T.C.
DOKUZ EYLÜL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND
GENOME INSTITUTE

PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF iNKT CELL SUBSETS IN VIVO

Zeynep Gülçe TANYOLAÇ

MOLECULAR BIOLOGY AND GENETICS

MASTER'S THESIS

IZMIR-2020

THESIS CODE: DEU.IBG.MSc/2017850028

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Supervisor: Dr. GERHARD WINGENDER

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DOKUZ EYLÜL ÜNİVERSİTESİ

İZMİR ULUSLARARASIBİYOTIP VE GENOM ENSTİTÜSÜ

ÇEVRİM İÇİ YÜKSEK LİSANS TEZ SAVUNMA SINAVI TUTANAĞI

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☒ Sınav kayıt altına alınmıştır.

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ABBREVIATIONS

α:	anti-
aa:	Amino Acid
αGalCer:	Alpha-galactosylceramide
AF488:	Alexa Flour 488
AF647:	Alexa Flour 647
AF700:	Alexa Flour 700
APCs:	Antigen presenting cells
APC:	Allophycocyanin
APC-Cy7:	Allophycocyanin-cyanine7
APC-eF780:	Allophycocyanin – eFlour 780
BSA:	Bovine serum albumin
Bcl6:	B cell lymphoma 6
BCR:	B cell receptor
BUV395:	Brilliant Ultraviolet 395
BV421:	Brilliant Violet 421
BV510:	Brilliant Violet 510
BV570:	Brilliant Violet 570
BV605:	Brilliant Violet 605
BV650:	Brilliant Violet 650
BV711:	Brilliant Violet 711
BV785:	Brilliant Violet 785
BV786:	Brilliant Violet 786
C:	Celsius
CD:	Cluster of differentiation
cm:	Centimeter
CTL:	Cytotoxic T cell
cRPMI:	Completed RPMI
CXCL13:	Chemokine (C-X-C Motif) ligand 13
DMSO:	Dimethylsulfoxid
DNA:	Desoxyribonucleic acid

DNase:	Desoxyribonuclease
DCs:	Dendritic cells
EDTA:	Ethylendiamintetraacetat
eF660:	eFlour 660
eFluor 450:	eFlour 450
EtOH:	Ethanol
Fc:	Fragment crystallizable
FACS:	Fluorescent activated cell sorting
FCS:	Foetal calf serum
FITC:	Fluorescein isothiocyanate
FBS:	Fetal Bovin serum
FITC:	Fluorescein isothiocyanate
FoxP3:	Forkhead box P3
FR4:	Folate receptor 4
g:	gram; acceleration of gravity (9.81 m/s2)
GC:	Germinal centers (GCs)
h:	hour
IFN:	Interferon
Ig:	Immunoglobulin
IL:	Interleukin
iNKT:	Invariant Natural Killer T
IL-10:	Interleukin 10
IL-12:	Interleukin 12
IL-13:	Interleukin 13
IL-17A:	Interleukin 17A

IL-21:	Interleukin 2
IL-4:	Interleukin 4
ILCs:	Innate lymphoid cells
Kb:	Kilobase
kD:	Kilodalton
L:	Liter
µ:	Micro- (10^{-6})
m:	Meter; milli- (10^{-3})
M:	Molar
MACS:	Magnetic activated cell sorting
MHC:	Major histocompatibility complex
Min:	Minute
mRNA:	messenger RNA
MW:	Molecular weight
n:	nano- (10^{-9})
NKT_{FH}:	Follicular helper NKT
NaN_3:	Sodium azide
NK1.1:	Natural Killer 1.1
OD:	Optical density
PBS:	Phosphate buffered saline
PE:	Phycoerythrin
PMA:	Phorbol 12-myristat 13-acetat
PB:	Pacific blue
PE-Cy5:	Phycoerythrin cyanine5
PE-Cy7:	Phycoerythrin cyanine 7
PE-Dazzle 594:	Phycoerythrin Dazzle 594

PE-eFluor610:	Phycoerythrin eFluor610
PerCP-Cy5.5:	Peridinin chlorophyll protein cyanine 5.5
PerCP-eF710:	Peridinin chlorophyll protein – eFluor 710
PLZF:	Promyelocytic leukemia zinc finger
RNA:	Ribonucleic acid
rpm:	Rotations per minute
RT:	Room temperature
RORγt:	RAR-related orphan receptor gamma T
s:	Second
SDS:	Sodium dodecyl sulphate
scWAT:	Subcutaneous white adipose tissue
Slamf6:	Signaling lymphocyte activation molecule 6
SSC-A:	Side scatter area
SSC-W:	Side scatter width
TCR:	T cell receptor
T_{FH}:	T follicular helper
Th:	T helper
TNF:	Tumor necrosis factor
Tris:	Tris- (hydoxymethyl) -aminomethan
Tbet:	T-box transcription factor
TRAIL:	Tumor necrosis factor-related apoptosis inducing ligand
U:	Unit
UV:	Ultraviolet light
v/v:	Volume per volume
Vol:	Volume
W:	Weight
w/v:	Weight per volume

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PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF iNKT CELL SUBSETS IN VIVO

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ABSTRACT

*i*NKT cells are a unique subset of T cells. Unlike T cells, they recognize glycolipids via MHC1 like molecule, CD1d. After activation, they can produce both Th1 and Th2 cytokines. *i*NKT cells have several subsets. These subsets are distinguished by the transcription factors they express and the cytokines they produce. NKT1, NKT2, and NKT17 cell express the transcription factor PLZF, TBET, and RORγt at different levels. NKT2 cells are known as the main producer of *i*NKT cell-derived Th2 cytokines. However, in this study, splenic NKT2 cells produced less IL-4 than NKT1 cells after activation with either αGalCer, or PMA/Ionomycin. Therefore, we hypothesize that peripheral NKT2 cells are hypo-responsive. Additional work on cell signalling (NUR77) confirmed this hypothesis. Knowledge of the main source of *i*NKT cell-derived Th2-cytokines is essential to understand the role *i*NKT cells can play in various diseases and to improve *i*NKT cell-related diagnosis and therapy.

Keywords: *i*NKT cell subsets, NKT2 cells

DOĞALA YAKIN T HÜCRE ALT GRUPLARININ KARAKTERİZASYONU

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ÖZET

Doğala yakın öldürücü T hücreleri (*i*NKT), T hücrelerinin farklı bir alt grubudur. T hücrelerinin aksine glikolipid yapısında olan antijenleri CD1d molekülü yardımıyla tanırlar. *i*NKT hücreleri aktive olduktan sonra Th1 ve Th2 sitokinlerini üretirler. Bu hücreler eksprese ettikleri transkripsiyon faktörlerine ve ürettikleri sitokinlere göre farklı alt gruplara ayrılırlar. NKT1, NKT2 ve NKT17 hücreleri PLZF, TBET ve RORγt transkripsiyon faktörlerini farklı seviyelerde eksprese ederler. NKT2 hücreleri *i*NKT hücre kaynaklı Th2 sitokinlerinin ana üreticisi olarak bilinirler. Fakat bu çalışmada dalak *i*NKT hücrelerinin NKT1 hücrelerine göre PMA yada αGalCer ile sitimule edildiklerinde Th2 sitokinlerinden olan IL-4 sitokinini daha az ürettiklerini gözlemledik. NUR77 markörü ile yapılan çalışma da NKT2 hücrelerinin TCR ile aktive olduklarını fakat sitokin üretemediklerini gözlemledik. Bu bulgular *i*NKT hücre alt gruplarının bazı hastalıkları anlamak açısından ve *İ*NKT hücre odaklı immun terapi geliştirebilmek için önemli bilgiler sağlamaktadır.

