98	IL-18Rβ
Others	Glu6, Lys53, Thr63	-

1.2.4.3 Immunoregulatory role of IL-18 and IL-18 binding protein (IL-18BP)

IL-18 exhibits features of other inflammatory cytokines, such as induces the expression of cell adhesion molecules, nitric oxide synthesis, and chemokine production and its effector role in immunoregulatory is rapidly expanding (140). IL-18 was firstly found to be important for inducing the Th1 response when co-stimulate with IL-12 or IL-15, which is characterized by the production of IFN-γ (6,8,9). Mechanically, IL-18 directly controls IFN-γ promoter by binding the AP-1 site in the IFN-γ promoter, while following the activation of co-stimulatory signals by CD28 in T cells, IL-12 only mediates its production (146). However, when combined with IL-2, IL18 enhances the production of IL-13 in cultured T cells and NK cells and can polarize Th2 response characterized by the production of IL-4, IL-5 and IL-10 receptor in murine experimental models (8,147). IL-18 also stimulates NK cells to produce IFN-γ and seems to play a distinct role in driving the polarization of a helper NK population which migrates to lymph node and contributes to the development of Th1 responses by interaction with DCs (148,149). Neutrophils is another cell that can produce IL-18, which increases their mobilization, up-regulation of CD11b, cytokine and chemokine production, granule formation, and oxidative burst following exposure to fMLP (150–152). Moreover, in combining with IL-23, IL-18 also has ability to polarize Th17 differentiation by enhancing the production of IL-17 in Th17 and γδT cells (153,154). Th17 polarizton affected by IL-18 and IL-18Rα may play roles in the inflammatory process in several chronic autoimmune diseases (8). IL-18 is shown to induce the production of IL-17 and IL-23 from lymphocytes of systemic lupus erythematosus (SLE) patients ex vivo (155).

The combination of IL-18 and IL-12 can induce the up-regulation of IL-18R α and β chain and the production of IFN-γ by $CD8^+$ T cells, NK cells, and activated B cells (8,127,156,157). It was also found that IL-18 has ability to induce the generation of GM-CSF, TNF-α, IL1β, IL-8 and IL-32 in peripheral blood mononuclear cells (8,158). This cytokines has a chemoattractive property to T cells, can increase the granule formations in neutrophils, and up-regulate the expression of Fas-ligand in T cells and NK cells, leading to the apoptosis of Fas expressed cells (8,158).

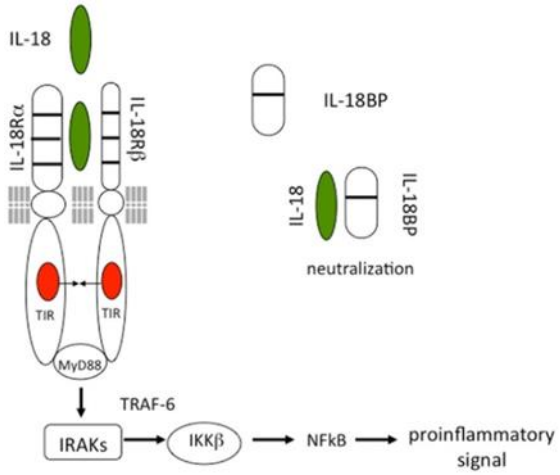


Figure 5. IL-18 signaling pathway (188). After activation, IL-18 triger the signaling cascade that lead to the stimulation of NKκB and proflammatory cytokines induction. The activity of IL-18 can be inhibited by IL-18BP.

Unsuitable regulation of IL-18 can result in potentially dangerous inflammatory responses. IL-18 can be regulated by IL-18BP, a soluble protein that inhibits the binding of IL-18 to its receptor. IL-18BP is a constitutively secreted protein, which built approximately 20-30 times more IL-18 and binds specifically to mature IL-18 only (8,127,159). It is considered as the natural mechanism of the body for attenuating the stimulation of Th1 cells, resulting in the absence of autoimmune disease (8,127,159). There are four isoforms of human IL-18BP involving IL-18BPa to d (8).Alternative mRNA splicing explains these four IL-18BP isoforms, which differ primarily in their C-terminal region (8). In fact, IL-18BPa isoform with an Ig domain and 29 aa C-terminal exhibits the greatest affinity to IL-18 followed by IL-18BPc (160). IL-18BPa and IL-18BPc neutralize > 95% of IL-18 at a molar excess of 2 (160). IL-18BPb and IL-18BPd with entire absence of the Ig domain resulting in the ineffective binding and neutralizing of human IL-18 (160). Both IL-18 and IL-18BP are up-regulated in inflammatory situations suggesting that IL-18BP appears to be up-regulated in pathologies with high IFN-γ level, suggesting that IL-18 indirectly increases the production of its own inhibitor in a feedback response (161).

1.2.4.4 IL-18 and cancers

Several researches revealed that IL-18 has anti-cancer properties through the stimulation of natural killer cells (NK cells), cytotoxic T lymphocytes, inhibits the growth and spread of cancer cells and increased the expression of Fas-ligand in immune cells for Fas expression and cancer cells killing (10–14). Overexpression of IL-18 in human tongue squamous cell carcinoma cell line led to cell death via apoptosis, increased caspase 3/7 activity, increased expression levels of IFN-γ and decreased the expression of cyclin D1 (11). IL-2 plus IL-18 combination also recover NKG2D expression, which is decreased by TGF-β in NK cell (162). Moreover, there are several successful studies in which IL-18 was used as a tumor suppressor protein in animal model and this protein could be applied to a patient safely (15–18). In an animal model, tumor regression activity of IL-18 can be achieved by administrating the cytokine into intraperitoneal (19–21), intravenous (19), intratumoral (22) and peritumoral (23) routes. This anti-tumor functions of IL-18 are mainly mediated through IFN-γ (24) which is generally known that IL-18 is an effective activator of IFN-γ production by T cells, NK cells and macrophages and this function can be synergistic in with IL-12 (25). Tse et al. showed that neutralizing antibody against IFN-γ completely eliminated IL-18-mediated anti-tumor effects (25). Consistently, Nagai et al. also demonstrated that IFN-γ played a role for the tumor inhibitory effects of IL-18 using a gene-transfer approach (26). Normal or RAG-1 or $CD4^+$ T cells and $CD8^+$ T depleted prostate cancer-bearing mice, in which the prostate cancer cells were engineered to express the a high level of IL-18, have a less cancer severity than anti-IL-18 antibody or anti-IFN-γ antibody injected mice and it was also found that $CD4^+$ T cells and $CD8^+$ T cells are highly recruited into tumor region than antibody injected mice, indicating the vital role of IL-18 in innate and adaptive immunity (24). In addition, when the TILs from cancer patient were treated with IL-18 and IL-12, and then pre-incubated with SGC-7901 gastric cancer cell line, which were then subcutaneously injected into BALB/C SCID mice, it was found that these mice have significantly longer survival rate than control, together with an increasing Th1 cytokines (163).

Combining IL-18 with other immunotherapy method has been widely studied to improve the efficiency of cancer treatment. Trial of IL-18 in combining with adoptive cell transfer showed that the addition of IL-18 into the step of treatment with DCs can destroy cancer cells more, both in vitro and in vivo (164,165). Using IL-18 as an adjuvant combination with other cytokines such as IFN-α, IL-15, IL-12 and IL-2 can also promote the productive DC-T cell interactions and the secretion of CCL19 to stimulate the naïve T cells to move to the lymph nodes and then are activated and proliferate in a colorectal cancer patient (166). It is also found that human IL18-IL2 fusion protein has ability to induce the production of IFN-γ in peripheral blood mononuclear cells (PBMCs) (15). In addition, when tested this fusion protein in colorectal cancer- or lung cancer bearing mice, the amounts of IFN-γ and cytotoxicity of NK cell are higher than the stimulation with only IL-18 or IL-2 or combine it together in separated protein (15).

Using IL-18 in combination with oncolytic virus is another strategy to deliver IL18 to the target tissue. Tumor-selective replicating adenovirus expressing IL-18 can enhance the cytopathic effects, apoptotic cell death and anti-angiogenesis event via decrease the expression of VEGF in renal cell carcinoma-bearing nude mice (167,168). Another group also expands this technique by engineering the oncolytic adenovirus to express both IL-18 and IL-12, which lead to the increasing survival rate of melanoma bearing mice and also enhance the recruitment of NK cells, $CD4^+$ T cells and $CD8^+$ T cells in tumor region (169).

Although IL-18 is a pro-inflammatory cytokine that causes inflammation, mice or rabbits receiving IL-18 does not have fever (170). Moreover, intravenous injection of IL-18 in patients with cancer, chills and fevers are not common and found that fevers were observed only in 3 of 21 patients at doses of 100 and 200 μg/kg (171). So, these trials showed that IL-18 can be safely used in phase I clinical study in cancer patients (16,17). As a result, this indicates the possibility of using IL-18 as a therapeutic agent to stimulate the immune system to fight cancer.

Since several proteins have been engineered for improving their activity, we now have been successful to produce engineered IL-18 that has increasing activity when testing with NK-92MI cell line. This IL-18 had substitution of the amino acid residue 6, from glutamic acid to lysine (E6K), together with residue 63, from

threonine to alanine (T63A), and this form of mutated IL-18 showed the higher activity with respect to native protein by inducing the expression of IFN-γ, about factors of 15-20. Since many reports have demonstrated that the anti-tumor functions of IL-18 are mainly mediated through IFN-γ (24–26), the result from previous experiment suggested that this double mutation form might result in a more potent antitumor response *in vivo*.

1.2.5 Protein expression system

Protein expression refers to the way in which proteins are synthesized, modified and regulated in living organisms and expression system refers to the factors that work together to yield a particular gene product such as a protein, ribozyme or RNA particle. In general, proteomics research involves investigating any aspect of a protein such as structure, function, modification, localization, or protein interaction. Given the size and complexity of proteins, chemical synthesis is not a viable option for this endeavor. Instead, living cells and their cellular machinery are usually used as the expression system to build and construct proteins made up of a gene, which is encoded by DNA, and the machinery needed to make mRNA from the DNA and translate it into a protein (172).

There is a wide variety of protein expression systems available, but the most popular includes bacteria, yeasts, mammals, and insects. Protein quality, functionality, production speed and yield are the most important factors to consider when choosing the right expression system for recombinant protein production (Table 2).

1.2.5.1 Bacteria

1.2.5.1.1 *Escherichia coli*

The most widely used microorganisms in recombinant protein production are bacteria, more specifically *E. coli*. One of the reasons is that this organism is very well known and established in each laboratory. Moreover, the expression of proteins in *E. coli* is easiest, quickest and cheapest. So, it is no surprise that *E. coli* systems are

also most commonly used for industrial and pharmaceutical protein production. *E. coli* system is the basis for efforts in protein engineering and high-throughput structural analysis (173–175).

E. coli is perfect to produce small proteins that do not require posttranslational modifications. But, being prokaryotes, they are not so suitable to synthesize big proteins or those from higher organisms. These proteins often show complex folding, requiring disulfide bonds or cellular chaperones for the folding which are not available in prokaryotes (175,176). Moreover, many of these proteins require additional modifications after synthesis that cannot be performed in prokaryotic organisms. In addition, bacteria possess pyrogens and endotoxins that must be totally eliminated from proteins to be injected in animals or humans. Moreover, proteins which are produced as inclusion bodies are often inactive, insoluble and require refolding.

As an alternative choices, some other approaches have been developed to reduce the amount of inclusion bodies produced, such as bacteria growing at temperatures below the standard. In addition, recent progress in the fundamental understanding of transcription, translation, and protein folding in *E. coli*, together with the availability of improved genetic tools, is making this bacterium more valuable than ever for the expression of complex eukaryotic proteins. Its genome can be quickly and precisely modified with ease, promotor control is not difficult, and plasmid copy number can be readily altered. This system also features alteration of metabolic carbon flow, avoidance of incorporation of amino acid analogs, formation of intracellular disulfide bonds, and reproducible performance with environmental control. *E. coli* can accumulate recombinant proteins up to 80% of its dry weight and survives a variety of environmental conditions.

Table 2. Comparison of expression systems (155)

Characteristics	***E. coli***	**Yeast**	**Insect cells**	**Mammalian cells**
Cell growth	rapid (30 min)	rapid (90 min)	slow (18-24 h)	slow (24 h)
Complexity of growth medium	minimum	minimum	complex	complex
Cost of growth medium	low	low	high	high
Expression level	high	low - high	low - high	low - moderate
Extracellular expression	secretion to periplasm	secretion to medium	secretion to medium	secretion to medium
Posttranslational modifications				
Protein folding	refolding usually required	refolding may be required	proper folding	proper folding
***N*-linked glycosylation**	none	high mannose	simple, no sialic acid	complex
***O*-linked glycosylation**	no	yes	yes	yes
Phosphorylation	no	yes	yes	yes
Acetylation	no	yes	yes	yes
Acylation	no	yes	yes	yes
gamma-Carboxylation	no	no	no	yes

1.2.5.1.1.2 Bacillus

Gram-positive bacteria, such as *B. subtilis*, have also been utilized for recombinant protein production, with the advantage that they can secrete large amounts of properly folded product and contain low concentrations of pyrogens (177). Industrial strains of *B. subtilis* are high secretors and host strains used for successful expression of recombinant proteins are often deleted for genes amyE, aprE, nprE, spoIIAC, srfC and transformed via natural competence (175). However, recombinant plasmids are not stable in *B. subtilis* and chromosomal integration is the only way to obtain a stable recombinant cell. Yields are lower than those of Gram-negative bacteria, due mostly to the high activity of endogenous proteases.

An exoprotease-deficient *B. licheniformis* host strain has been specifically tailored for heterologous gene expression. It is asporogenous and gives high extracellular expression levels with minimal loss of product due to proteolytic cleavage subsequent to secretion. Vectors for *B. licheniformis* include both integrating as well as autonomously replicating forms. The essential elements of these vectors are the strong promoter, efficient terminator, suitable signal sequence, and a selection marker. Extensive studies on plasmid stability have shown the vector to be recombinationally stable and segregationally stable during fermentation in the absence of selective pressure.

Heterologous proteins successfully expressed in Bacillus systems include IL-3EGF and esterase from *Pseudomonas*. Homologous proteins include *Bacillus stearothermophilus* xylanase, naproxen esterase, amylases and various proteases (175).

1.2.5.2 Yeast

Yeasts, the single-celled eukaryotic fungal organisms, are often used to produce recombinant proteins that are not produced well in *E. coli* because of problems dealing with folding or the need for glycosylation. Yeast system provides advantages of both prokaryotic and eukaryotic expression systems including: high expression level, low cost, ease of scaling, and proper protein folding.

1.2.5.2.1 *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae* was the first yeast species to be manipulated for recombinant protein expression, and many proteins have been produced in it. Due to its many applications, excellent knowledge of *S. cerevisiae* molecular biology and physiology has accumulated (178). However, glycosylation by *S. cerevisiae* is often unacceptable for mammalian proteins because the *O*-linked oligosaccharides contain only mannose whereas higher eukaryotic proteins have sialylated *O*-linked chains. Furthermore, the yeast over-glycosylates *N*-linked sites leading to reduction in both activity and receptor-binding, and may cause immunological problems (176).

Like other yeasts, *S. cerevisiae* can secrete recombinant proteins to the culture medium. Moreover, intracellular proteins are usually properly folded. As other eukaryotes, yeasts are also capable of performing most posttranslational processing typical of mammalian cells. Products on the market which are made in *S. cerevisiae* are insulin, hepatitis B surface antigen, urate oxidase, glucagons, granulocyte macrophage colony stimulating factor (GM-CSF), hirudin, and platelet-derived growth factor.