Anahtar Sözcükler: *i*NKT hücre grupları, NKT2 hücreleri

1. INTRODUCTION AND OBJECTIVES

Invariant natural killer T (*i*NKT) cells are a unique subset of T cells that recognize glycolipids rather than peptides. They produce copious amounts of different cytokines after activation. At steady-state, there are four different *i*NKT cell subsets that resemble $CD4^+$ T cell subsets. They are characterized based on the transcription factors they express and the cytokine they produce: NKT1 cells are characterized as $PLZF^{low}$ $Tbet^{high}$ $ROR\gamma t^{neg}$ cells and produce T_H1 cytokines, NKT2 cells are $PLZF^{high}$ $Tbet^{low}$ $ROR\gamma t^{neg}$ and produce T_H2 cytokines, and NKT17 cells are $PLZF^{int}$ $Tbet^{low}$ $ROR\gamma t^{high}$ and produce T_H17 cytokines. NKT10 cells express IL-10, but the master transcription factor has not been defined yet. However, flow cytometric staining for transcription factors or cytokines is destructive in nature as it requires fixation of the cells and, consequently, precludes further functional studies. Therefore, it would be important to distinguish *i*NKT cell subsets in a nondestructive manner via surface markers. Furthermore, it has been suggested that NKT2 cells are the main producer of IL-4. However, cytokine production of splenic *i*NKT cells did not differ between BALB/c and C57BL/6 mice although BALB/c mice have more NKT2 cells. Therefore, it is important to clarify the cytokine production of NKT2 cells.

Three major aims were addressed in this project:

1) To establish the *in vivo* distribution of *i*NKT cells subsets: Data on the *in vivo* distribution of *i*NKT cell subsets are still limited and, for example, no information on the adipose tissue or the bone marrow is available. Therefore, we isolated cells from several organs in mice and measured the frequency of iNKT cell subsets by flow cytometry.

2) To identify *i*NKT cell subsets via surface markers: To measure *i*NKT cells without the need of fixation, we aimed to establish a set of surface markers that could be used instead of transcription factors. To this end, we compared the expression of various surface markers on *i*NKT cell subsets identified via transcription factors. We hypothesized that it is possible to identify *i*NKT cell subsets via surface markers.

3) To determine the functional characteristics of NKT2 cells: After antigen stimulation, *i*NKT cells rapidly produce cytokines. It was suggested that NKT2 cells are the main producers of *i*NKT cell-derived IL-4. However, based on cytokine data from splenic

*i*NKT cells from BALB/c and C57BL/6 mice, we hypothesized that NKT2 cells are not the main producer of the T_H2 cytokine IL-4. To measure the cytokine production of *i*NKT cell subsets, we stimulated splenic *i*NKT cells with αGalCer, PMA/ionomycin, or with αCD3ε antibodies for 4-5 hours and analysed their cytokine production by flow cytometry.

2. LITERATURE SUMMARY

2.1. The immune system

Mammals are exposed to various microorganisms throughout their life. In some way, they need to defend their body against organisms that threaten their normal homeostasis. These organisms include pathogens, like bacteria and viruses, but also commensals. The immune system evolved to eliminate pathogens and to control commensals (Alam R., 1998). The immune system protects the body by detecting structural features of the pathogen that identify it as different from host cells. The immune response is commonly divided into two different arms: innate immune and adaptive immune system. The innate immune system is the first to respond to microorganisms. Usually, most of the pathogenic organisms are handled by the innate immunity without causing any disease. Furthermore, the innate immune system calls the adaptive immune system into play, which offers a more diverse response (Chaplin D., 2010, Janeway, 2012).

2.2. Innate immune cells

Innate immune cells are the ones that attack first when a pathogen invades the body and they have different mechanisms to handle the pathogen (Cronkite D. and Strutt T., 2018). Some innate immune cells are derived from myeloid stem cells, including monocytes, macrophages, dendritic cells, neutrophils, eosinophils, basophils, and mast cells. Monocytes circulate in the blood for about 72 hours before they differentiate into either macrophages or dendritic cells. During an infection, monocytes, macrophages, and granulocytes (neutrophils, eosinophils, basophils, mast cells) migrate to the infection site and destroy the pathogen. This is achieved on the one the hand by releasing the toxic content of their granules (specialized intracellular vesicles) onto the pathogen. On the other hand, the pathogen can be ingested and digested intracellular, a process called phagocytosis that is performed in particular by macrophages. Macrophages also participate in antigen presentation to adaptive immune cells, which means that they are able to present protein fragments (peptides) from the pathogen on their surface. Adaptive T cells (see 2.3.2) can recognize these peptides and become activated. Similar to macrophages,

dendritic cells can also phagocytes pathogens. However, their main function is antigen presentation. Due to this function, dendritic cells, macrophages as well as adaptive B cells (see 2.3.1) are also called professional antigen presenting cells (APC) (Marshall J., Warrington R., 2018). In contrast to myeloid cells mentioned before, Natural killer (NK) cells are lymphoid cells as they derived from the common lymphoid progenitor. They can recognize and destroy abnormal cells, like virus-infected cells or tumour cells. The killing of the target cells by NK cells is either achieved via the release of cytotoxic proteins in their lytic granules or via specialized surface receptors that can trigger a programmed cell death, called apoptosis, in the target cells (Janeway, 2012 and Abbas AK., 9th edition).

2.3 Adaptive immune cells

The innate immune system protects the body against infections. However, it only can protect the body against pathogens via recognition of common pathogenic structures for which specific receptors have been evolved. In contrast, each adaptive immune cell bears one unique antigen binding receptor for antigens. This greatly increases the range of antigens that can be recognized by the immune system and allows the adaptive immune cells to fight with the “unknown”, i.e. novel pathogenic structures. Once adaptive immune cells receive a stimulating signal via their unique receptor, they rapidly proliferate and respond with various effector functions. Adaptive immune cells use receptors that allow them to recognize an almost unlimited number of antigens. However, for every particular receptor only a few cells are present initially in the body. Although these cells rapidly proliferate after activation this requires time, which makes their response slower than innate response. Furthermore, the adaptive immunity offers a long-lasting memory, which greatly improves the response to the pathogen upon a secondary encounter. These memory cells are kept in a dormant state after the initial clearance of the pathogen and allow a more effective response during a secondary encounter with the same pathogen. There are two main types of adaptive immune cells, B and T cells.