1.2.5.2.2 *Pichia pastoris*

As single cell protein production was not economically attractive, *Pichia pastoris* was proposed as a host for recombinant protein production in the 1980s (179). Very high cell densities have been obtained, up to 100 g of dry weight per liter, and also high protein concentrations, up to 1 g/L of secreted recombinant protein (180). *P. pastoris* produces high levels of mammalian recombinant proteins in the extracellular medium. Production of serum albumin in *S. cerevisiae* amounted to 0.15 g/L whereas in *P. pastoris,* the titer was 10 g/L (181)

P. pastoris is a methyltropic yeast, and can use methanol as its only carbon source. The growth of *P. pastoris* in methanol-containing medium results in the dramatic transcriptional induction of the genes for alcohol oxidase (AOX) and dihydroxyacetone synthase (182). After induction, these proteins comprise up to 30% of the *P. pastoris* biomass. Investigators have exploited this methanol-dependent gene induction by incorporating the strong, yet tightly regulated, promoter of the *alcohol oxidase I* (*AOX1*) gene into the majority of vectors for expressing recombinant proteins. The *P. pastoris* expression vectors integrate in the genome whereas by contrast, *S. cerevisiae* vectors use the more unstable method of replicating episomally. The length of time to assess recombinant gene expression with the *P. pastoris* method is approximately 2-3 weeks which includes the transformation of yeast, screening the transformants for integration, and an expression time course (182).

Glycosylation is less extensive in *P. pastoris* than in *S. cerevisiae* due to shorter chain lengths of *N*-linked high-mannose oligosaccharides, usually up to 20 residues compared to 50–150 residues in *S. cerevisiae* (Table 3). *P. pastoris* also lacks α-1, 3- linked mannosyl transferase which produces α-1, 3-linked mannosyl terminal linkages in *S. cerevisiae* and causes a highly antigenic response in patients. Moreover, two groups have completed extensive engineering to create *P. pastoris* strains that produce complex *N*-linked glycan structures comparable to those produced by mammalian cells (183). However, only the strains developed by Roland Contreras' group are available to investigators and must be licensed through Research Corporation Technologies.

Table 3. Comparison of two yeast systems (184).

Expression system	Advantages	Disadvantages
Saccharaomyces cerevisiae	• Good expression levels • Choice of secreted or cellular expression • Low cost • Simple culture conditions • Scalable • Able to perform most eukaryotic post-translational modifications • Efficient protein folding • Endotoxin-free	• Likely lower expression than with *Pichia pastoris* • Secretion likely lower than with *Pichia pastoris* • Glycosylation still different to mammalian cells • Tendency to hyperglycosylate proteins • N-glycan structures considered allergenic
Pichia pastoris	• High expression levels • Low cost • Simple culture conditions • Relatively rapid growth • Scalable • Choice of secreted or intracellular expression • Protein secretion efficient and allows simple purification • Extensive posttranslational modification of proteins • Efficient protein folding • N-glycosylation more like higher eukaryotes than with *Saccharomyces cerevisiae* • Low native protein secretion	• Use of methanol as inducer is a safety (fire) hazard at scale • Glycosylation still different to mammalian cells

1.2.5.3 Baculovirus/insect cells

Insect cells are a higher eukaryotic system than yeast and are able to carry out more complex posttranslational modifications than the other 2 systems. They also have the best machinery for the folding of mammalian proteins and, therefore, give you the best chance of expressing soluble protein of mammalian origin. The most commonly used vector system for recombinant protein expression in insects is the baculovirus. The most widely used baculovirus is the nuclear polyhedrosis virus (*Autographa californica*) which contains circular double-stranded DNA, is naturally pathogenic for lepidopteran cells, and can be grown easily in vitro.

Baculovirus is routinely amplified in insect cell lines derived from the fall armyworm *Spodoptera frugiperda* (Sf 9, Sf 21), and recombinant protein expression is completed either in the aforementioned lines or in a line derived from the cabbage looper *Trichoplusia ni* (High-Five) (185). The baculovirus-assisted insect cell expression offers many advantages. They can give the proper protein folding and S–S bond formation and have almost eukaryotic posttranslational modifications without complication, including phosphorylation, N-glycosylation and O-glycosylation, correct signal peptide cleavage, proper proteolytic processing, acylation, palmitylation, myristylation, amidation, carboxymethylation, and prenylation (175). Moreover, the most common promoters used with baculovirus expression, polH and p10 promoters, induce a high level of expression in the very late phase of the baculovirus infection. Recent information indicates that the baculovirus insect cell system can produce 11 g/L of recombinant protein (186). They are easy to scale up with high density suspension culture with no limitation on protein size. The disadvantages of insect cells are the higher costs and the longer duration before get protein (usually 2 weeks).

1.2.5.4 Mammalian cells

Even though many mammalian proteins have been successfully produced in bacteria, yeast, or insect cells, mammalian cell cultures are still the system of choice if mammalian-specific posttranslational modifications are required. Mammalian expression systems are often used for production of proteins requiring mammalian posttranslational modifications. Recombinant protein produced by mammalian cells rose from 50 mg/L in 1986 to 4.7 g/L in 2004 mainly due to media improvements yielding increased growth (186). Most labs use HEK (human embryonal kidney) or CHO (Chinese hamster ovary) cell lines for preparative expression of more complex proteins. Both cell lines can be used for both transient and stable cell line expression which is more time consuming due to the generation of stable cell lines but offers higher productivity and less variation if long-term production of a target protein is required.

Compared with other eukaryotic cell expression system, the proteins from target gene expressed in mammalian cell are nearly the same with the nature proteins in the term of structure, glycosylation style and the way of glycosylation, and can be assembled into a multi-subunit protein. Moreover, the mammalian cell can be cultivated in suspension way or in serum-free medium with high density which the volume could reach to 1,000 L and even more.

Mammalian systems do have some drawbacks. The secreted foreign proteins is poor and its processes are expensive (175). Moreover, mammalian cell processes can be contaminated by viruses.

1.2.6 Molecular dynamic (MD) simulation

1.2.6.1 Basic principle of MD simulation

Molecular dynamics (MD) simulation is a computational method to virtualize molecular systems based on Newtonian physics, whose successive positions and velocities can be calculated from the forces that act on all particles (187,188). Nowadays, this method is widely used to produce the actual conformations over the time evolution of real molecules, such as proteins and other biological macromolecules (188), and also kinetic and thermodynamic information (187). A general process of approximation requires only three inputs:an initial structural coordinate, a potential, and algorithms for propagation (189). The initial coordinate or a computer model of the molecular system can be obtained from nuclear magnetic resonance (NMR), crystallographic (X-ray), or homology-modeling data. The potential is calculated from an energy equation based on force field, a parameterization of the energy surface of the protein (189).

Force field can be used to predict two groups of molecular characteristics: the first is bonded interaction, which derived from bond stretching, bending of valence bond angle, and the direction of dihedrals, which are modeled using a sinusoidal function that approximates the energy differences between eclipsed and staggered conformations. The second is nonbonded interaction that evaluates electrostatic data, and dispersion by using the Lennard-Jones 6-12 potential and Coulomb's law (188,190). Therefore, to obtain the actual behavior of real molecules in motion, the force field will be exploited mimic the ideal stiffness and lengths of the springs that describe chemical bonding and atomic angles, determining the best partial atomic charges used for calculating electrostatic-interaction energies, identifying the proper van der Waals atomic radii, and so on (188). Currently, many different force field models have been available for MD simulation such as CHARMM (191), AMBER (192), and GROMACS (193). Although each force field could generally give similar results (188), some found that with different force field for simulation, different structural trends emerged (194). Therefore, the selection of force field is usually a combination of personal preference and type of molecular simulation suite.

Once the force calculation of each particle in the system is adjusted, the next crucial step is the modeling of the accelerations and velocities, and to update the atom positions after a solvent model is upon according to Newton's laws of motion. After integration of movement is completed numerically, a simulation of time step shorter than the fastest movements of the molecule can be modeled, often by only 1-2 femtosecond (fs), in full atomic detail with modern computers and modern algorithms. This process is then repeated, typically millions of times (188,189,195). Moreover, if the system is more simplified, the much longer time steps are possible, and consequently the effective length of the simulations is possibly extended (195).

Since MD simulation requires so many calculation and algorithm, the requirement of computer cluster or supercomputer is essential if not hundreds of processors in parallel (188). The most popular simulation software packages, which come together with their force field, for example AMBER (192), CHARMM (191), GROMAC (193), and NAMD (196), are compatible with the Message Passing Interface (MPI), a system of computer-to-computer messaging, which can greatly reduce the computation time when multiple processors operate simultaneously (188,195). The major benefit of the MD simulation is an ability to model the experimental conditions in which a typical biological question is addressed, and many studies has shown that when these simulations were compared with experimental data, the result have shown good agreement between MD approach and experimental measurements of macromolecular dynamics (197–199).

Although MD simulation has many advantages for applying to a number of scientific questions, it stil has some limitation. First of all, the force field used in the study cannot apply to some other chemical compounds, for example the drug molecule, which has the enormously large size of the chemical space, number of functional groups and their combinations, therefore, it is very difficult to develop the special parameters for all of molecule (200,201). The second is that MD simulation need a person who has expertise in this field for processing and analyzing all of steps since a lot of parameters for different methods in different software packages need to be set up carefully (202). Moreover, the search problem has also been described because of the rough energy surface of biomolecular systems and the difficulty of finding the global minimum (201). Other problems have also been addressed, for

example, the need of a large number of configurations for biomolecular modeling governed by statistical mechanics or the unavailability of enough experimental data resulting in a hard interpretation, and are sometimes not precise enough (201).

1.2.6.2 MD simulation and application

1.2.6.2.1 Structure prediction

Proteins are an inherently dynamic biological molecule that in physiological environment can adapt themself from one conformation state to another form beyond those found in the crystal structures in the Protein Data Bank (PDB) (203). The functions of a protein usually depend on these conformational states, and the understanding of protein function and regulation is very important for medical and pharmacological research. Therefore, structure prediction has been one of the ~~most~~ major questions addressed in these areas. Currently, MD simulation are frequently applied to identify conformational changes, to define equilibrium distributions among different conformations, and to investigate changes of protein structure resulted from mutation or ligand binding related to drug design (204).

One of study in therapeutic drug design is the prediction of function and regulation of kinases, a class of enzymes that have been used as therapeutic targets for cancer and autoimmune diseases. The investigation of structural changes in the activation and inactivation states of kinases showed the intermediate forms that can be targeted for drug development. For example, the conformational transition pathway of the epidermal growth factor receptor (EGFR) cytoplasmic kinase domain has been investigated via MD simulation combining with another method (205). Faraldo-Gomez and Roux also used MD simulations to characterize the regulation of Src family tyrosine kinases, which depends on an inactivation process in which the auxiliary domains (206). Moreover, some other enzyme structural changes have also been studied. Gasper et al. discovered the two interrelated allosteric pathways of pro- and anti-coagulant activities of thrombin using conventional MD and accelerated MD (AMD) simulations (207). Moreover, conformational changes of cytochrome P450

(CYP) after ligand entering were also indentified by using MD simulation and other techniques (208,209).

1.2.6.2.2 Membrane transport

The movement of substances through membrane channels plays an important role in maintaining cellular constitution and transmission of biochemical signals. However, the knowledge about the regulation of transport efficiency and substrate selectivity has not been well illustrated. Furthermore, a dynamical scenario of multisteps in the transport cycle, and the transition between them, remain unresolved. Since MD simulation can be used to track all atoms inside the molecules under the modeling system, this let MD simulation take advatages to be used as an approach for a study of membrane transport process (204). Nowadays, MD simulations have been used as a solution to answer many questions about membrane transport process. The membrane transports that have been well characterized such as aquaporins (210,211), potassium channels (212,213), and glutamate transporters (214,215).

Aquaporins function as a cytoplasmic channel consisting of six transmembrane α-helices and two half-helices that conduct water, small linear alcohols, and gases. The simulations of aquaporin has been used to be an ideal example of investigation for molecular modeling since reported simulations date back several years ago (216). Simulations had shown that aquaporins need the coorperation of several water molecules in motion of orientation and translocation. Characterization of aquaporin selectivity has also been made by utilizing MD simulation. The high selectivity barrier against proton transport has been revealed by the combination of classical steered MD simulations with advanced quantum chemistry calculations (217). Moreover, an involvement of gas conduction (O_2, CO_2, and NO) in aquaporin has been also studied by MD simulation, and the result showed that the central tetrameric pore of aquaporins can be readily used by either gas molecule to permeate the channel (218).

1.2.6.2.3 Protein folding

The challenging topics in protein folding may be the determination of the native structure of the protein and the elucidation of the pathways of protein folding (204). Currently, MD simulation can be used to answer both topics particularly the direct observation of the dynamics of a folding event in atomic detail. A protein that has been exemplified as a good model for protein folding simulation is tendamistat, a α-amylase inhibitor derived from *Streptomyces tenda*, investigated for the role of disulfide bonds on the stability and folding kinetics (219,220). Basically, tenmidostat function need two-state folding behavior, and this behavior is sustained by disulfide bond. The study showed that the removal of the C11-C27 or C45-C73 disulfide bond resulting in the loss of protein folding and stability (219). This was supported by MD simulation based on a Gō-like model, which was used to study the folding processes of protein tendamistat both native and disulfide mutants (220). This study showed that the folding behaviors of disulfide mutants lead to a large decrease in the thermodynamical stability and folding dynamic of folding nuclei (220).

Another example of MD application in protein folding is metal coupled protein folding since many proteins need a metal ion as a cofactor for their functions. The MD method needs to be modified for revealing the detailed mechanism of this type of protein folding. Li et al. has developed a theoretical model for zinc coupled folding of Cys2His2 type Zinc-finger by modified MD method (221). The the result showed that the Zn(II) binds to the peptide at the very beginning of the folding state at the two conserved cystein residues before the two conserved histidine residues (221). Moreover, the ligand exchange is required for their ion coupled since other non-specific ligands can bind to zinc ion before Zn(II) binding (221,222). For the folding process, modified MD simulation showed that the folding began with a hydrophobic collapse and then with the formation of the β-hairpin and the α-helix in a rate-limiting step since this step needed to overcome a high barrier (222).

1.2.6.2.4 Ligand binding

Generally, most signaling pathways in our body need the interaction between ligands and receptors. Modern molecular targeted therapy in various diseases also employs this interaction as a therapeutic strategy. MD simulation is powerful enough to predict the scenario that ligands bind spontaneously to proteins without any knowledge of the binding site (223–225). This leads to the most practical application in molecular recognition of structure-based drug design. Although the atomic structure of molecules produced by NMR, X-ray crystallography, and homology modeling provide valuable information of the structures, they are just a rigid form and cannot be used for molecular recognition study since drug binding are very dynamic processes. Therefore, using MD simulation could mimic/mock virtual screening with a clear advantage.

One compelling example of drug design based on binding affinity calculations from MD simulation is an identification of HIV reverse transcriptase inhibitor. The technique used in this study is Monte Carlo simulations that closely related to MD simulation, and the researchers discovered many molecules that proved experimentally active as highly potent anti-HIV agents with activities in protecting human T-cells from HIV infection (226). Another example is the screening of inhibitor of erythropoietin-producing hepatocellular (Eph) carcinoma receptor tyrosine kinases, which play an important role in angiogenesis and blood vessel remodeling in tumor progression. A novel class of low-micromolar inhibitors of EphA3 was identified with the binding energy smaller than -10 kcal/mol (227). Moreover, the researchers from the same group has identified 10 low-micromolar inhibitors which correspond to six distinct chemotypes to the zeta-chain associated protein kinase 70 kDa (ZAP70), which is implicated in inflammatory and autoimmune diseases by using an explicit solvent molecular dynamics simulation with restraints on the backbone atoms (228).

1.3 Objectives

1. To test the activity of human IL-18 produced by yeast.
2. Try to enhance the activity of this protein by mutagenesis.
3. To test anti-cancer capability of the protein through IFN-γ induction assay
4. To test the effect of IL-18 mutants on cancer tumorigenesis compared with wild-type IL-18.
5. To investigate the effect of IL-18 mutants on tumor infiltrating leukocytes compared with wild-type IL-18.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials and Equipment

Materials	**Manufacturers**
NK-92MI cell line	ATCC, USA
CT-26WT	ATCC, USA
RPMI 1640 medium	Gibco, USA
α-MEM medium	Gibco, USA
Fetal bovine serum	Gibco, USA
Horse serum	Gibco, USA
Penicillin-Streptomycin	Gibco, USA
GeneJET RNA Purification Kit	Thermo Scientific, USA
GeneJET Plasmid Miniprep Kit	Thermo Scientific, USA
Maxima H Minus First Strand cDNA Synthesis Kit	Thermo Scientific, USA
pTZ57R/T cloning vector	Thermo Scientific, USA
pPICZα A Pichia Vectors	Invitrogen, USA
E. coli DH5α strain	RBC Bioscience, Taiwan
Pichia pastoris KM71	Invitrogen, USA
LB broth	Sigma-aldrich, USA
Zeocin	Invitrogen, USA
*EcoR*I enzyme	Thermo Scientific, USA
*Xba*I enzyme	Thermo Scientific, USA
*Dpn*I enzyme	Thermo Scientific, USA
*Sac*I enzyme	Thermo Scientific, USA
Platinum *Pfx* DNA polymerase	Invitrogen, USA
TopTaq Master Mix Kit	QIAGEN, Germany
Yeast nitrogen base without amino acid	Amresco, USA
Dextrose	Amresco, USA
Peptone	Amresco, USA

Sorbitol	Amresco, USA
K_2HPO_4	Amresco, USA
KH_2PO_4	Amresco, USA
$Na_2HPO_4 \cdot 2H_2O$	Amresco, USA
$NaH_2PO_4 \cdot H_2O$	Amresco, USA
Biotin	Amresco, USA
Methanol	Merck Millipore, Germany
Glycerol	Merck Millipore, Germany
HisTrap HP column	GE Healthcare, USA
Imidazole	Merck Millipore, Germany
NaCl	Merck Millipore, Germany
Tris	Merck Millipore, Germany
Sodium dodecyl sulfate	Merck Millipore, Germany
Ammonium persulfate	Merck Millipore, Germany
TEMED	Merck Millipore, Germany
Glycine	Merck Millipore, Germany
Bromophenolblue	Merck Millipore, Germany
Acetic acid	Merck Millipore, Germany
Dithiothreitol (DTT)	Merck Millipore, Germany
Amicon Ultra4 centrifugal filter unit	Merck Millipore, Germany
Bovine serum albumin	Sigma-aldrich, USA
Bradford protein kit	Merck Millipore, Germany
Polyvinylidene difluoride (PVDF) membrane	Bio-rad, USA
Acrylamide/Bis-acrylamide	Bio-rad, USA
Mouse anti-IL-18 antibody	R&D system, USA
HRP-conjugated goat anti-mouse antibody	R&D system, USA
Recombinant human IL-12	R&D system, USA
IFN-γ ELISA kit	R&D system, USA
96-well plates	SPL Lifesciences, Korea
Cell culture flask	SPL Lifesciences, Korea
Petri dish	Merck Millipore, USA

Serological pipette	NEST, China
Primers	Sigma-aldrich, USA
50 ml tube	Eppendoft, USA
15 ml tube	Nunc, USA
Centrifuge tube	Sorenson, USA
BALB/c mice	National Laboratory Animal Center, Thailand
TAE buffer	Thermo Scientific, USA
Agarose	Amresco, USA
100 bp DNA ladder	Thermo Scientific, USA
1 Kb DNA ladder	Thermo Scientific, USA
Pre-stained protein ladder	Thermo Scientific, USA
10x DNA loading dye	QIAGEN, Germany
PCR tube	Corning, USA

Equipment	**Manufacturers**
Micropipette	Eppendoft, USA
Centrifuge	Kubota, Japan
CO_2 incubator	Shel lab, USA
Incubator	Shel lab, USA
Microplate reader	Molecular device, USA
Incubator shaker	Eppendoft, USA
Biological safety cabinet class II	Esco, Singapore
CCD camera	Vilber, France
Thermal Cycler	Bio-rad, USA
Microwave	Sharp, Japan
Autoclave	Hirayama, Japan
Waterbath	Memmert, Germany

2.2 Methods

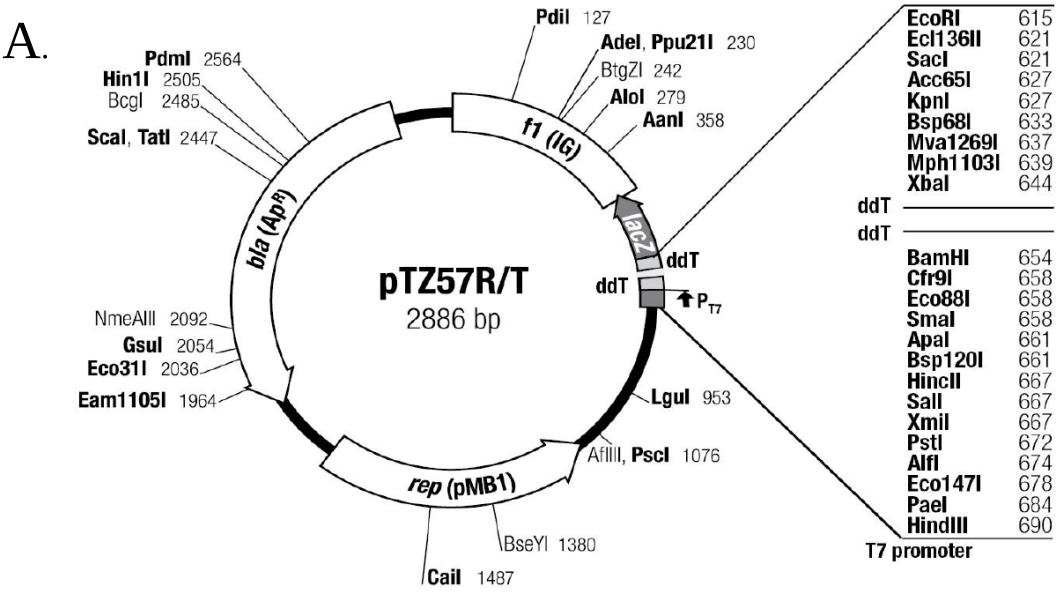
2.2.1 Cloning and mutagenesis

The *IL-18* open reading frame (ORF) was selected based on the *IL-18* gene transcript in the NCBI Reference Sequence: NM_001562.3. PCR primers (Table 4) were designed to amplify IL-18 lacking the pro-IL-18 sequence. Total RNA was extracted from colon tissue sample randomly using GeneJET RNA purification kit (Thermo Scientific, USA) approved by the Human Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University. After the quality verification of RNA by spectrophotometry (Nano drop, Thermo Scientific, USA), RNA was reversely transcribed into first-strand cDNA by using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) with both Oligo$(dT)_{18}$ and random hexamer primers. Mature sequence of IL-18 amplicon was generated from cDNA using the primers listed in Table 4 and conditions shown in Table 5. The first amplicon was tagged with partial TEV protease cleavage site at C-terminus for eliminating histidine tag after protein production by using primer IL-18Fw/IL-18Rv1.

The product was then purified and subsequently used as a template for complete TEV cleavage site generation in the second PCR amplification by using IL-18Fw/IL- 18Rv2 primers (Table 4). After that, the IL-18 without pro-peptide was cloned into pTZ57R/T cloning vector (Thermo Scientific, Fig. 6) and transformed into *E. coli* DH5α (RBC Bioscience, Taiwan). Firstly, the competent cells were pre-chilled on ice and mixed with 5-10 µl of a ligation reaction. The tube was incubated on ice for 30 min and heat shock was performed by incubation for 90 seconds at 42°C before placing the tubes immediately on ice for at least 2 min. 1 mL SOC medium was added to the tube and the incubation was performed for 1 hour at 37°C in incubator shaker before 6,000 rpm centrifugation, the removal of 0.8 mL medium and the growing of the suspension on a LB agar plate containing 100 µg/mL of ampicillin. After overnight incubation, the positive colonies were randomly selected to further plasmid extraction and the IL-18 mature sequence was confirmed by automated DNA sequencer.

To construct the yeast expression vector pPICZα-IL18WT, the mature sequence of IL-18 was sub-cloned into pPICZαA (Invitrogen, Fig. 7) at the *EcoR*I and *Xba*I sites and then transformed into *E. coli* DH5α. The transformants were selected on LB medium containing 25 μg/mL of Zeocin (Invitrogen). A plasmid was extracted and the nucleotide sequence of the mature IL-18 was confirmed by DNA sequencing. The mutagenesis was performed based on a previous report that the binding sites of IL-18 were divided into three regions (27). To create higher binding affinity to its receptors based on increment in the major force at target sites, the substitutions were performed at the following sites in the IL-18 mature form: E6K, M33Q, M60Q and T63A (Fig. 8). Since Met33 and Met60 in site I and II are surrounded by other charged amino acids such as Asp, Arg and Lys, a methionine was replaced by a glutamine (Q) to increase the site polarity, based on an implication that these site I and II require dipole/electrostatic force to facilitate IL-18 binding. Moreover, according to the report that a substitution of E6 with an alanine and a lysine can enhance IL-18 activity, E6K was also taken in this study as a comparative standard. Thr63 is another interesting residue, reported for increased activity when it was alanine-substituted. The plasmid pPICZα-IL18WT was used as a template for the mutagenized PCR by using the primer indicated in Table 4. For E6K+T63A, the template plasmid was pPICZα-IL18E6K. The nicked mutagenized plasmids were amplified with. Platinum Pfx DNA polymerase (Invitrogen) and purified. To remove the plasmid template, the purified PCR fragments were digested with *Dpn*I (Thermo Scientific) and transformed into *E. coli* DH5α. The transformants were selected and plasmids were verified as described above.

A.



B.

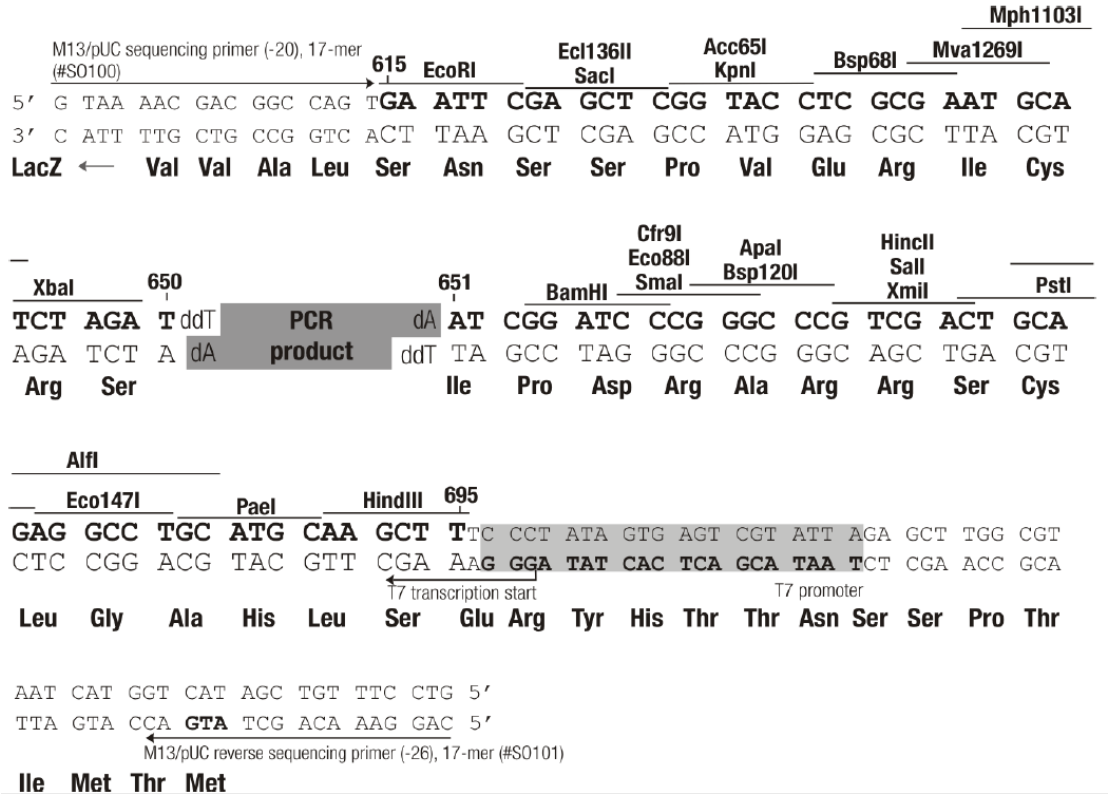
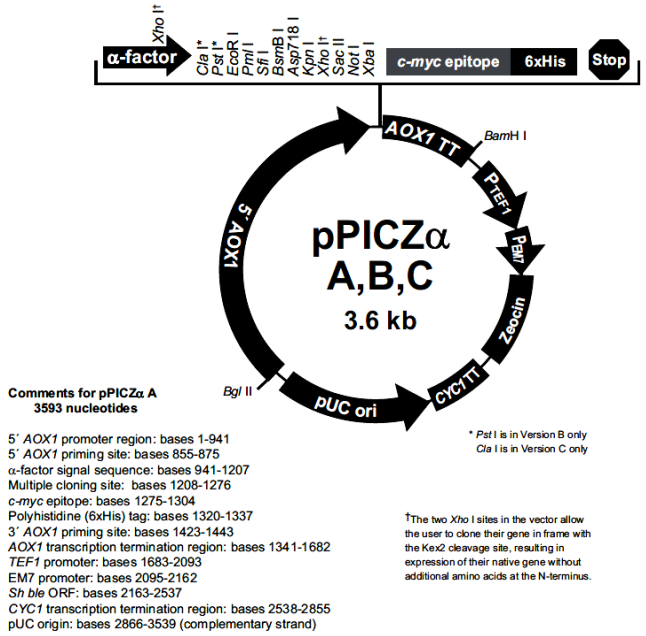


Figure 6. Cloning vector used in this study (A.) Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated. (B.) DNA sequence of MCS region.

A.



B.

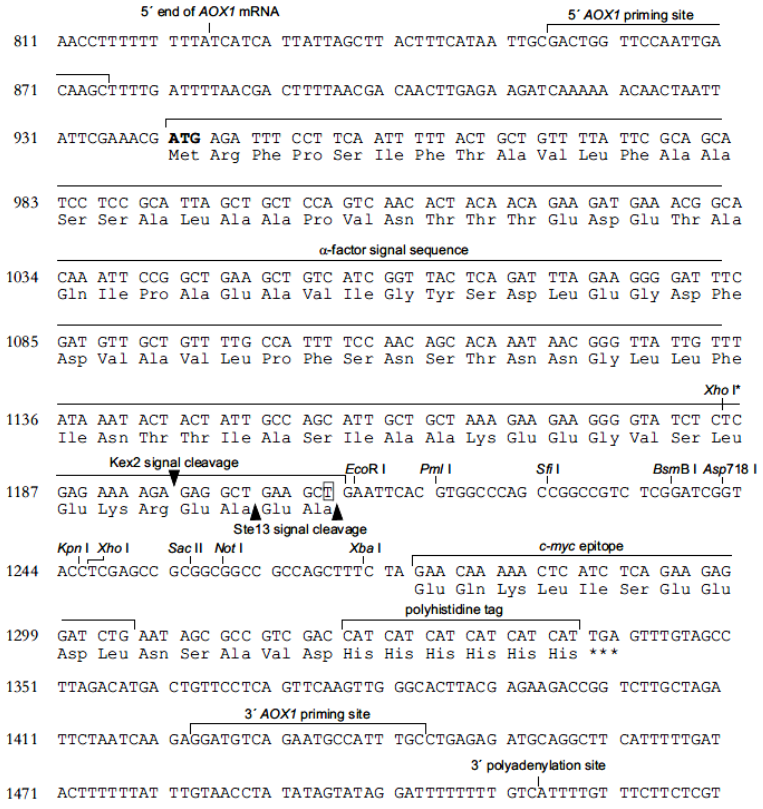


Figure 7. Expression vector used in this study (A.) The summary of the features of the pPICZα A, B, and C vectors (B.) The multiple cloning site for pPICZα A.