2.3.1. B cells

The unique receptor on B cells is called B cell receptor (BCR). This BCR can directly bind and recognize antigens, like pathogenic surface molecules, that are outside of host cells. The main function of B cells is the production of antibodies, which are a soluble secreted form of the BCR (Chaplin D., 2010). After they become activated, some B cells differentiate into plasma cells that produce large amounts of these antibodies. The four main subtypes of antibodies, called IgA, IgG, IgE and IgM each have different roles in immunity. Antibodies can bind to pathogens in order to tag them for efficient recognition by innate immune cells and other defence mechanisms. Also, they can inactivate pathogens or toxins directly by binding to them and blocking essential parts. Lastly, they facilitate phagocytosis by APCs, which augments the antigen presentation to T cells (Marshall J., Warrington R., 2018). During infection, some of the B cells become memory cells and these cells stay in the body long after the infection is eliminated. Memory B cells do not secrete antibodies and can survive independently of the initial stimulating antigen. Therefore, memory B cells provide long-lasting immunity and when the same pathogen invades the body, memory cells respond quickly to the antigen, allowing a faster and more robust response (Chaplin D., 2010).

2.3.2. T cells

Since B cells and antibodies only recognize antigens that are outside of the cell, there is another type of adaptive immune cells that supports the elimination of infected host cells. This type of cells is called T cells and their unique receptor is called T cell receptor (TCR). The TCR consists of an α- and a β- chain and each chain consists of variable (V) and constant (C) region. The V-region contains the antigen binding side of the TCR and two main mechanisms evolved to ensure an enormous diversity in this region. On the one hand, the V-region of the α- and β-chains are assembled from two or three parts that are encoded by different gene segments: V and J genes for the α-chain and V, D, and J genes for the TCRβ chain. As there are many different options for each of these segments to choose from, the random process of combining them can generate many different TCR chains. On the other hand, when these

segments are joined, additional mechanisms exist that can randomly add and change the nucleotide sequence at the junction. This greatly increases the diversity of the finished TCR chains. The TCR gene rearrangement generates functional TCRs and occurs in the thymus during the development of the T cells. Unlike the V-region of the TCR, the C-region of TCRα and TCRβ is derived from only one C gene segment (Alcover A., Alarcon B. and Bartolo V., 2017). In contrast to the BCR, the TCR does not recognize antigens directly, but only in the context of specialised host molecules that act as a tray for the bound antigens. These molecules are called major histocompatibility complex (MHC) molecules and two main classes are distinguished, called MHC class I and MHC class II molecules. MHC class I molecules bind and present peptides derived from intracellular proteins to T cells that bear the CD8 glycoprotein. Such $CD8^+$ T cells are involved in the cytotoxic destruction of abnormal host cells, similar to NK cells, using similar mechanism. In contrast, MHC class II molecules present peptides derived from extracellular proteins to T cells that bear the CD4 glycoprotein. CD4+ T cells are responsible for orchestrating the adaptive immune response. They can influence the activity of other immune cells by releasing small signaling proteins, called cytokines. There are several functional distinct types of $CD4^+$ T cells, also called subsets. They include, among others, T_H1, T_H2, T_H17, T_H22, T_H9, T_{regs}, and T_{FH} cells (Golubovskaya V. and Wu L., 2016, Janeway, 2012). These subsets have different roles in immunity as different pathogens require different immune responses. This can be achieved as each $CD4_+$ T cell subset produces a unique blend of cytokines, which boost one particular type of immune response. For example, T_H1 cells respond against intracellular pathogens, like bacteria or viruses by producing cytokines like IFNγ and TNF. These cytokines greatly increase the killing power of e.g. macrophages and NK cells and help to eliminate the pathogens (Marshall J., Warrington R., 2018). Furthermore, previously activated T cells can also differentiate into memory cells. Like memory B cells, such memory T cells can survive in the body independently of the initial stimulating antigen and provide long-lasting immunity.

2.4. Innate-like T and B cells

The immune system is commonly divided into innate and adaptive immunity. However, some cells cannot be easily classified as innate or adaptive immune cells. Similar to adaptive immune cells, they bear either a TCR or a BCR. However, they quickly exert effector functions, like innate immune cells. Examples of these innate-like T and B cells are, γδ T cells, invariant natural killer T (*i*NKT) cells, mucosal-associated invariant T (MAIT) cells, and marginal zone B (MZB) cells (Verykokakis M., Zook E. and Kee B., 2014).

2.4.1. Invariant Natural Killer T (*i*NKT) cells

Invariant natural killer T (*i*NKT) cells are unique subsets of T cells, capable of recognizing unique antigens through their distinct TCR (Lantz O. et. al., 1994). The antigen-presenting molecule they engage is different and they recognize lipid antigens rather than peptides (Bendelac A., 1995). For this reason, they can be called "unconventional T cells". *i*NKT cells express an invariant TCRα chain which means the α-chains of all *i*NKT cells use an identical Vα14-Jα18 pair in mice or a Vα24-Jα18 pair in humans. As an outcome, basically every human has the same *i*NKT cell TCRα chain. *i*NKT cells differentiate during T cells development at the double positive ($CD4^+$ $CD8^+$) thymocyte stage in the thymus. *i*NKT cells are characterized by the recognition of glycolipids presented by CD1d, which is a nonpolymorphic MHC class I-like molecule (Barg et al., 2007). Moreover, it presents lipids to *i*NKT cells and most cells express CD1d molecule on their surface (Brenner M. and Brigl M, 2004). In line with their innate features, *i*NKT cells are "poised effector" cells and produce large amounts of cytokines rapidly after activation (Brennan P., Brigl M. and Brenner M, 2013). The first described glycolipid antigen presented by CD1d to activate *i*NKT cells was α-galactosylceramide (αGalCer), which is also the best studied antigen (Borg N. et. al., 2007). Because of its potent nature, αGalCer has been used in clinical trials for cancer treatment and is still under development (Cerundolo V. et. al., 2009). After activation, *i*NKT cells secrete different cytokines and promote T_H1, T_H2, and T_H17 responses (Godfrey D. and Mitchell K., 2004). However, the impact of activated *i*NKT cells on the immune response after their activation can be unpredictable, as it can be either

pro-inflammatory or anti-inflammatory (Brigl M. and Brenner B., 2010). Furthermore, *i*NKT cells can be multi-functional, promoting for example either they can promote tumour rejection and tolerance or exacerbating autoimmune reactions (Godfrey D. and Mitchell K., 2004).