Table 4. Primers used in this study.

Primer name	Primer sequence
IL-18Fw	5’-GAATTCTACTTTGGCAAGCTTGAATCTAAATTATCAG-3’
IL-18Rv1	5’-*GTAGAGGTTCTC*GTCTTCGTTTTGAACAGTG-3’
IL-18Rv2	5’-TCTAGATA*TCCCTGGAAGTAGAGGTTCTC*GTCTTC-3’
Pic-FF	5’-TTGTCAGGAACACGATGAAACCAGGATGCC-3’
E6K-Fw	5’-TACTTTGGCAAGCTT*AAG*TCTAAATTATCAGTC-3’
E6K-Rv	5’- GACTGATAATTTAGA*CTT*AAGCTTGCCAAAGTA-3’
M33Q-Fw	5’-CCTCTATTTGAAGAT*CAG*ACTGATTCTGACTG-3’
M33Q-Rv	5’- CAGTCAGAATCAGT*CTG*ATCTTCAAATAGAGG-3’
M60Q-Fw	5’- AGCCAGCCTAGAGGT*CAG*GCTGTAACTATCTC-3’
M60Q-Rv	5’- GAGATAGTTACAGC*CTG*ACCTCTAGGCTGGCT-3’
T63A-Fw	5’- AGAGGTATGGCTGTA*GCA*ATCTCTGTGAAGTGT-3’
T63A-Rv	5’- ACACTTCACAGAGAT*TGC*TACAGCCATACCTCT-3’

The underlined bases are recognition sites for *EcoR*I and *Xba*I, respectively. The italicized sections of the bases are the codons translated for the Tobacco Etch Virus (TEV) protease cleavage site. Base mutations are italicized and underlined

Table 5. PCR condition used in this study

Step	Temperature	Time
1	95°C	5 minnutes
2	95°C	30 seconds
3	55°C	45 seconds
4	72°C	60 seconds
	Go to step 2 for 19 times	
5	72°C	10 minnutes
6	4oC	Until keep in refrigerator

A.

```
        10          20          30          40
YFGKLESKLS  VIRNLNDQVL  FIDQGNRPLF  EDMTDSDCRD
        50          60          70          80
NAPRTIFIIS  MYKDSQPRGM  AVTISVKCEK  ISTLSCENKI
        90         100         110         120
ISFKEMNPPD  NIKDTKSDII  FFQRSVPGHD  NKMQFESSSY
       130         140         150
EGYFLACEKE  RDLFKLILKK  EDELGDRSIM  FTVQNED
```

B.

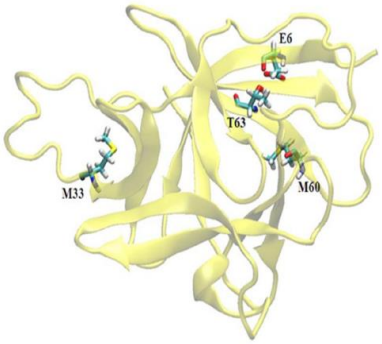


Figure 8. Sequence and structure of human IL-18. (A) Amino acid sequence of mature human IL-18. The red and underlined residues are the target amino acid residues that were mutated in this study. (B) 3D structure of mature human IL-18. The residues used in the experiment are shown.

2.2.2 Protein production

Plasmid pPICZα-IL18WT and other mutagenized plasmids were linearized with *Sac*I (Thermo Scientific) enzyme, which is specific to the site at AOX1 promoter of the plasmid and the digested condition is shown in Table 6. Plasmid was purified and introduced to *Pichia pastoris* KM71 (Invitrogen) by electroporation (Bio-Rad). The transformants were selected on YPD medium containing 100 µg/mL of Zeocin (Invitrogen) and plasmid integration was verified by PCR method with the IL-18Rv2 and Pic-FF primers (Table 4). Protein expression of recombinant *Pichia pastoris* KM71 bearing the target gene in its genome was performed by preparing the yeast inoculum in 5 mL YPD broth and incubation at 30°C at 250 rpm overnight. The inoculum was then transferred into 200 mL BMGY medium (2.0% peptone, 1.0% yeast extract, 100mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acid, 0.4µg/mL biotin, 1.0% Glycerol) with an initial OD_{600} of 0.2 and incubated at 30°C at 250 rpm until the OD_{600} reached 5–6. All cells were harvested by centrifugation and transferred to 20 mL BMMY medium (2.0% peptone, 1.0% yeast extract, 100mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base without amino acid, 0.4µg/mL biotin, 2.0% methanol) at 30°C at 250 rpm for 48 hours. The volume of the culture should be no more than 10-30% of the total flask volume to ensure sufficient aeration. To maintain the induction, methanol was added every 24 hours to give a final concentration of 2%. The supernatant was collected to confirm the existence of recombinant IL-18 by SDS-PAGE and Western blotting.

Table 6. Condition used in *Sac*I digestion

Reagent	Volume	Condition
10x buffer SacI	2 µL	Incubation at 37°C for 16-18 hours
SacI (10U/µL)	1 µL	
Plasmid	1 µg	
Nuclease free water	Adjust to 20 µL	

2.2.3 SDS-PAGE and Western blotting

The protein samples were reduced and denatured by boiling in 6X SDS-PAGE loading buffer (375 mM Tris-HCl, 9% SDS, 50% glycerol, 0.03% bromophenol blue) in a volume ratio of 5:1 at 95-100˚C for 10 minutes before cooling down on ice for 5 minutes. Centrifugation at 14000 rpm was then performed before running the samples on 12% SDS-polyacrylamide gels according to Table 7 and transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After electroblotting at 35 volts for 16 hours in transfer buffer using a Transblot unit (Bio-Rad), the membrane was blocked TBST (137 mM sodium chloride, 20 mM Tris, 0.1% Tween-20, pH 7.6) containing 3% (w/v) BSA (Sigma-Aldrich) for 1 hour at room temperature, followed by detection with specific mouse anti-IL-18 (R&D systems) in 1:3,000 dilution and incubation at room temperature for 1 hour. The antibody was removed and the PVDF membrane was washed three times for 5 min each in TBST with gentle agitation. Horse radish peroxidase-conjugated goat anti-mouse (R&D system) was added at a dilution of 1:10,000 in TBST containing 3% (w/v) BSA and incubated for 1 hour with gentle agitation at room temperature. The sheet was then washed three times in TBST and antigen-antibody complexes were detected by the addition of Luminata Forte Western HRP substrate (Millipore).

Table 7. The components for 1 SDS-PAGE gel

Components	**Stacking gel (5%)**	**Resolving gel (12%)**
Water	1.75 mL	1.920 mL
30% Acrylamide	0.413 mL	2.4 mL
0.5 M Tris-HCl, pH 6.8	0.312 mL	-
1.5 M Tris-HCl, pH 8.8	-	1.56 mL
10% SDS	0.025 mL	0.06 mL
10% APS	0.025 mL	0.06 mL
TEMED	0.006 mL	0.006 mL

2.2.4 Protein purification

The secreted IL-18 protein was purified by HisTrap HP column (GE Healthcare) according to manufacturer's protocol. Briefly, the culture supernatant was loaded into a 4 mL size Ni^{2+}- Sepharose HisTrap affinity column equilibrated with 5 column volume of binding buffer at pH 7.4 which contained 20 mM sodium phosphate, 0.5 M NaCl and 20 mM imidazole. The native proteins were washed out with 12 column volume of washing buffer that contained 50 mM imidazole and the target protein was eluted with buffer containing 400 mM imidazole. The recombinant protein was desalted and concentrated by Amicon Ultra4 centrifugal filter unit (Millipore) and diluted in PBS. Protein concentration was determined spectrophotometrically according to Bradford using bovine serum albumin (BSA) as a standard. Moreover, the purified protein was sent for MS analysis for identification.

2.2.5 IFN-γ inducing assay

The NK-92MI cells were maintained in complete α-MEM medium supplemented with 12.5% FBS and 12.5% horse serum at 37°C in 5% CO_2 humidified air. For the assays, NK-92MI cells were suspended at 0.5×10^6 cells per ml in complete α-MEM medium and stimulated in 0.2 ml volumes in 96-well plates with 0.5 ng/ml of IL-12 and different concentrations (200, 100, 50, 25 and 12.5 ng/mL) of recombinant IL-18, or the five mutants. After 16-20 h at 37°C in humidified air with 5% CO_2, the culture supernatants were collected for IFN-γ measurement by the ELISA method according to manufacturer's protocol (R&D system).

2.2.6 Cell lines, animal model and assessment of treatment effect

Mouse CT26-WT colon cancer cells (American Type Culture Collection; CRL-2638) were obtained from ATCC. CT26-WT is a N-nitro-N-methylurethane induced BALB/c murine colon carcinoma cell line, which was grown in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine at 37°C in 5% CO_2.

Male 7- to 9-week-old BALB/cMlac mice were obtained from the National Laboratory Animal Center and maintained in Southern Laboratory Animal Facility, Faculty of Science, Prince of Songkla University. All animal experiments were conducted according to the guidelines of the Ethics Committee of Prince of Songkla University. During the logarithmic growth phase, CT26-WT cells were harvested and screened with trypan blue to allow preparation of single-cell suspensions with 90 % viability before inoculation. Each group of mice was subcutaneously injected with $2x10^6$ CT26-WT cancer cells/100 µL of PBS in the left hind flank. Survival time, tumor volume, and physical symptoms were monitored after inoculation. The CT26 mice were randomized into 5 groups of 13 mice each (10 mice for examination of survival time, and 3 mice for analysis of immune cells). The five treatment groups will be as follows: group A (control group), untreated control mice; group B (vehicle group); group C (IL-18WT group); group D (IL-18E6K group); group E (IL-18E6K+T63A). The experimental treatment was begun after tumor volume reached 100 mm^3, and refer the treatment-starting day as day 0, and we used only 2 types of IL-18 that exhibited the highest activity to reduce the number of mice and compared to each other.

Group B mice receiving PBS solution injections served as a vehicle control. Group C, D and E mice received 50 µg/kg daily of IL-18WT, IL-18E6K and IL-18E6K+T63A, respectively, 10 days continuously. Tumor volume was measured by caliper measurements every day. After 10 days of treatment, mice were sacrificed, and tumor and spleen were collected for flow cytometry, weight, and size analysis.

2.2.8 Leukocyte isolation

For splenocyte isolation, spleens were harvested and placed in complete RPMI medium at room temperature during transportation. Spleens were homogenized into single cell suspensions by gently mashing the spleen through the cell strainer using the plunger end of the syringe, and filtered through a 70 µm cell strainer, which was rinsed with 5 mL RPMI medium. Cells were centrifuged at 300g for 10 minutes and were incubated in 3 mL of red blood cell lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA) for 5 minutes at room temperature. After RBC lysis, 20 mL

of complete RPMI medium was added and cells were centrifuged again as before and resuspended in complete RPMI medium

For tumor infiltrating leukocyte isolation, tumors were removed from euthanized mice on day 11 after treatment, and were kept in complete RPMI medium during transportation. After that, tumors were dissected into small fragments, and incubated in complete RPMI containing 1 mg/mL type IV collagenase (sigma aldrich), 0.1 mg/mL hyaluronidase (sigma aldrich), 20U/mL DNase I (sigma aldrich) at 37 °C for 45 min with agitation. The cell suspension was then filtered through a 70 μm cell strainer on 50 mL tube, which was rinsed with 5 mL RPMI medium. The single cell suspension was centrifuged at 300g for 10 minutes to get the cell pellet before washing with complete RPMI medium and incubating with 5 mL RBC lysis buffer for 5 minutes. The cells were washed again and resuspended in 15 mL of 40% Percoll (GE healthcare) in RPMI. The suspension was underlaid on 15 mL 80% Percoll and centrifuged for 25 min at 400 *g* with the lowest descending and ascending rates. Leukocytes were collected from the interphase between two concentrations.

2.2.9 Flow cytometry

The isolated leukocytes were counted by using hemacytometer, adjusted at 2×10^6 cells per mL, and incubated in complete RPMI medium containing cell activation cocktail (Biolegend), which included 20 ng/mL phorbol 12-myristate-13-acetate (PMA) and 1 μg/ml ionomycin and 5 μg/mL brefeldin A for 4 hours at 37 °C in CO_2 incubator. The cells were washed with 25 mL cell staining buffer (Biolegend) two times before adjusting at $2x10^7$ cells/mL and incubating with 10 ng/μL TruStain fcX™ (Biolegend) for 10 minutes on ice. Cell surface antibodies were then added according to details in Table 8 and the incubation was then performed on ice for 20 minutes in the dark environment. After two time washing with cell staining buffer, the cells were fixed with 5x volume of fixation buffer (Biolegend) for 20 minutes at room temperature in the dark environment. The cells were subsequently washed once with cell staining buffer and twice with intracellular staining permeabilization wash buffer (Biolegend) before staining with fluorescence conjugated antibodies against intracellular cytokines in permeabilization buffer according to Table 8 for 20 minutes

at room temperature in the dark environment. The double stained cells were then washed two times with intracellular staining permeabilization wash buffer and resuspended in cell staining buffer before flow cytometry analysis using BD FACSCalibur™ platform (BD Biosciences)

Table 8. Antibodies used in this study

Antibody	**Provider**	**Clone**	**Volume used (µL)**
PE Rat anti-mouse IL-4	Biolegend	11B11	1.25
PE Rat IgG1, κ isotype	Biolegend	RTK2071	1.25
APC Rat anti-mouse IFN-γ	Biolegend	XMG1.2	4
APC Rat IgG1, κ isotype	Biolegend	RTK2071	4
FITC Rat anti-mouse CD4	Biolegend	GK1.5	0.5
FITC Rat IgG2b, κ isotype	Biolegend	RTK4530	0.5
FITC Rat anti-mouse CD8a	Biolegend	53-6.7	2
FITC Rat IgG2a, κ isotype	Biolegend	RTK2758	2
Anti-mouse CD16/32	Biolegend	93	2

2.2.10 Molecular dynamic (MD) simulation

Three dimensional structure of full length IL-18 (157 amino acids) was obtained from Research Collaboratory for Structural Bioinformatics (RCSB) protein data bank (www.rcsb.org), PDB identification code 1J0S. As 1J0S is an NMR structure, 4^{th} conformer was chosen on a basis of the lowest root-mean-square deviations (RMSDs) among 20 conformers. All hydrogen atoms in the structure were then removed and the protonation state of amino acid at pH 7 was determined using PROPKA webtools (229). The missing hydrogen atoms, with corrected protonation state, were then added again using Leap module in AMBER12 package (230). Four mutated IL18 (E6K, M30Q, M60Q and T63A) were prepared using Visual Molecular Dynamics (VMD) package (231) and Leap module as auxilliary tools. All IL protein was finally energy-minimized using steepest descent method, under AMBER10 nonpolarizable force field parameters, for 2000 steps.