During thymic development, *i*NKT cells start to express a variety of surface markers, co-stimulatory molecules and transcription factors that identify them (Constantinides M. and Bendelac A., 2013). An essential important transcription factor for *i*NKT cells development is promyelocytic leukemia zinc finger (PLZF) (L. Wu et al., 2009). Furthermore, several subsets of *i*NKT cells have been described that are analogous to CD4+ T cell subsets (Lee et al, 2013). There are four distinct *i*NKT cell subsets at steady state, namely NKT1, NKT2, NKT17, and NKT10 cells, based on the transcription factors they express and their function (Lee et al, 2013 and Sag et al., 2014). Each subset expresses the transcription factors PLZF, TBET (T-cell-specific T-box transcription factor), and RORγt (Rar-related orphan receptor gamma t) at different levels. NKT1 cells are identified as $PLZF^{low}$ $Tbet^{high}$ $ROR\gamma t^{neg}$, NKT2 cells and NKT10 cells are identified as $PLZF^{high}$ $Tbet^{low}$ $ROR\gamma t^{neg}$, and lastly NKT17 cells are identified as $PLZF^{int}$ $Tbet^{neg}$ $ROR\gamma t^{high}$ (Lee et al, 2013, Sag et al., 2014). In addition to these subsets, there is another subset called follicular helper NKT (NKT_{FH}) cells. They arise after activation in the periphery and express the transcription factor BCL6 (B-cell lymphoma 6) (Chang et al., 2012). Unlike conventional T cells, *i*NKT cell subsets do not recirculate in the body. They are tissue resident cells based on different chemokine receptors they express (Brailey P. et. al., 2020). Moreover, *i*NKT cells are found in lymphoid and non-lymphoid tissues at different proportions (Crosby C. and Kronenberg M., 2018). For example, NKT1 cells are enriched in mouse liver, NKT2 cells are enriched in mesenteric lymph nodes and NKT17 cells are abundant in lungs and inguinal lymph nodes. Finally, NKT10 cells are highly abundant in adipose tissue at steady state (Crosby C. and Kronenberg M., 2018). These *i*NKT cell subsets have different roles in mediating immune functions. After activation, NKT1 cells produce the Th1-cytokine IFNγ and also secrete the Th2-cytokine IL-4, which sets them apart from T_H1 cells, which only produce Th1-cytokines. (Brailey P. et al., 2020). NKT2 cells are known as the producer of IL-4 and IL-13 and they initiate T_H2 response when they are activated. NKT17 cells produce

IL-17 and are responsible for T_H17 response (Lee Y. et. al., 2015). IL-10 producing NKT10 cells have a regulatory phenotype and they can greatly expand after antigen activation (Sag et. al., 2014). NKT_{FH} cells are responsible for providing help to B cells to induce antibody responses against lipid antigens similar to conventional T_{FH} cells (Brailey P. et al., 2020, Crosby C. and Kronenberg M., 2018, Clancy-Thompson et. al., 2017, Chang et al., 2012).

3. MATERIALS AND METHODS

3.1. Type of The Research:

Experimental study.

3.2. Location and the time frame of the research:

All experiments were performed at the Izmir Biomedicine and Genome Center, between 2017 and 2020.

3.3. Research population, sampling and experimental groups:

Not applicable.

3.4. Research Materials:

Different organs were obtained from BALB/c, which were housed in IBG's vivarium (May 2018 - February 2020). The CD1d/□GalCer- tetramers (labelled with BV421 or PE) were kindly provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA, USA). All other reagents, including chemicals, kits, antibodies, and consumables, were obtained from commercial sources as outlined below.

3.5. Research Variables:

Variables of our study are cell frequencies and the expression of surface markers, cytokines, and transcription factors.

3.6. Data Collection Tools:

3.6.1 Laboratory Materials

3.6.1.1 General Laboratory Equipment

- Autoclave (Systec, VX-150, 166L, SN:8373)

- Biological safety cabinet (ThermoFisher Scientific, class II type A2- SN:41806507)
- Cell analyser, LSR FortessaX20 (BD Biosciences, #647780S1, SN:H647780S1001, model no:N/A)
- Centrifuge (IKA, model: mini G, SN: 100046858)
- Centrifuge 5702 (Eppendorf, SKU: Z606936EU, SN: 5702DR5336846)
- Centrifuge microCL 17R (ThermoFisher Scientific, # 75002455, SN: 41793777)
- CO2 incubator (MEMMERT, INCOmed 246 0°C/+50°C, SN: 0215-0125)
- Freezer, -20°C (Bosch, 161x70 cm, SN: GSN51AW30)
- Ice maker (HOSHIZAKI, model number: FM-300AKE-N, SN:E11212)
- Magnet, EasySep–immunomagnetic column-free magnet (StemCell Technologies, #18000)
- Microscope, inverted (Carl Zeiss, model: Axio Vert.A1, SN: 3849001095)
- Pipette pump, electrical (Isolab, 0.1-200 ml, # 010.01.006)
- Pipette stand, carrousel (GILSON, SKU: F161401)
- Pipette, multi-, pipet-lite L8 -200XLS+ (Rainin, # 17013805)
- Pipette, single channel, PIPETMAN classic P2 (GILSON, SKU: F144801)
- Pipette, single channel, PIPETMAN G starter kit, P20G, P200G, P1000G (GILSON, SKU: F167900)
- Refrigerator, +4°C (Bosch, model: KSV36AI31/09)
- Thermal cycler, SimpliAmp (ThermoFisher Scientific, # A24811, SN: 228002716)
- Vortex (ThermoFisher Scientific, LP vortex mixer, SN: EAKT18071, # 88880018)
- Water bath (Nüve laboratory & sterilization technology, model no: NB 9, SN: 02-2531)
- Water purification system (ThermoFisher Scientific, model: Smart2Pure 3 UV/UF, SN:41531232)

3.6.1.2. Vivarium Laboratory Equipment

- Anaesthesia machine (E-Z systems, model: classic, # EZ-150C)
- Biological safety cabinet (LABGARD, class II, model no: NU-S677-400E, SN:182313100617)
- Charcoal filter canister (E-Z systems, ReFresh, # EZ-258)
- Forceps (graefe), 10.16 cm long serrated slight curve 0.8 mm tip (ROBOZ, # RS-5135)
- Forceps (semken), 1x2 teeth; 1.6 mm tip width; 15.24 cm length (ROBOZ, # RS-5248)
- Induction chamber (E-Z systems, sure-seal mouse chamber 10.16 cm long x 10.16 cm width x 10.16 cm height, # EZ-177)
- Scissors (delicate operating), 12.065 cm straight sharp/sharp (ROBOZ, # RS-6702)
- Scissors (light operating), 12.7 cm curved sharp/sharp (ROBOZ, # RS-6753)
- Scissors (operating), straight; sharp-blunt; 15.24 cm length (ROBOZ, # RS-6818)

3.6.1.3 Consumables

- Parafilm: Wrap, 4" Wide; 125 Ft/Roll (#PM-996)
- Gloves, examination (Small, Medium, Large): MyGlove (Labmarker Dis Ticaret Ltd. Sti., #9604 0203)
- Aluminium foil, 30m, (Migros)
- Tube, 50mL (polypropylene): canonical bottom, (Greiner Bio-One, #227261; Sarstedt, #62.547.254; Orange Scientific, #4440100N)
- Tube, 15mL (polypropylene): canonical bottom (Greiner Bio-One, #188271)
- Tube, 5mL (polypropylene): round bottom (Stem Cell Technologies, #352063)
- Pasteur pipette, glass, 225mm (ISOLAB, #084.01.002)