The minimized protein was neutralized by either sodium (Na^+) or chloride (Cl^-) ion and solvated by TIP3P water molecules along with NaCl pair, yielding concentration of 0.15 M NaCl solution. This protein-solution system was equilibrated in isothermal ensemble (NVT), using Langevin Dynamics as a thermostat at 310 K (37^oC). The harmonic potential was applied to all position of IL-18 with a force constant of 200, 100, 50, and 20 kcal/mol $^{\circ}A^2$ and 400-ps-NVT simulation was finally obtained, comprised of four 100-ps-MD sessions for each force constant, with a time step of 1 fs. The system was switched to 155 isobaric/isothermal (NPT) ensemble with a time step of 2 fs. A temperature of 310 K and pressure of 1.013 bar (1 atm) were regulated using weak coupling algorithm (232), in order to mimic *in vivo* environment. The NPT simulation was carried out for 40 ns by Particle mesh Ewald molecular dynamics simulator (PMEMD), implemented in AMBER12 package. The first 10ns was omitted as an equilibration phase. The 3000 equidistant snapshots from the last 30 ns simulation was taken for a configurational average and analysis. Simulations of wild-type and 4 mutated ILs followed the identical protocol. All IL-180's structures herein were analysed using ptraj module in AMBER12 and illustrated using VMD package.

For structural comparison between the wildtype and engineered recombinant IL-18, molecular dynamics simulation was performed. This was carried out using the program package GROMACS 4.0.5. (193) or Amber 10 (196) running on a Linux cluster which allow calculation and investigation of the conformational characteristics of the protein at every step during the simulation in natural environment.

Herein, the simulation of IL-18 began from the reported NMR structure of human IL-18 (pdb no. 1J0S) as a starting input. The system of IL-18 in 0.15 M NaCl solution at 310 K (37^oC) was started in order to mimic the *in vivo* environment. The study of wild type and mutants follows the identical simulation protein. After that, the structure of mutants and wildtype IL-18 was analysed.

2.2.11 Statistics processing

All data are presented as the mean±SD. For comparisons between the two groups, the data was analyzed by using the 2 tailed independent t-test. Survival data were plotted using the method of Kaplan–Meier and were analysed by log-rank test. All analysis was performed by using SPSS, version 24.0 (The International Business Machines Corporation, NY, USA). P values of <0.05 is considered to be significant. *, $p< 0.05$; **, $p< 0.01$; ***, $p< 0.001$.

CHAPTER 3

RESULTS

3.1 Vector construction and mutagenesis

To construct recombinant plasmid for protein production, primers were designed for IL-18 open reading frame (ORF). As shown in Table 4, IL-18Fw sequence bind to the first amino acid of mature IL-18 form. Initial optimization using TopTaq DNA polymerase demonstrate specific band at annealing temperature of 55°C when the amplicon was loaded on 2% agarose gel and visualized by UV light with expected size (506 bp) (Fig. 9). PCR product was then ligated into pTZ57R/T vector by TA-cloning method and transformed to competent DH5α *E. coli* bacteria using heat shock principle. The insertion was then verified by two methods. First, by the antibiotic ampicillin as only the clone containing the vector would survive. The second method, depended on PCR amplification of the IL-18 ORF to confirm that the colonies grown on agar plates were positive clones for the inserted gene. The pTZ57R/T vector has 3 prime-T overhangs at its insertion site which facilitate ligation and cloning of an amplicon that has complementary 3 prime-A overhangs provided to both sides by TopTaq DNA polymerase. IL-18 ORF was subsequently sub-cloned into pPICZαA expression vector excised by using *Xba*I/*Eco*RI restriction sites, and the verification was performed by PCR and sequencing. DNA sequence analysis revealed 100% homology with the published sequence of human IL-18 (Fig. 10).

The recombinant expression vector has a size of about 4030 bp insert including zeocin resistant gene and C-terminus six tandem histidine (6xHis) residues to facilitate purification on HISTRAP HP columns. At the N-terminus, AOX1 promoter from *P. pastoris* was added for methanol-induced expression of the gene, and α-factor secretion signal from *Saccharomyces cerevisiae* was also included in the vector as a secretion signal of the recombinant protein. This lead to the release of the protein into culture supernatant, which make it easy to purify the recombinant protein.

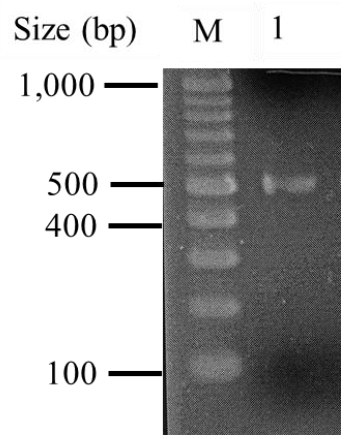


Figure 9. Gel electrophoresis of the amplified mature IL-18 fragment. Lane M, DNA ladder (Thermo Scientific GeneRuler 100 bp Plus DNA Ladder); Lane 1, amplified IL-18

```
  1 ATGGCTGCTGAACCAGTAGAAGACAATTGCATCAACTTTGTGGCAATGAAATTTATTGAC  60
  1 -M--A--A--E--P--V--E--D--N--C--I--N--F--V--A--M--K--F--I--D-  20

 61 AATACGCTTTACTTTATAGCTGAAGATGATGAAAACCTGGAATCAGATTACTTTGGCAAG 120
 21 -N--T--L--Y--F--I--A--E--D--D--E--N--L--E--S--D--Y--F--G--K-  40

121 CTTGAATCTAAATTATCAGTCATAAGAAATTTGAATGACCAAGTTCTCTTCATTGACCAA 180
 41 -L--E--S--K--L--S--V--I--R--N--L--N--D--Q--V--L--F--I--D--Q-  60

181 GGAAATCGGCCTCTATTTGAAGATATGACTGATTCTGACTGTAGAGATAATGCACCCCGG 240
 61 -G--N--R--P--L--F--E--D--M--T--D--S--D--C--R--D--N--A--P--R-  80

241 ACCATATTTATTATAAGTATGTATAAAGATAGCCAGCCTAGAGGTATGGCTGTAACTATC 300
 81 -T--I--F--I--I--S--M--Y--K--D--S--Q--P--R--G--M--A--V--T--I- 100

301 TCTGTGAAGTGTGAGAAAATTTCAACTCTCTCCTGTGAGAACAAAATTATTTCCTTTAAG 360
101 -S--V--K--C--E--K--I--S--T--L--S--C--E--N--K--I--I--S--F--K- 120

361 GAAATGAATCCTCCTGATAACATCAAGGATACAAAAAGTGACATCATATTCTTTCAGAGA 420
121 -E--M--N--P--P--D--N--I--K--D--T--K--S--D--I--I--F--F--Q--R- 140

421 AGTGTCCCAGGACATGATAATAAGATGCAATTTGAATCTTCATCATACGAAGGATACTTT 480
141 -S--V--P--G--H--D--N--K--M--Q--F--E--S--S--S--Y--E--G--Y--F- 160

481 CTAGCTTGTGAAAAAGAGAGAGACCTTTTTAAACTCATTTTGAAAAAAGAGGATGAATTG 540
161 -L--A--C--E--K--E--R--D--L--F--K--L--I--L--K--K--E--D--E--L- 180

541 GGGGATAGATCTATAATGTTCACTGTTCAAAACGAAGACTAG                   582
181 -G--D--R--S--I--M--F--T--V--Q--N--E--D--*-                  193
```

Figure 10. DNA and amino acid sequences of coding IL-18. The underline sequence is the prosequence of IL-18. The prosequence is not necessary for IL-18 function, and will be eliminate by casepase-1 before secreting outside from cell.

3.2 Protein production and purification

Production of recombinant IL-18 protein was optimized by altering various concentrations of methanol. The result showed that after three different methanol concentrations and the expression levels were tested with SDS-PAGE, 2% methanol resulted to an optimal condition for IL-18 production (Fig. 11). Upon induction from pPICZα-IL18-containing *P. Pastoris* and based on the amino acid sequence, we observed a 22 kDa band consistent with the calculation of molecular weight of recombinant IL-18, and this was absent in the empty pPICZα (data not shown). Therefore, these data indicated that optimal IL-18 production was achieved following induction with 2% methanol, and this condition were applied to the production of all engineered IL-18.

After optimization of IL-18 production in *P. pastoris*, a nickel resin column, HITRAP HP, was used to purify all of recombinant IL-18 protein. The optimal concentration of imidazole for binding was 20 mM and 400 mM for the elution in phosphate buffer pH 7.4. The IL-18 fraction was then concentrated and desalted by Amicon Ultra4 centrifugal filter unit resulting in the higher concentration of recombinant IL-18 in PBS buffer. Following these procedures, from 20 mL of BMMYculture medium, we obtained a purified IL-18, which achieved a purity of 95% as revealed by SDS-PAGE (Fig. 12B). Then all types of purified recombinant proteins were identified by Western blot analysis. The result demonstrated that the recombinant protein could bind with the anti-human IL-18 monoclonal antibody, and after the 22 kDa protein bands were submitting to Proteomics International Pty Ltd. for LC-MS/MS analysis, tryptic digestion of 22 kDa protein produced 5 peptide fragments that covered 28.66% of the mature IL-18 (Fig. 13A and 13B). These data indicated that the purified protein was mature IL-18.

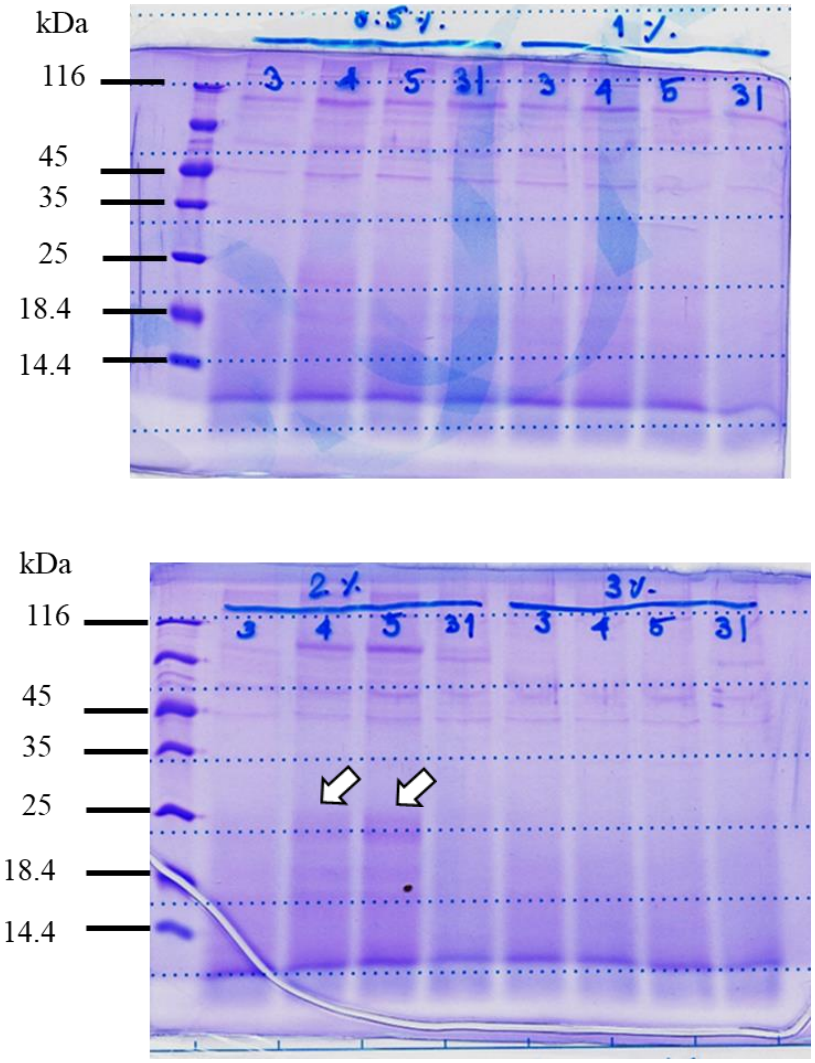


Figure 11. SDS-PAGE supernatant culture of *P. pastoris* after protein induction with 4 concentrations of methanol, 0.5%, 1%, 2% and 3%. 3, 4, 5 and 31 represent the clone number of *P. pastoris*. The arrows indicate IL-18 protein bands found in supernatant.

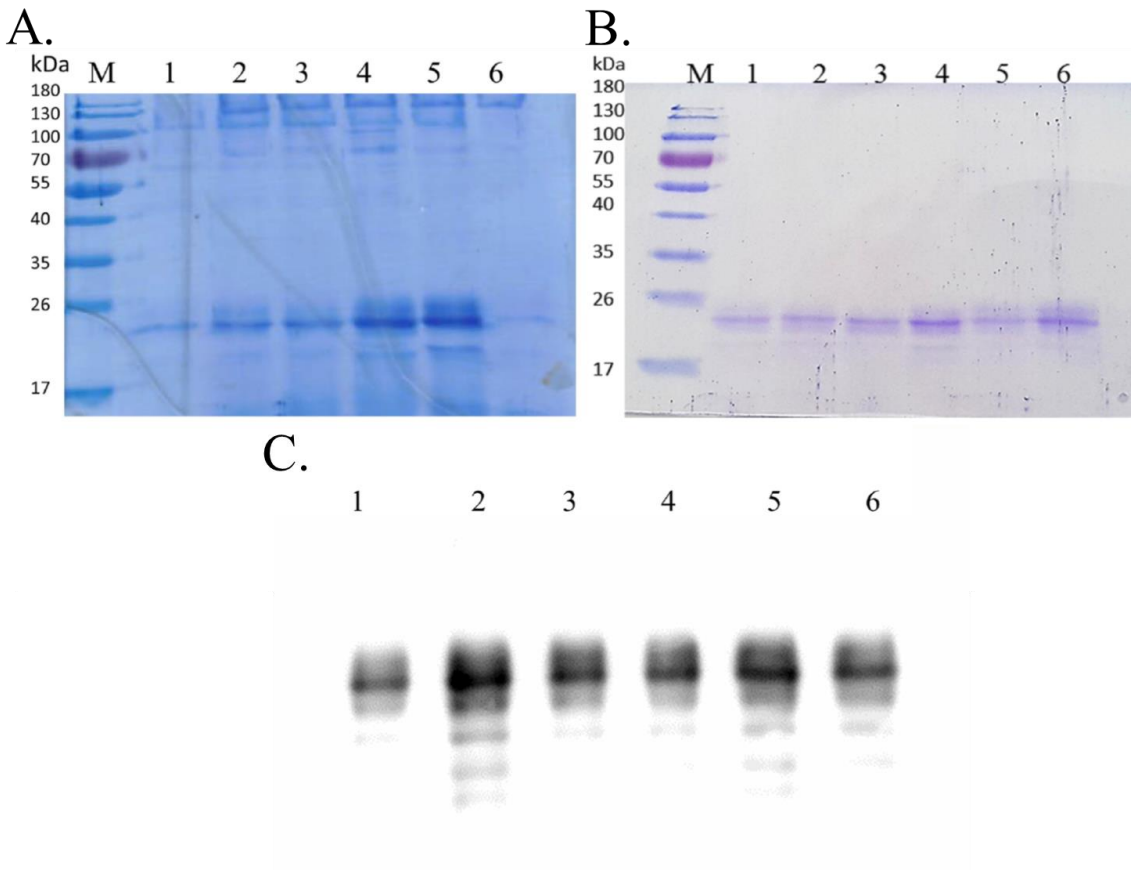


Figure 12. SDS-PAGE and Western blot analysis of recombinant IL-18. (A) SDS-PAGE analysis of secreted proteins from the *P. pastoris* KM71 before purification. (B) SDS-PAGE analysis of recombinant IL-18 after purification with HisTrap affinity column, (C) Western blot analysis of recombinant IL-18 protein. All samples were loaded onto 12% SDS-PAGE. Lane: M=Molecular mass standard (Thermo Scienctific), 1 = wild-type, 2 = E6K, 3 = M33Q, 4 = M60Q, 5 = T63A and 6 = E6K+T63A.