- Pasteur pipette, polyethylene, 3mL, non-sterilized (ISOLAB, #084.02.001)
- Syringe, single use with needle, 5mL (Genject)
- Filter syringe, 33mm x 0.22µm, (CHROMFILTER, #S33-CA22-S)
- Reservoirs, reagent – disposable, 55mL, non-sterilized (ISOLAB, #006.03.055)
- Microplate, 96-well F- bottom (Greiner Bio-One, #655081)
- Petri dish, 10 cm (ISOLAB, #081.01.100)
- Petri dish, 5 cm (ISOLAB, #081.01.060)
- Multi-well plate, cell culture 96 well (Greiner Bio-One, #655180)
- Cell culture multi-well plate, 48 well (Greiner Bio-One, #677180)
- Cell culture multi-well plate, 24 well (Greiner Bio-One, #662160)
- Cell culture multi-well plate, 6 well (Greiner Bio-One, #657160)
- Flask, cell culture, 250mL (Greiner Bio-One, #658175)
- Flask, cell culture, 50mL (Greiner Bio-One, #690175)
- Reaction tube, 2mL with attached cap (Greiner Bio-One, #623201)
- Reaction tube, 1.5mL with attached cap (Greiner Bio-One, #616201)
- Reaction tube, 0.5mL with attached cap (Greiner Bio-One, #667201)
- Pipet tips, 1mL (Rainin, #17001977)
- Pipet tips, 250mL, LTS for multichannel (Raining, #17000506)
- Pipet tips, 200mL (Corning Inc., #4845)
- Pipet tips, 10mL (Rainin, #17004280)
- Pipettes, serological, 25mL, sterile (Greiner Bio-One, #760180; SPL Lifesciences, #91025)
- Pipettes, serological, 10mL, sterile (Greiner Bio-One, #607180; SPL Lifesciences, #91010)
- Pipettes, serological, 5mL, sterile (Greiner Bio-One; #606180; SPL Lifesciences,

#91005)

3.6.1.4 Chemicals, Reagents, And Commercial Kits

- 2-Mercaptoethanol (HSCH2CH2OH, sigma, #M6250-1L, [55 µM], stored at 4°C)
- CytoFix/Perm (BD Biosciences, #554722, stored at 4°C)
- Dynabeads mouse pan B (B220) (ThermoFisher Scientific, #11441D)
- Ethanol Technical Grade (Interlab, #TK.200650.05001)
- Fetal bovine serum (FBS, Biochrome, #s0115, heat inactivated, stored at -20°C)
- GolgiPlug (BD Biosciences, #555029, brefeldin A [1 mg/ml] in DMSO, stored at 4°C)
- GolgiStop (BD Biosciences, #554724, monensin [2 mg/ml] in EtOH, stored at 4°C)
- Ionomycin (C41H72O9, Sigma, #I9657-1MG, [1 mg/mL] in DMSO, stored at -20°C)
- L-Glutamine 100X (ThermoFisher Scientific, #25030081, [200 mM] stored at –20°C)
- Lymphoprep (StemCell Technologies, #07801, stored at 4°C)
- PE positive selection kit II, EasySep™ mouse (StemCell Technologies, #18554)
- Penicillin-streptomycin (10,000 units/mL penicillin and 10,000 µg/mL of streptomycin in in 0.85% saline, ThermoFisher Scientific, #15140122, stored at –20°C)
- Percoll plus 1L GE healthcare (Sigma, #17-5445-01, stored at RT)
- Phorbol 12-myristate 13-acetate (PMA, C36H56O8, Sigma, #P8139-1MG, [1 mg/mL] in DMSO, stored at –20°C)
- Phosphate buffered saline (10X PBS, ThermoFisher Scientific, # 70011036, stored at 4°C)

- Phosphate buffered saline (PBS, ThermoFisher Scientific, #10010023, stored at 4°C)
- RPMI 1640 without L-Glutamine (ThermoFisher Scientific, #42401018, stored at 4°C)
- Sodium azide (NaN_3, Sigma, #S2002-500G, stored at RT)
- Transcription factor buffer set (BD Biosciences, #562574, stored at 4 °C)
- α-Galactosylceramide (αGalCer, $C_{50}H_{99}NO_9$, Avanti Polar Lipids, #867000P, [1 mg/mL] in DMSO, stored at -20°C)

3.6.1.5 Antibodies for Flow Cytometry

Table 1: Antibodies used in the study

Specificity	Clone	Species	Isotype	Conjugate	Ordering number	Company
CD127	A7R34	Rat	IgG2a, κ	APC-eF780	47-1271-82	eBioscience
CD127	A7R34	Rat	IgG2a, κ	BV510	135033	BioLegend
CD127	A7R34	Rat	IgG2a, κ	BV605	135041	BioLegend
CD127	A7R34	Rat	IgG2a, κ	BV650	135043	BioLegend
CD16/32	2.4G2	Rat	IgG2b	Purified	70-0161-M001	Tonbo Bioscience
CD19	MB19-1	Mouse	IgA, κ	APC	17-0191-82	ThermoFisher Scientific
CD19	1D3	Rat	IgG2a, κ	Biotin	30-0193-U500	Tonbo Biosciences
CD19	6D5	Rat	IgG2a, κ	BV510	115546	BioLegend
CD19	6D5	Rat	IgG2a, κ	BV570	115535	BioLegend

CD19	MB19-1	Mouse	IgA, κ	PE	12-0191-82	ThermoFisher Scientific
CD19	6D5	Rat	IgG2a, κ	PE-Cy5	115510	BioLegend
CD19	6D5	Rat	IgG2a, κ	Pacific Blue	115523	BioLegend
CD19	1D3	Rat	IgG2a, κ	PerCP-Cy5.5	65-0193-U100	Tonbo Biosciences
CD196 / CCR6	29-2L17	Armenian hamster	IgG	BV605	129819	BioLegend
CD1d	1B1	Rat	IgG2b, κ	PE	123510	BioLegend
CD1d	1B1	Rat	IgG2b, κ	Purified	559438	BD Biosciences
CD278 (ICOS)	C398.4A	Armenian hamster	IgG	PerCP-Cy5.5	313518	BioLegend
CD278 (ICOS)	15F9	Armenian hamster	IgG	PE	12-9940-81	ThermoFisher Scientific
CD278 (ICOS)	15F9	Syrian hamster	IgG	PerCP-eF710	46-9940-82	eBioscience
CD3ε	145.2C11	Armenian hamster	IgG1, κ	APC	20-0031-U100	Tonbo Biosciences
CD3ε	145-2C11	Armenian hamster	IgG1, κ	BUV395	563565	BD Biosciences
CD3ε	145-2C11	Armenian hamster	IgG1, κ	PE-Cy5	100310	BioLegend
CD3ε	145-2C11	Armenian hamster	IgG1, κ	PE-eFluor610	61-0031-82	ThermoFisher Scientific
CD3ε	145-2C11	Armenian hamster	IgG1, κ	PerCP-Cy5.5	45-0031-82	ThermoFisher Scientific
CD3ε	17A2	Rat	IgG2b, κ	Purified	70-0032-	Tonbo

					U500	Biosciences
CD4	RM4-5	Rat	IgG2a, κ	AF647	100530	BioLegend
CD4	RM4-5	Rat	IgG2a, κ	AF700	100536	BioLegend
CD4	RM4-5	Rat	IgG2a, κ	APC	17-0042-81	ThermoFisher Scientific
CD4	RM4-5	Rat	IgG2a, κ	eVolve605	83-0042-42	eBioscience
CD4	RM4-5	Rat	IgG2a, κ	PE	100512	BioLegend
CD4	RM4-5	Rat	IgG2a, κ	BV570	100542	BioLegend
CD4	RM4-5	Rat	IgG2a, κ	BV605	563151	BD Biosciences
CD4	GK1.5	Rat	IgG2b, κ	Pacific Blue	100428	BioLegend
CD44	IM7	Rat	IgG2b, κ	APC	17-0441-82	ThermoFisher Scientific
CD44	IM7	Rat	IgG2b, κ	PE	12-0441-83	ThermoFisher Scientific
CD44	IM7	Rat	IgG2b, κ	V500	560780	BD Biosciences
CD45.1	A20	Mouse	IgG2a, κ	AF700	110724	BioLegend
CD45.1	A20	Mouse	IgG2a, κ	BV785	110743	BioLegend
CD8α	53-6.7	Rat	IgG2a, κ	APC-eF780	47-0081-82	ThermoFisher Scientific
CD8α	53-6.7	Rat	IgG2a, κ	BV570	100740	BioLegend
CD8α	53-6.7	Rat	IgG2a, κ	BV650	100741	BioLegend
CD8α	53-6.7	Rat	IgG2a, κ	eFluor 450	48-0081-80	eBioscience
CD8α	53-6.7	Rat	IgG2a, κ	eVolve655	86-0081-42	ThermoFisher Scientific

CD8α	53-6.7	Rat	IgG2a, κ	PE-Cy5	100710	BioLegend
CD8α	53-6.7	Rat	IgG2a, κ	PE-Cy7	25-0081-82	ThermoFisher Scientific
CD8α	53-6.7	Rat	IgG2a, κ	PerCP-Cy5.5	100734	BioLegend
CD8α	53-6.7	Rat	IgG2a, κ	PerCP-eF710	46-0081-82	ThermoFisher Scientific
CD8α	53-6.7	Rat	IgG2a, κ	Biotin	30-0081-U500	Tonbo Biosciences
CD8α	53-6.7	Rat	IgG2a, κ	BV711	100748	BioLegend
CD8β	eBioH35-17.2	Rat	IgG2b, κ	FITC	11-0083-85	ThermoFisher Scientific
CD8β	eBioH35-17.2	Rat	IgG2b, κ	PE	12-0083-81	ThermoFisher Scientific
GATA3	L50-823	Mouse	IgG1, κ	BV711	565449	BD Biosciences
GATA3	L50-823	Mouse	IgG1, κ	PE-Cy7	560405	BD Biosciences
IFNγ	XMG1.2	Rat	IgG1, κ	BV650	505831	BioLegend
IFNγ	XMG1.2	Rat	IgG1, κ	BV785	505838	BioLegend
IFNγ	XMG1.2	Rat	IgG1, κ	APC	20-7311-U100	Tonbo Biosciences
Nur77	12.14	Mouse	IgG1, κ	AF488	4347883	ThermoFisher Scientific
Nur77	12,14	Mouse	IgG1, κ	PE	12-5965-80	ThermoFisher Scientific
PLZF	9E12	Armenian hamster	IgG	PE	145804	BioLegend

PLZF	9E12	Armenian hamster	IgG	PE-Cy7	145806	BioLegend
Slamf6 / Ly108	330-AJ	Mouse	IgG2a, κ	APC	134608	BioLegend
Slamf6 / Ly108	330-AJ	Mouse	IgG2a, κ	Pacific Blue	681304	BioLegend
Tbet	4B10	Mouse	IgG1, κ	AF647	644804	BioLegend
Tbet	O4-46	Mouse	IgG1, κ	BV786	564141	BD Biosciences
Tbet	O4-46	Mouse	IgG1, κ	AF488	561266	BD Biosciences
TCRβ	H57-597	Armenian hamster	IgG	FITC	109206	BioLegend
TCRβ	H57-597	Armenian hamster	IgG	PE	12-5961-83	eBioscience
TCRβ	H57-597	Armenian hamster	IgG	Purified	70-5961-U100	Tonbo Biosciences
TCRβ	H57-597	Armenian hamster	IgG	AF700	109224	BioLegend
TCRβ	H57-597	Armenian hamster	IgG	PerCP-Cy5.5	65-5961-U100	Tonbo Biosciences
TCR γ/δ	eBioGL3	Armenian hamster	IgG	FITC	11-5711-82	eBioscience
TCR γ/δ	UC7-13D5	Armenian hamster	IgG	FITC	11-5811-85	ThermoFisher Scientific
TNF	MP6-XT22	Rat	IgG1, κ	FITC	554418	BD Biosciences
TNF	MP6-XT22	Rat	IgG1, κ	BV711	506349	BioLegend

3.6.1.6 Other Reagents for Staining

Table 2: Other reagents for staining

Clone	Species	Isotype	Conjugate	Ordering number	Company
Polyclonal	Rat	IgG	Unconjugated	012-000-003	Jackson ImmunoResearch
Polyclonal	Mouse	IgG	Unconjugated	015-000-003	Jackson ImmunoResearch

3.6.1.7 Other Fluorescent Reagents for Flow Cytometry

Table 3: Other fluorescent reagents for flow cytometry

Specificity	Clone	Species	Isotype	Conjugate	Ordering Number	Company
N/A	N/A	N/A	N/A	Live/Dead-Blue	13-0868-T100	Tonbo Biosciences
CD1d-tetramer	N/A	Mouse	N/A	BV421	N/A	NIH tetramer core facility
CD1d-tetramer	N/A	Mouse	N/A	PE	N/A	NIH tetramer core facility

3.6.2. Methods

3.6.2.1. Animal Handling and Experimentation

3.6.2.1.1 Mouse Handling

To perform *in vivo* experiments and to collect mouse organs for the subsequent purification of immune cells, the proper handling of mice is essential.

Materials:

- Polycarbonate cage, with wire lid and drinking bottle

Solutions:

- 70% ethanol

Method:

The surface of the mouse cage was sterilized with 70% ethanol, then placed inside of the laminal flow of the procedure room. To open the cage, the lid and the drinking bottle were removed. The mouse was lifted out of its cage by gently holding the tail.

3.6.2.1.2 *Mouse Euthanasia by Cervical Dislocation*

Cervical dislocation is the preferred method for mouse euthanasia, as it is fast, minimizes the pain for the animal, and avoids chemical contamination of the tissue, as it might occur with the use of chemical euthanasia.

Materials:

- Scissors (operating), straight; sharp-blunt; 15.24 cm length.

Method:

The mouse was held by the tail and placed on the wire grid of the cage. Scissors were placed on its neck, behind of the ears, with enough pressure to prevent movement. Then, the tail was pulled while keeping pressure on the neck with the scissors to dislocate the cervical vertebrate. Death was verified by a lack of reflexes following toe pinch.

3.6.2.1.3 Recovery of Mouse Organs

To analyse and study immune cells, the organs of interest need to be removed from the animals to allow the purification of cells.