A.

```
        10          20          30          40
YFGKLESKLS  VIRNLNDQVL  FIDQGNRPLF  EDMTDSDCRD
        50          60          70          80
NAPRTIFIIS  MYKDSQPRGM  AVTISVKCEK  ISTLSCENKI
        90         100         110         120
ISFKEMNPPD  NIKDTKSDII  FFQRSVPGHD  NKMQFESSSY
       130         140         150
EGYFLACEKE  RDLFKLILKK  EDELGDRSIM  FTVQNED
```

B.

m/z	Molecular weight (Mw)	Position	Peptide
294.1987	586.3828	9-13	LSVIR
558.3131	1114.6117	45-53	TIFIISMYK
453.2596	904.5046	59-67	GMAVTISVK
554.6230	1660.8473	80-93	IISFKEMNPPDNIK
513.2738	1024.5331	97-104	SDIIFFQR

Figure 13. LC-MS/MS analysis of purified recombinant mature IL-18. (A) Tryptic peptide map of recombinant mature IL-18 produced by yeast *P. pastoris*. The identified peptides are shown in red, (B) Identified tryptic peptide fragment derived from purified IL-18.

3.3 IFN-γ inducing assay

In order to test the biological efficacy of each type of IL-18, NK92-MI cell line, which can represent as a human natural killer (NK) cell, was used to evaluate the capability of the engineered IL-18 to stimulate the production of IFN-γ in the presence of IL-12 as a co-stimulant in this cell line. NK92-MI cells were stimulated with various concentration of each type of recombinant IL-18 in the presence of low constant concentration of commercial recombinant human IL-12 for 16-18 hours. The supernatant was then collected for analysis using ELISA kit for IFN-γ measurement.

The results showed that at the concentration of 12.5 ng/mL, IL-18WT, M33Q, M60Q and T63A could not induce the production of IFN-γ from NK92-MI cells, whereas E6K and E6K+T63A IL-18 influenced the significantly different levels of IFN-γ, especially the combination between E6K and T63A impacted about a 17x greater IL-18 activity than the wild-type (Fig. 14). Interestingly, at the concentration of 25 and 50 ng/mL, only E6K, T63A and E6K+T63A were observed to stimulate the production of IFN-γ from NK92-MI cell line significantly while others did not show this activity. Moreover, at the higher concentrations, although WT IL-18 activity could be found at 100 and 200 ng/mL, and M33Q was observed only at 200 ng/mL,

and the M60Q forms did not show any IFN-γ induction activity at all concentrations (Fig. 14). Obviously, higher levels of activity of E6K, T63A and E6K+T63A were observed when the concentrations of recombinant proteins were increased to 50, 100 and 200 ng/mL. The activities of E6K, T63A and E6K+T63A forms were about 9.3, 3.9 and 16.4 times higher than wild-type IL-18 at a concentration of 200 ng/mL.

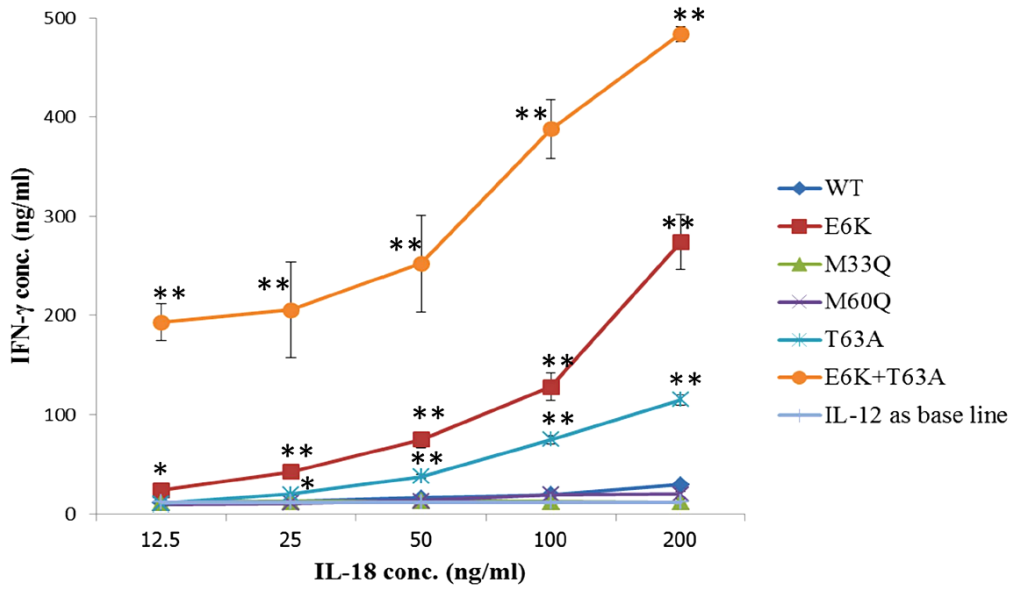


Figure 14. IFN-γ induction assay with the purified recombinant IL-18. NK-92MI cells were treated with various concentrations of recombinant purified IL-18 for 24 hours. IFN-γ was measured in the supernatant by ELISA. The illustrated results represent the mean SD of three independent experiments. *p < 0.05 and **p < 0.01, compared with the levels of IFN-γ that were simulated with wild-type IL-18.

3.4 Molecular dynamic simulation

To explore the structural and functional behavior of mutants, MD simulation of aqueous IL-18 at 37°C for 70 nanoseconds was performed by using an 1J0S pdb file as a template. This simulation was generated to determine the structure alterations of the engineered protein when compared to the native. All energetic parameters, such as temperature, pressure, energy and density, became stable throughout the simulations. From 70 ns MD simulation, trajectory files were analyzed for structural impact by RMSD and RMSF since a standard analysis for measuring the protein stability and fluctuations of residues in MD simulation is RMSD and RMSF of all heavy atoms, respectively, with respect to its native structure. The calculation of RMSD for all the Cα atoms of native and mutant IL-18 were calculated from the initial structure and as showed in Fig. 15A, simulation of native IL-18 proteins showed the slightly different RMSD compared to the NMR structure derived from protein data bank with backbone RMSD ~ 2 Å -2.3 Å, the lowest among all MD simulations.

The result showed that the inspection of RMSD plot indicates the constant convergence of all types of IL-18 with a range near to 3.5 Å, except M33Q, throughout the simulation. Specifically, all mutants except E6K and M60Q revealed the higher deviation after ~ 500 ps when compared to native, as presented in Fig. 15A. Although M60Q showed the close RMSD to native at the begining, it became higher after 1800 ps indicating the unstability of this kind of mutant when the time was past. On the other hand, RMSD of E6K+T63A, which showed the higher value at the initial simulation, was observed to become closer to native after 3000 ps. Obviously, M33Q showed the highest RMSD throughout the simulation even in the beginning stage while E6K showed the closest RMSD when compared to the other mutants. In other word, this suggested that most of mutations caused a vivid conformational change in the IL-18 structure, while E6K and double mutation preserved the tertiary IL-18 structure.

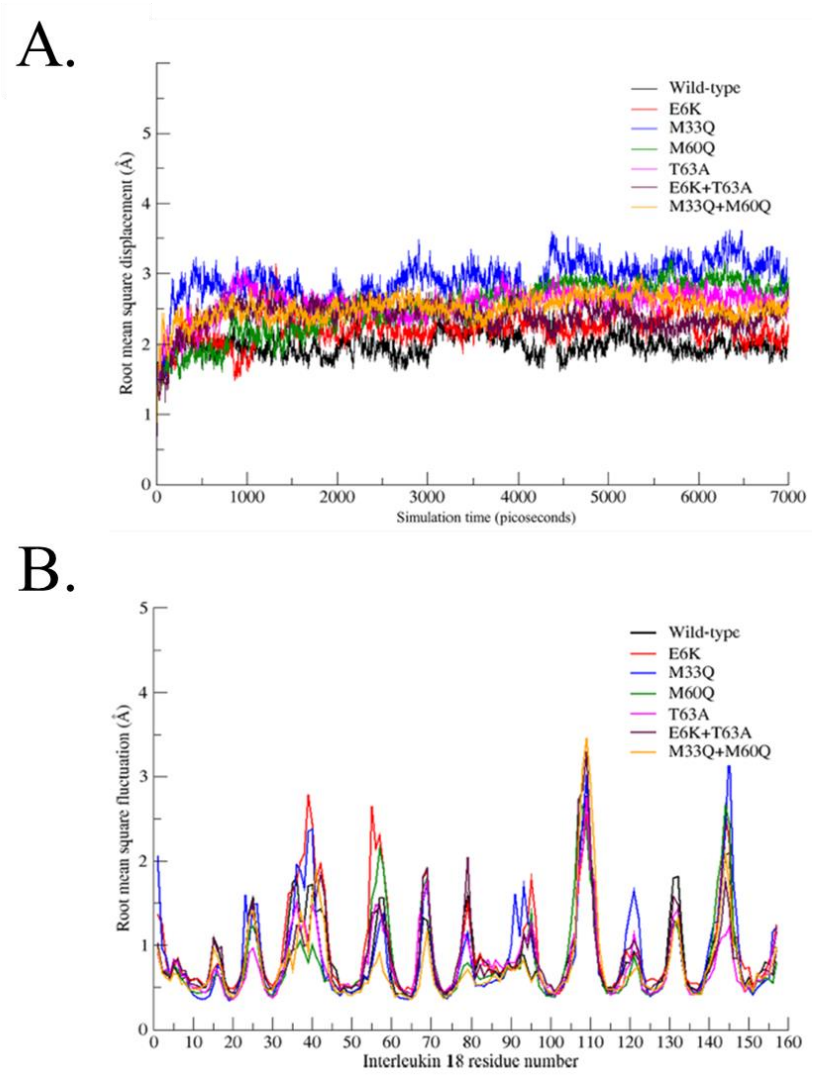


Figure 15. Root-mean-square displacement (RMSD) and root-mean-square fluctuation (RMSF) results from MD simulation (A.) RMSD in Angstrom units from MD simulations of all IL-18 proteins, relative to the initial coordinates, the 3rd conformer of 1J0S NMR structure. (B.) RMSF from IL-18 MD simulations.

Apart from the structure similarity, Figure 15 showed RMSF plot of Cα atoms measured the dynamic behavior of residues and protein flexibility plotted against protein residue order (1st-159th for IL-18), (Fig. 15B), with reference to the starting template (1J0S.pdb). The result demonstrated that all IL-18 proteins share a similar RMSF pattern in most of the entire structure but differences in degree. Analysis of RMSF score revealed that E6K mutation make the protein highest in flexibility at residue 30-45 and 50-60 as compared to the other mutants. M33Q could also promote the higher flexibility pattern in most of region while M60Q, T63A and E6K+T63A showed the moderate differences of the flexibility in some area (Fig. 15B).

To study in more details with the aspect of structural changes and receptor interactions, the conformations of IL-18 in the wild-type and mutant states were obtained from the MD trajectories (Fig. 16 and 17). Interestingly, when all mutation trials were performed, there were 2 important regions that showed the different fashions. The first was the loop that contains residues 31-45 which some of these residues seemed to play a role in site I interaction at the IL-18Rα pocket (Fig. 16). E6K and E6K+T63A showed conformational changes at this area, especially D37, R39, D40 and N41 that changed directly forward to the IL-18Rα pocket (Fig. 17). M33Q, M60Q and T63A showed larger structural alterations with little difference in RMSDs. In M33Q, the direction of loop was changed whereas T63A demonstrated a new loop direction towards the receptor (Fig. 16).

Another region affected by mutations is residues 52-63 containing loop, which consists of the functional K53, a key residue for receptor binding site II of IL-18. MD simulation revealed that the secondary structure of this loop was transformed into a sheet after M33Q mutation was introduced (Fig. 16). Apart from M33Q, E6K also influenced on this loop since the closer contact of K53 with the corresponding amino acid in IL-18Rα could be found (Fig. 17), although molecular dynamics simulation revealed that wild-type IL-18 and E6K shared a similar general structure, namely β-sheets and α-helices (Fig. 17). Interestingly, when E6K and T63A were combined, the loop change caused a novel direction of K53 which could lead to the interaction of K53 of IL-18 to T299 and V300 deeply in the IL-18Rα pocket (Fig. 17). D54, S55, Q56, R58 and G59 were also affected by the loop change which may be due to the

combination of the mutations, shown in Fig. 16B. This result directly indicates that the mutations have affected the conformation of binding regions in different fashion.

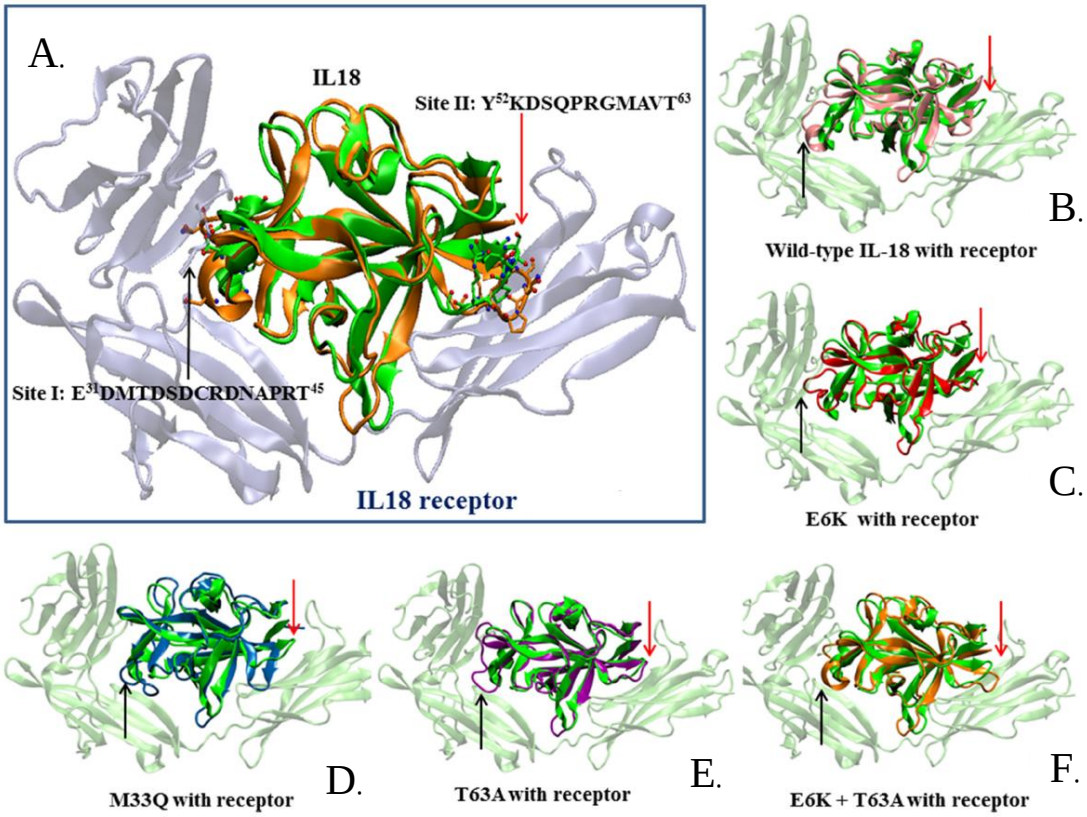


Figure 16. IL18-IL18R complex: (A) Shows the IL-18 crystal structure (green) of the complex, aligned with the MD structure of a double-mutated IL-18. The residues of IL-18 interactions with site I and site II in IL-18Rα are indicated. (B)-(F) Illustrate all average structures of simulated IL-18 with respect to IL-18 in the IL18-IL18R complex crystal. The arrows indicate the loop of IL-18, which directly interacts with IL-18R.