Materials:

- Forceps (graefe), 10.16 cm long serrated slight curve 0.8 mm tip
- Forceps (semken), 1x2 teeth; 1.6 mm tip width; 15.24 cm length
- Scissors (delicate operating), *12.07* cm straight sharp/sharp
- Scissors (light operating), 12.7 cm curved sharp/sharp
- Scissors (operating), straight; sharp-blunt; 15.24 cm length
- 15 ml Falcon tubes
- Forceps (graefe), 10.16 cm long serrated slight curve 0.8 mm tip
- Forceps (semken), 1x2 teeth; 1.6 mm tip width; 15.24 cm length
- Scissors (delicate operating), *12.07* cm straight sharp/sharp
- Scissors (light operating), 12.7 cm curved sharp/sharp
- Scissors (operating), straight; sharp-blunt; 15.24 cm length
- 15 ml Falcon tubes

Solutions:

- PBS
- 70% ethanol
- P2X7 receptor inhibitor A-804598 (Selleckchem) [10 µM]

Method:

- *Spleen:* The sacrificed mouse was placed on its right side on a clean paper. The fur was sterilized with 70% ethanol in order to prevent contamination. An incision was made perpendicular to the body axis below the rib cage. The skin around of the incision was pulled to opposite directions with both hands to expose the peritoneum. Another incision was made in the peritoneal wall above the spleen. The spleen is a

red-elongated organ that is found near the greater curvature stomach under the omentum and is usually visible through the peritoneal wall. The spleen was lifted with curved graefe forceps out of the peritoneum and detached from the body by cutting the connecting tissue with a pair of scissors. The spleen was placed into a 15 ml falcon tube containing approximately 5 ml PBS.

- *Thymus*: The sacrificed mouse was placed on its back on a clean paper. The fur was sterilized with 70% ethanol in order to prevent contamination. An incision was made perpendicular to the body axis below the rib cage. The skin around the incision was pulled to opposite directions with both hands to expose the peritoneum. The lower tip of the sternum was held with forceps and the ribs were cut left and right along the sternum to the top of the rib cage. The ribs were lifted to lateral sides to expose the lungs. The thymus is a white-coloured bi-lobed organ located behind the heart in the anterior superior thorax. With curved graefe forceps, the lobes of the thymus were grasped and removed from the body by cutting the connecting tissue with a pair of scissors. The thymus was placed into a 15 ml falcon tube containing approximately 5 ml PBS.

3.6.2.1.4 Cell Isolation

Cell isolation is a procedure to isolate single cell suspensions from tissue samples. There are different techniques used, depending on the organ and the composition or purity of the intended cell suspension.

3.6.2.1.4.1 Density gradient separation with Lymphoprep

Following Lymphoprep density-gradient centrifugation, dead cells are found at the bottom of the tube, due to their shrinking and increased granularity. Therefore, the living cells can easily be harvested at the interphase. The removal of dead cells before the in vitro stimulation improves the detection of cytokines produced by *i*NKT cells. (Sag D., *et al*., 2017) since dead cells can induce *i*NKT cells apoptosis. (Lee H, *et al*. 2010)

Materials:

- 50 ml Falcon tubes

- 70 µm mesh
- 5 mL round bottom FACS tubes
- 3 mL syringe plunger
- 15 ml Falcon tubes
- Pasteur pipettes

Solutions:

– PBS

– Complete medium: Supplement RPMI-1640 medium with 5% (vol/vol) heat-inactivated FBS, 100 U/mL penicillin-streptomycin, 0.05% (vol/vol) 2-Mercaptoethanol, 4 µM L-glutamine

– Lymphoprep (stored at RT)

Method:

The cell pellet was re-suspended in 2 ml of complete medium and transferred into a 5 mL round bottom FACS tube. The cell suspension was underlaid slowlywith 1,5 ml Lymphoprep using a 1 ml pipette tip through a glass Pasteur pipette reaching the bottom of the FACS tube. The gradient was centrifuged at 500 *g* for 20-30 min at RT without acceleration or break. Using a 1 ml pipette tip, the interphase containing the live leukocytes was recovered twice and transferred into a new 15 ml falcon tube. The cells were washed once with 150 µl PBS (400 g, 5-10 min, 4 °C).

3.2.6.3 In Vitro Stimulation of Single Cell Suspensions

*i*NKT cells produce large amounts of different cytokines after stimulation. Since cytokines typically are secreted proteins, they must first be trapped inside the cell with a protein transport inhibitor. The two most commonly used protein transport inhibitors are monensin and brefeldin A. Both interrupt the intracellular transport processes leading to an accumulation of the cytokine in the Golgi complex, allowing the detection of cytokines inside the producing cells (Schuerwegh, *et.al*., 2001).

Materials:

- Flat-bottom 24-well plates
- V-bottom 96-well plates

Solutions:

- GolgiPlug (Brefeldin A [1 mg/ml] in DMSO, stored at 4 °C)
- GolgiStop (Monensin [2 mg/ml] in EtOH, stored at 4 °C)
- PMA ([1 mg/mL] in DMSO)
- Ionomycin ([1 mg/mL] in DMSO)
- Complete medium: Supplement RPMI-1640 medium with 5% (vol/vol) heat-inactivated FBS, 100 U/mL penicillin-streptomycin, 0.05% (vol/vol) 2-mercaptoethanol, 4 µM L-glutamine (stored at 4 °C)

Method:

Cells were resuspended in complete medium and stimulated in flat-bottom 24 well-plates at 2 x 10^6 cells in 500 µl. The cells were stimulated either with

(i) PMA [50 ng/mL] and ionomycin [500 ng/mL]; (ii) αGalCer [100 ng/mL]; or with plate-coated αCD3ε Ab. The cells were stimulated in the presence of GolgiPlug (final concentration 0.5 µl/mL), GolgiStop (final concentration 0.33 µl/mL) for 4 hours (PMA/ionomycin; αCD3ε Ab) or for 5 hours (αGalCer) at 37°C in a CO2 incubator.

3.6.2.4 Cell Staining for Flow Cytometry

3.6.2.4.1 Cell Surface Staining for Flow Cytometry

Cell surface markers are expressed on the cell surface and can be used to define cell types. To this end, the surface markers are labelled with fluorochrome-conjugated antibodies and analysed by flow cytometry.

Materials:

- V-bottom 96-well plates

Solutions:

- PBS

- FACS buffer: PBS, 1% FBS, 0.1% NaN_3 (stored at 4°C)
- CytoFix/Perm (stored at 4°C)

Method:

Cells were stained in V-bottom 96-well plates with up to 3 x 10^6 cells per well 50 µL FACS buffer per well of master mix was prepared containing pre-titrated amounts of the antibodies, plus 5 µl/mL of α-CD16/32-Ab (clone 2.4G2), unconjugated IgGs (1:1, 10 µg/mL mix of mouse IgG and rat IgG). The master mix was added to the cells, resuspended, and incubated for 15-30 minutes on ice in the dark. After incubation, the cells were centrifuged and the pellet was washed twice with 150 µl PBS (400 g, 5 min, 4°C). Then, 150 µl of CytoFix/Perm buffer per well was added to the cell pellet and immediately re-suspended. They were fixed for 10 min on ice or at 37 °C in the dark and then washed twice with 150 µl PBS (400 g, 5 min, 4°C).