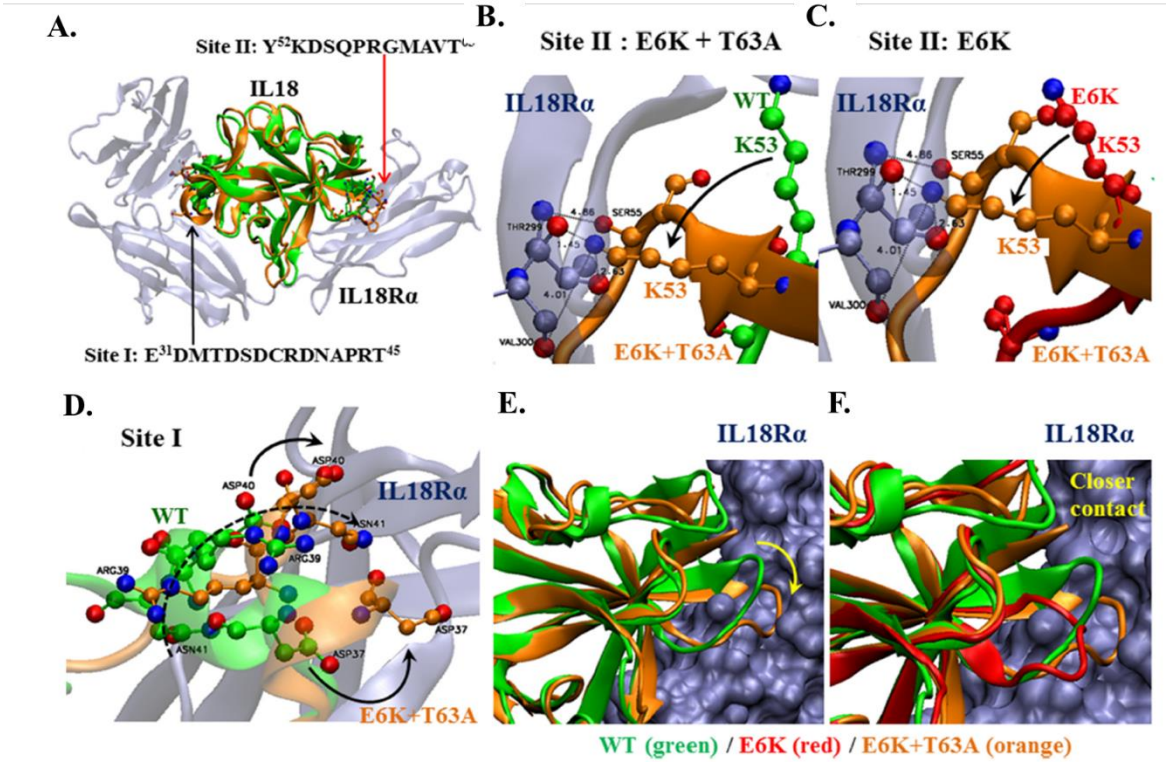


Figure 17. Interaction of mutant IL-18 with IL-18R at site II. (A.) Shows the site II interface between IL-18 and IL-18Rα. (B.) Shows the molecular interaction between the protein and receptor. In the mutant, K53 (IL-18) moves to bind to T299 and V300 of the receptor, whereas in the wild-type structure, this K53 resides far from these two residues. (C.) Shows E6K+T63A mutation facilitate the loop in the IL18 to make closer contact with the receptor, compared to the wild-type and E6K cases. (D.) To (E.) Shows a comparison of the IL18 structure at site II between WT IL18 (green) and E6K+T63A IL18 (orange). The mutated IL18 structure (orange) clearly comes closer to site II. (F.) The comparison of WT, E6K and E6K+T63A IL-18 at the binding site.

3.5 Anti-tumor effect of recombinant IL-18 *in vivo*

To evaluate the anti-tumor effect of recombinant IL-18 in the animal model, BALB/c mice were injected with CT26-WT cell line subcutanously to create tumor bearing mice. Mice were separated randomly into the 4 different treatment regimens as follows: native IL-18, E6K, E6K+T63A and vehicle. Therapy was instituted according to the schema in Fig. 18A, starting when tumors reached 100 mm^3, tumor bearing mice were injected with each type of recombinant IL-18 once a day for 10 days intratumorally. The result showed that, after receiving 50 µg/kg of recombinant protein, the average survival was 31.1±2.7 (mean±SD) days in the vehicle group, 44.3±3.4 days in the native IL-18 treated group, 49.4±2.3 days in the E6K group, and 61.0±7.1 days in the double mutation treatment group (Table 9). This indicated the improved long-term survival was affected from different types of recombinant IL-18 since mice treated with all types of recombinant IL-18 (n=10) survived significantly longer than vehicle group (n=10) (Fig. 18B) with the p-value=0.008 for native IL-18, and p<0.0001 for both E6K and E6K+T63A suggesting in vivo efficacy of recombinant IL-18. However, there are no significantly different between groups of recombinant IL-18 treated mice (Table 9), although mice treated with E6K+T63A showed the longest of survival time (61±7 days).

Table 9. Statistical analysis of survival rate of each group of mice treated with different regimens.

Intervention	Means Survival Time (days)		Pairwise Comparisons (Log Rank (Mantel-Cox) analysis)							
	Estimate	Std. Error	E6K+T63A		E6K		Native		Vehicle	
			Chi-Square	p-value	Chi-Square	p-value	Chi-Square	p-value	Chi-Square	p-value
E6K+T63A	61.0	7.1	-	-	1.510	0.219	3.182	0.074	16.195	0.000*
E6K	49.4	2.3	1.510	0.219	-	-	0.570	0.450	15.312	0.000*
Native	44.3	3.4	3.182	0.074	0.570	0.450	-	-	7.002	0.008
Vehicle	31.1	2.7	16.195	0.000*	15.312	0.000*	7.002	0.008	-	-

* p<0.0001

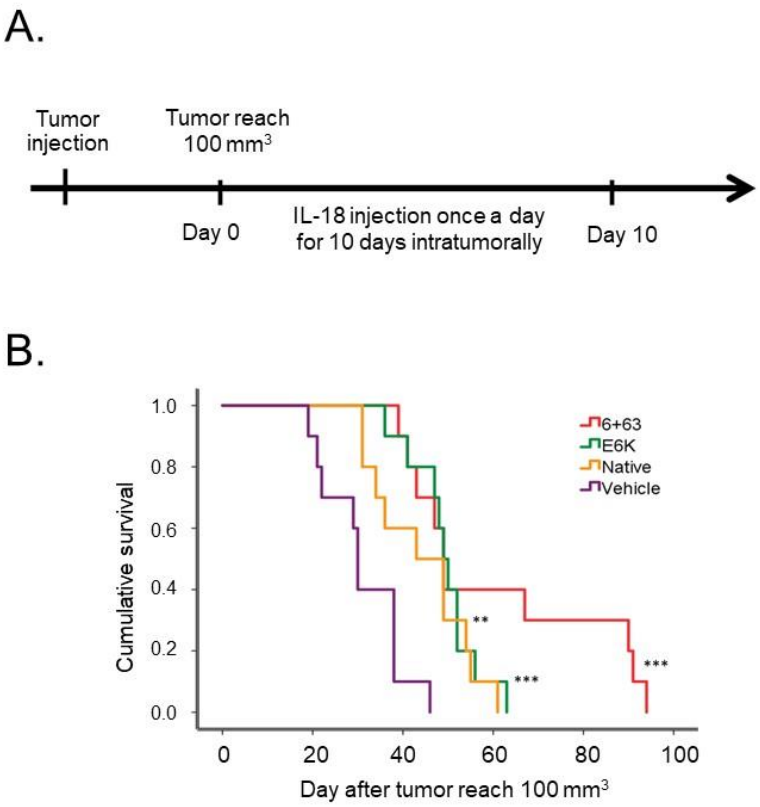


Figure 18. Mice treated with recombinant IL-18 showed the improved survival rate from colon tumor model rechallenge. (A.) The sheamatic of treatment. (B.) Kaplan-Meier analyses of overall survival in each group of mice. n=10. *p<0.05, **p<0.01, ***p<0.001.

In addition to the survival analysis of each regimen groups, we also investigated whether the tumor growth was affected by different types of IL-18. An experimental model was generated as previously described. The results showed that the double mutation (E6K+T63A) and the E6K groups had a significant inhibitory effect on tumor volume when compared to the vehicle group ($p<0.001$ at day 10 of treatment) and native protein treated group ($p<0.001$ and $p<0.05$, respectively) (Fig. 19A and 19B). Tumor growth in mice receiving intratumoral administration of native IL-18 was also slower than vehicle group with the p-value of 0.019 (Fig. 19A and 19B). Indeed, E6K IL-18 induced approximately 50% reduction in tumor volume, and

the combination of E6K with T63A had highly inhibitory effect (around 70% reduction in tumor volume) (Fig. 18B).

The tumor weight was also measured after 10 days of treatment. As shown in Fig. 18C, a considerable decrease in tumor weight was observed in mice treated with recombinant IL-18. The tumors were significantly smaller when tumor bearing mice were injected with all types recombinant IL-18 (Fig. 19C and 19D). Obviously, tumor weight from E6K+T63A treated group showed the 5 and 3.6 times lower levels than tumor from vehicle and native IL-18 treated groups, respectively. This quantification of the tumor size and weight confirmed that E6K+T63A IL-18 significantly reduced xenograft tumor volume (Fig. 19A and 19B) and tumor weight (Fig. 19C and 19D).

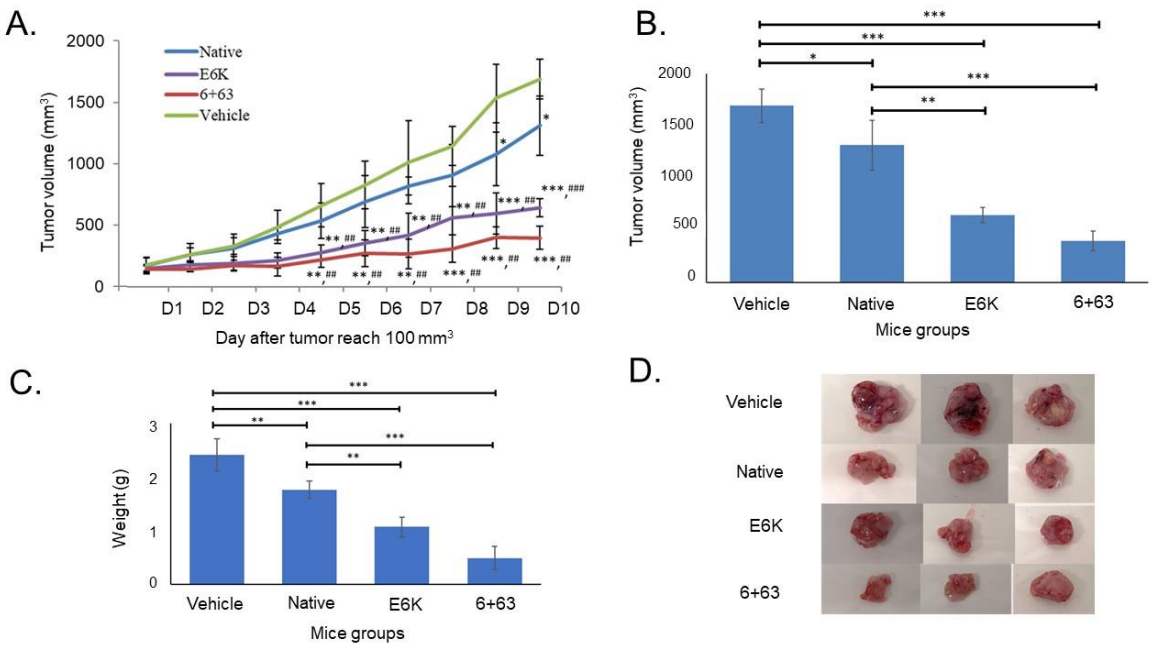


Figure 19. Intratumoral recombinant IL-18 injection suppressed tumor growth *in vivo*. (A) Groups of ten tumor bearing mice were challenged with each type of recombinant IL-8. Tumor size was measured every day starting on d 1 after IL-18 treatment. (B–D) The tumor volumes and weights were measured. The image represents tumor growth at 10 days after IL-18 treatment. Mean±SD. n=5. *p<0.05, **p<0.01, ***p<0.001.

3.6 Tumor microenvironment analysis

Finally, to determine that our recombinant IL-18 indeed induced T cell activity in the context of anti-tumor immunity, we investigated the proposion of different type of T cells. The phenotype of lymphocytes in tumors of CT26-WT bearing mice after recombinant IL-18 treatment was examined. After treatment with each regimen, tumors mass was then collected, and mononuclear cells were then isolated by Percoll gradient centrifugation. All cells were then subjected to flow cytometer after anti-body staining against CD4, IL-4 and IFN-γ. Th1, Th2 and CTL cells were identified as $CD4^{+}IFN\text{-}\gamma^{+}$, $CD4^{+}IL\text{-}4^{+}$ and $CD8^{+}IFN\text{-}\gamma^{+}$, respectively, by flow cytometry and gating on viable cells. The results presented in Fig. 20 showed that the percentage of Th1 and CTL were present in tumor mass of immunized mice, and the use of E6K+T63A IL-18 in the therapy resulted in significantly increased number of both Th1 and Th2 cells ($p<0.05$), as was found for pentamer staining (Fig. 20A and 20C). However, there was no any different in the number of Th2 cells frome mice treated with all types of IL-18 as compare to vehicle group (Fig. 20B). These data demonstrate directly that E6K+T63A IL-18 can induced the strongest functional Th1 and CTL response, and correlate with the *in vivo* tumor therapy data.

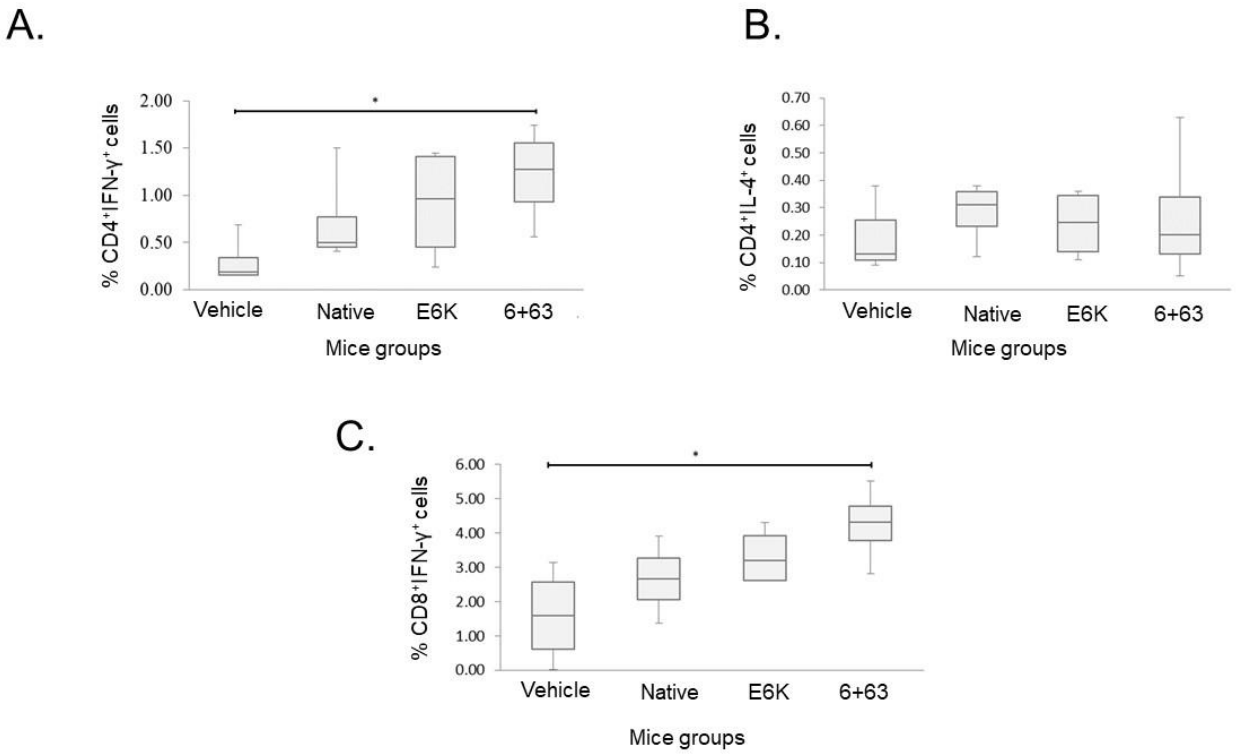


Figure 20. Flow cytometry analysis of immune cells in tumor microenvironment. (A.) Th1 cells, (B.) Th2 cell and (C.) CTL.

CHAPTER 4

DISCUSSION

In this study, we tried to engineer IL-18 by altering amino acids of the molecule in order to improve IL-18 activity using single point mutagenesis, which based on their receptor binding residues and polarity. The proteins were expressed in yeast system that gave us the low cost and no denaturing agents or refolding steps presented in the *E. coli* expression system. In current study, the methylotrophic yeast *P. pastoris*, was chosen with pPICZαA as the expression vector. This system is much easier to carry on and cheaper in cost compared with bacterial expression systems. In addition, as *P. pastoris* is a eukaryotic microorganism, it provides the ability for producing soluble recombinant proteins with correct folding and post-translational modifications (233,234). Therefore, it is not surprise that many proteins produced in *P. pastoris* are similar to those produced in mammalian cells.

Several researches revealed that IL-18 has potential as an anti-cancer agent. This includes the stimulation of NK cells, cytotoxic T lymphocytes, inhibits the growth and spread of cancer cells and increases the expression of Fas-ligand in immune cells for Fas expression cancer cells killing (10–14). Moreover, there are many reports demonstrating that the anti-tumor functions of IL-18 are mainly mediated through IFN-γ (24), which is generally known that IL-18 is an effective activator of IFN-γ production by T cells, NK cells and macrophages and this function can be synergistic with IL-12 (25). Tse et al. showed that neutralizing antibody against IFN-γ completely eliminated IL-18-mediated anti-tumor effects (25). Consistently, Nagai et al. also demonstrated that IFN-γ played a role for the tumor inhibitory effects of IL-18 using a gene-transfer approach (26).

Since IL-18 has anti-tumor capability, there are many models to use this protein in experiment trials with the modified type. Du et al. showed that when IL18-IL2 fusion protein was produced and tested in colorectal cancer- or lung cancer bearing mice, IL18-IL2 fusion protein showed a synergetic effect on tumor regression with the amounts of IFN-γ and cytotoxicity of NK cell that are higher than the

stimulation with only IL-18 or IL-2 or combine it together in separated protein (15). Trial of IL-18 in combining with adoptive cell transfer showed that the addition of IL-18 into the step of treatment with DCs can induce more cancer cells death both in vitro and in vivo (164,165). In oncolytic virus trials, tumor-selective replicating adenovirus expressing IL-18 enhanced the cytopathic effects, apoptotic cell death and anti-angiogenesis event via decease the expression of VEGF in renal cell carcinoma-bearing nude mice (167,168). The another group also expanded this technique by engineering the oncolytic adenovirus that expressed both IL-18 and IL-12, which led to the increasing survival rate of melanoma bearing mice and enhanced the recruitment of NK cells, $CD4^+$ T cells and $CD8^+$ T cells in tumor region (169). In animal models, mice or rabbits receiving IL-18 did not show any fever (170). In addition, intravenous injection of IL-18 in patients with cancer, chills and fevers were not common, and found that fevers were observed only in 3 of 21 patients at doses of 100 and 200 μg/kg (171). So, these trials showed that IL-18 can be safely used in cancer patients (16,17). This indicates the possibility of using IL-18 as a therapeutic agent to stimulate the immune system to fight cancer.

Due to several proteins have been engineered for improving its activity, we substituted some IL-18 binding residues which have been reported in experimental research for this propose. In this study, we engineered IL-18 by the altering binding residues in order to improve its activity using single point mutagenesis. Since Met33 and Met60 in sites I and II are surrounded by other charged amino acids such as Asp, Arg and Lys, a methionine was replaced by a glutamine to increase the site polarity, based on an implication that these sites require dipole/electrostatic forces to facilitate IL-18 binding. Moreover, according to the report that a substitution of E6 with an alanine and a lysine (28) can enhance IL-18 activity, E6K was also taken in this study as a comparative standard. T63 is another interesting residue, reported for increased activity when it was alanine-substituted (190). We also combined both types of previous activity enhanced mutations for evaluating the synergistic effect acquired from the polarity change (E6K) and increasing flexibility (T63A). When 4 mutations of IL-18 were performed and IFN-γ inducing assay using NK-92MI cell was tested, the combination of E6K and T63A showed the highest IFN-γ inducing activity, while E6K and T63A showed the second and third strongest capability of IFN-γ induction

with 9.3 and 3.9 times higher than stimulation by wild-type IL-18 at the concentration of 200 ng/mL in combination with 1 ng/ml of IL-12 (Fig. 14). E6K+T63A also showed the 225 and 136 times higher IFN-γ inducing activity when compared to the previous report of engineered IL-18, E6A+T53A and E6K from *E. coli* system (28,235). However, the activity of M33Q and M60Q, the novel types of mutations we done in the experiment were decreased. This data suggested that all types of changed residues led to the alteration of IL-18 structure or charge area that affected the affinity of IL-18 for its receptor. Recently, the recognition model between IL-18 and its receptor has been crystallized and reported (236). We used this 3D structure obtained from RCSB protein data bank, PDB code: 3WO4, for analyzing how E6K and T63A mutation influence the ligand-receptor binding affinity. The structural change was also evaluated by molecular dynamic simulation. For E6K, the region of IL-18Rα that surrounds E6 contains amino acid residues showing acidic surface composed of E244, E245 and D246 of IL-18Rα, which the distances are between 5.36-7.45 A^o (Fig. 17). It is possible that when E6 was substituted by K, this maybe caused an increasing both cationicity and surface area of this residue. The appropriate distances between K6 and surrounding acidic surface may be occurred leading to the formation of salt bridge that resulted in a higher affinity between IL-18 and IL-18Rα. Molecular dynamics simulation also revealed that when E6 was replaced by K, the higher activity occurred with the good overall structural alignment, showed by root-mean-square deviation (RMSD) values (Fig. 15A). The protein also illustrated the same 3D structures with β-sheets and α-helices in similar arrangement and distribution. This may indicate that the increasing activity of this mutation was affected only by charge transformation.

Interestingly, in the case of T63A, although T63 is located on a region which is not exposed by any residues of IL-18R, this mutant showed 3 times increasing activity. It may be explained by structural changes implicated on its activity enhancement since we observed that in molecular dynamics simulation analysis the system was not stable after 1 ns, confirming the conformational change in this mutant. This hypothesis is in accordance with Swencki-Underwood et al. (29), who elucidated that T63 is one of the first buried residues of a loop Y^{52}KDSQPRGMAVT^{63} that contains K53, which plays a role as a key residue for receptor binding in site II of IL-18 (236). The T63A substitution could make the loop more flexible which may allow

other amino acids within it to interact with the receptor (29). Moreover, according to a recently work (236), *N*-linked glycosyl chain in N297 of IL-18Rα that is close to the β4-β5 loop of IL-18 play a crucial role in bridging D3:D3 domains of IL-18 and IL-18Rα through electrostatic and hydrophobic interaction. The more flexibility of this loop due to T63A substitution could facilitate the easier and stronger these interactions of the domains.

In contrast to T63A, although molecular dynamics simulation showed the conformation changes in M33Q, this type of substitution caused the complete loss of biological activity (Fig. 14). As methionine is a hydrophobic amino acid that prefers to be buried in hydrophobic region, it plays a simple structural role in stabilizing protein hydrophobic core structure instead of reactive function (237). In accordance with molecular dynamic results, M33Q could give structural destabilization through affecting residue 33 that is buried in hydrophobic core. This hydrophobic bury which contain V11, V19, F21, M33, C38, A42 and I46 as components (236), may play a role in stabilizing the site I binding structure of IL-18 since M33Q might change the interaction mediated by α-helix I in site 1 of IL-18. Moreover, previous work indicated that Met-aromatic interaction in the distance of 5-6 A^o played the role in protein structure stabilization, which can be found in approximately one-third of all known protein structures (238). In IL-18 crystallized structure, there is an aromatic amino acid, F21, that might cause the sulfur-aromatic interaction between these 2 residues. Therefore, M33Q might break this intermolecular bond, and may lead to the loss of function of IL-18.

M60 was considered as binding residue in site II (236,239). In 3D structure, this residue is buried on the surface of IL-18 that is surrounded by other hydrophobic residues from both IL-18 itself and IL-18Rα (236). These include L5, M51, G59 and M60 of IL-18, and V246, I247 and A301 of IL-18Rα, which the distances of the residues are between 3.68-5.81 A^o. Since this region is formed from both IL-18 and its receptor, it might play an important role in keeping a protein biologically active through hydrophobic contact that help the ligand-receptor recognition. As demonstrated by molecular dynamics simulation, the structure of IL-18 that is substituted from M60 to Q60 showed a slightly conformation change that imply us this mutagenesis affect the structure and may led to the loss of ligand recognition.

This might be due to Q substitution broke hydrophobic interface buried in IL-18 protein and consequently make the complete loss of hydrophobic contact between site II of IL-18 binding site and IL-18Rα.

In addition to *in vitro* assay, we also investigated the functional effect of E6K and E6K+T63A IL-18 on anti-tumor immune response *in vivo* compared to native protein and control groups. Based on previous work, the route of administration of IL-18 also play an important role on the therapeutic effect of cytokine since IL-18 only slowed tumor progression when administered directly into the tumor mass or if the cytokine was secreted by tumor cells indicating the chaging tumor microenvironment can be an effective target for tumor treatment (24). This was also confirmed by several studies that intratumoral injection of immune-modulatory compounds was used as delivery approach, such as IL-12 (240), viral vectors encoding cytokine genes IFN-γ (241) or IL-12 (242). Therefore, to improve the efficacy of cytokine, we chose the intratumoral injection as a delivery method. After 10 days of treatment with recombinant proteins, the mean tumor volume and weight of E6K and double mutation groups in CT26-WT colon cancer models were observed obviously smaller than that in control group. Compared with native protein, E6K and E6K+T63A demonstrated better effects on tumor regression. These results suggested the combination of E6K and T63A showed a synergetic effect on tumor in immune-competent mice.

IL-18 is a potent inducer of IFN-γ production by T cells and NK cells (25). Thererfore, to evaluate the anti-tumor effect of each type of recombinant IL-18, the number of Th1, Th2 and CTL in tumor mass were then investigated, and these cells can be identified as $CD4^+IFN\text{-}\gamma^+$, $CD4^+IL\text{-}4^+$ and $CD8^+IFN\text{-}\gamma^+$, respectively, in accordance with the role of IFN-γ induction. In tumor-bearing mice models, although there was no any significantly different in Th2 number, the presence of E6K+T63A IL-18 induced significantly greater infiltrations of $CD4^+IFN\text{-}\gamma^+$ T cells and $CD8^+IFN\text{-}\gamma^+$ cells in tumor mass (Fig. 20A and 20C) compared to vehicle group supporting a role of our recombinant engineered IL-18 on anti-tumor immunity. This scenario is not surprising given that IL-18 can recruit T cells to the site (243), promote the polarization of Th1 cells, induce the proliferation and enhances the cytotoxicity of T cells (25) and induces the maturation of dendritic cells (244).

Taken together, we successfully produced IL-18 protein using *P. pastoris*, which is the system that is simply, safety and efficient, and may facilitate further studies of IL-18 in the near future. Moreover, we have demonstrated that point mutations of IL-18 truly affected its biological function, which further regulated the immunity and can be applied to use as an agent for immunotherapy. E6 and T63 can be modified for improving the activity of IL-18. Some extent and synergistic effects are observed when these two mutations are combined. We also revealed the molecular insights into how mutations affected IL-18 activity in terms of protein conformation, flexibility, and intra-protein and protein-receptor interactions. In animal model with immunocompetent mice, intratumoral injection of E6K+T63A IL-18 had the ability to inhibit tumor growth. This scenario was mediated by induction of local Th1 and CTL via IL-18. These results indicated that recombinant E6K+T63A IL-18 exhibited promising potential in the treatment of cancer, and in the future other experiment, such as IL-18BP inhibition assay or stability testing assay, should be investigated to characterize this novel type of IL-18.

CHAPTER 5

CONCLUSION

In this study, we have developed the engineered type of human IL-18 using site-directed mutagenesis based on protein-receptor interaction. The amino acids of the protein have been changed to other types, resulting in E6K+T63A IL-18 protein. For protein production, yeast expression system has been selected for IL-18 expression since this system can mimic the eukaryotic protein folding together with the low cost and high effectiveness. After protein purification, we have done to test the ability of this engineered IL-18 in cell culture, and the results showed the higher activity of E6K+T63A IL-18 than native IL-18 around 16 times. Molecular dynamic (MD) simulation was use to investigate the role of inter- and intra-molecular alterations of the protein after mutations were performed, and it has been revealed that the mutations affected the key residues containg region of IL-18 leading to the effective interaction between our engineered IL-18 and its receptors. Although these structural changes occurred with the higher activity, the overall structure still be the same. Animal study was also performed to evaluate the influence of this protein to the anti-tumor immunity. We chose the intratumoral injection as a rout since this delivery method has been reported to be more effective than systemic delivery. The results demonstrated that mice treated with our engineered IL-18 exhibited the lowest tumor volume, and our protein significantly expanded the survival preriod of this group of mice. This may be mediated by the recruitment of Th1 and CTL to the tumor mass effected by our engineered IL-18 since flow cytometry revealed the higher proportion of Th1 and CTL cells in the tumor region when compared to the control group. This all confirmed the performance of our engineered type of IL-18 as a cancer therapeutic candidate.

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Educational Attainment

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B. Sc. (Microbiology) First class Honors	Prince of Songkla University	2012

Scholarship Awards during Enrolment

1. PSU-Ph.D. Scholarship
2. Student exchange awards at University of Miyazaki, Japan

List of Publication and Proceeding

1. **Saetang J,** Boonpipattanapong T, Palanusont A, Maneechay W, Sangkhathat S. Alteration of leptin and adiponectin in multistep colorectal tumorigenesis. Asian Pac J Cancer Prev. 2016;17(4): 2119-23.
2. **Saetang J**, Puseenam A, Roongsawang N, Voravuthikunchai SP, Sangkhathat S, and Tipmanee V. Immunologic functions and molecular insight of recombinant IL-18. PLoS One. 2016 Aug 2;11(8):e0160321.
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4. Theerakitthanakul K, **Saetang J**, Kruatong J, Graidist P, Raungrut P, Kayasut K, Sangkhathat S. Senescence Process in Primary Wilms' Tumor Cell Culture Induced by p53 Independent p21 Expression. J Cancer. 2016 Sep ;7(13):1867-1876.
5. **Saetang J**, Sangkhathat S. Role of innate lymphoid cells in obesity and metabolic disease (Review). Mol Med Rep. 2017 Nov 13. doi: 10.3892/mmr.2017.8038. [Epub ahead of print]