3.6.2.4.2 Intracellular Staining for Flow Cytometry

To stain intracellular molecules, like cytokines or transcription factors, the cells need to be fixed in suspension and then permeabilized before the detection antibody is added. This fixation/permeabilization treatment allows the antibody to pass through the plasma membrane into the cell interior, while maintaining the morphological characteristics.

i. Transcription Factors

Materials:

- V-bottom 96-well plates
- 5 ml round bottom FACS tubes
- 70 µm mash

Solutions:

- PBS
- FACS buffer: PBS, 1% FBS, 0.1% NaN_3 (stored at 4°C)
- 1X TF Fix/Perm buffer (stored at 4°C)

- 1X TF Perm/Wash buffer (stored at 4°C)

Method:

Surface-stained cells were pelleted (400 g, 5 min, 4°C), resuspended in 150 µl of TF Fix/Perm buffer and incubated for 75 min at 4°C in the dark. Then, the cells were washed twice with TF Perm/Wash buffer (400 g, 5 min, 4°C). The intracellular staining cocktail was prepared using 50 µl per sample of Perm/Wash buffer containing pre-titrated amounts of the antibodies for the transcription factors and unconjugated IgGs (1:1, 10 µg/mL mix of mouse IgG and rat IgG). The intracellular staining cocktail was added to the cell pellet, the cells were re-suspended and incubated overnight at 4°C in the dark. Afterwards, the cells were washed once with TF Perm/Wash buffer, re-suspended with 150 µl TF Perm/Wash buffer and incubated five minutes on ice to permit unbound antibodies to diffuse out of the cells. The cells were washed once more with 150 µl TF Perm/Wash buffer and once with FACS buffer (400 g, 5 min, 4°C), then re-suspended in 150 µl of FACS buffer and filtered through a 70 µm mesh into a 5 mL round bottom FACS tube.

ii. Cytokines

Materials:

- V-bottom 96-well plates
- 5 ml round bottom FACS tubes
- 70 µm mesh

Solutions:

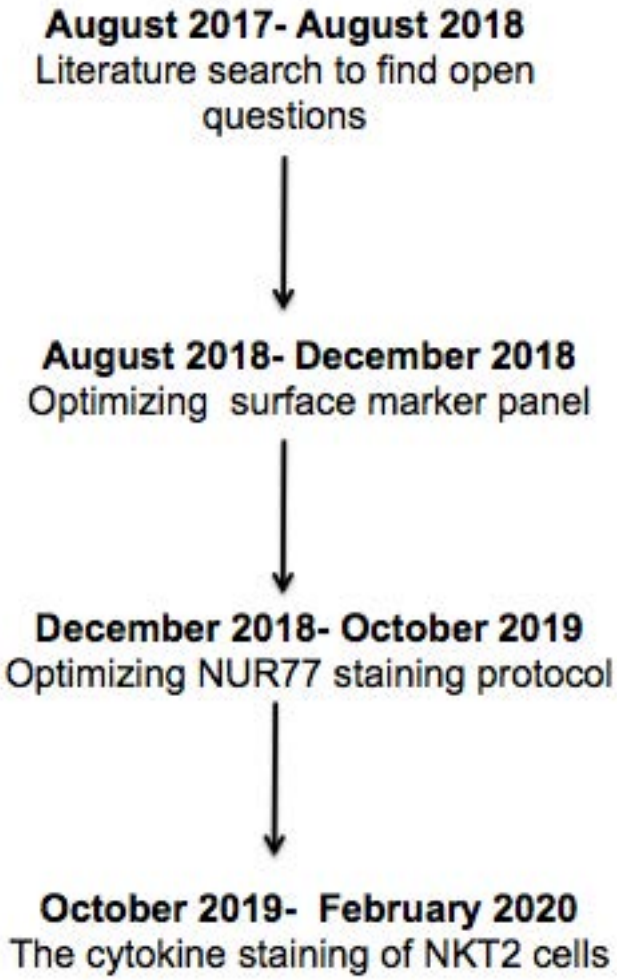
- PBS
- FACS buffer: PBS, 1% FBS, 0.1% NaN3 (stored at 4°C)
- CytoFix/Perm buffer (stored at 4°C)
- 1X Perm/Wash (stored at 4°C)

Method:

Surface-stained cells were pelleted (400 g, 5 min, 4°C), resuspended in 150 µl of CytoFix/Perm buffer and fixed for 10 minutes at 37 °C. Then, the cells were washed

twice with Perm/Wash buffer to permeabilize the cells (500 g, 5 min, 4°C). The intracellular cytokine staining (ICCS) cocktail was prepared with 50 µl per test of Perm/Wash buffer containing pre-titrated amounts of the antibodies for the cytokines and unconjugated IgGs (1:1, 10 µg/mL mix of mouse IgG and rat IgG). The ICCS cocktail was added to the cell pellet, the cells were re-suspended and incubated for 30 min or overnight at 4°C in the dark. Then, the cells were washed once with Perm/Wash buffer (500 g, 5 min, 4°C), re-suspended with 150 µl Perm/Wash buffer, and incubated five minutes on ice to permit unbound antibodies to diffuse out of the cells. The cells were washed once more with Perm/Wash buffer and once with FACS buffer (500 g, 5 min, 4°C). Then, the cells were re-suspended with 70 µl FACS buffer and filtered through a 70 µm mesh into a 5 mL round bottom FACS tube.

- **Research Plan**



- **Evaluation of Data**

Statistical analysis: Results are expressed as the mean ± SEM. Statistical comparisons were drawn using a 2-tailed Student's *t* test (Excel; Graphpad Software). *P* values less than 0.05 were considered statistically significant and are indicated as *$P < 0.05$, ** $P < 0.01$, and ***$P < 0.001$ in the figures. Graphs were generated with GraphPad Prism.

- **Limitation of the Research**

There were no limitations in our study.

4. RESULTS

4.1 The gating strategy of *i*NKT cell subsets

Splenocytes from BALB/c mice were prepared for flow cytometric analysis for transcription factor staining of *i*NKT cell subsets. To identify *i*NKT cells and their subsets, following gating strategy was employed. Lymphocytes were gated by FSC-Area vs. SSC-Area parameters **(Figure 1A)**. Cell doublets and aggregates were eliminated based on their high FSC-Width vs. FSC-Area **(Figure 1B)** and SSC-Width vs. SSC-Area **(Figure 1C)** values. Dead cells were eliminated by exclusion of cells labelled with a live/dead marker **(Figure 1D)**. Similarly, B cells, positive for CD19 or CD45R/B220 were excluded **(Figure 1E)**. Since mouse *i*NKT cells are negative for CD8α (Bendelac et al., 1997), $CD8\alpha^+$ positive cells were also excluded (data not shown). *i*NKT cells constitutively express the activation marker CD44 (Bendelac et al., 2007), thus we gated $CD44^{high}$ cells **(Figure 1F)**. $CD3\varepsilon i^{int}$ CD1d/αGalCer-tetramer+ cells are defined as *i*NKT cells **(Figure 1G)**. Finally, *i*NKT cell subsets were gated according to their PLZF, TBET, and RORγt expression. NKT17 cells are defined as $TBET^-$ $ROR\gamma t^+$ cells **(Figure 1H)**. The $ROR\gamma t^-$ were divided into $PLZF^{high}$ NKT2 cells and $TBET^+$ $PLZF^{low}$ NKT1 cells (data not shown).

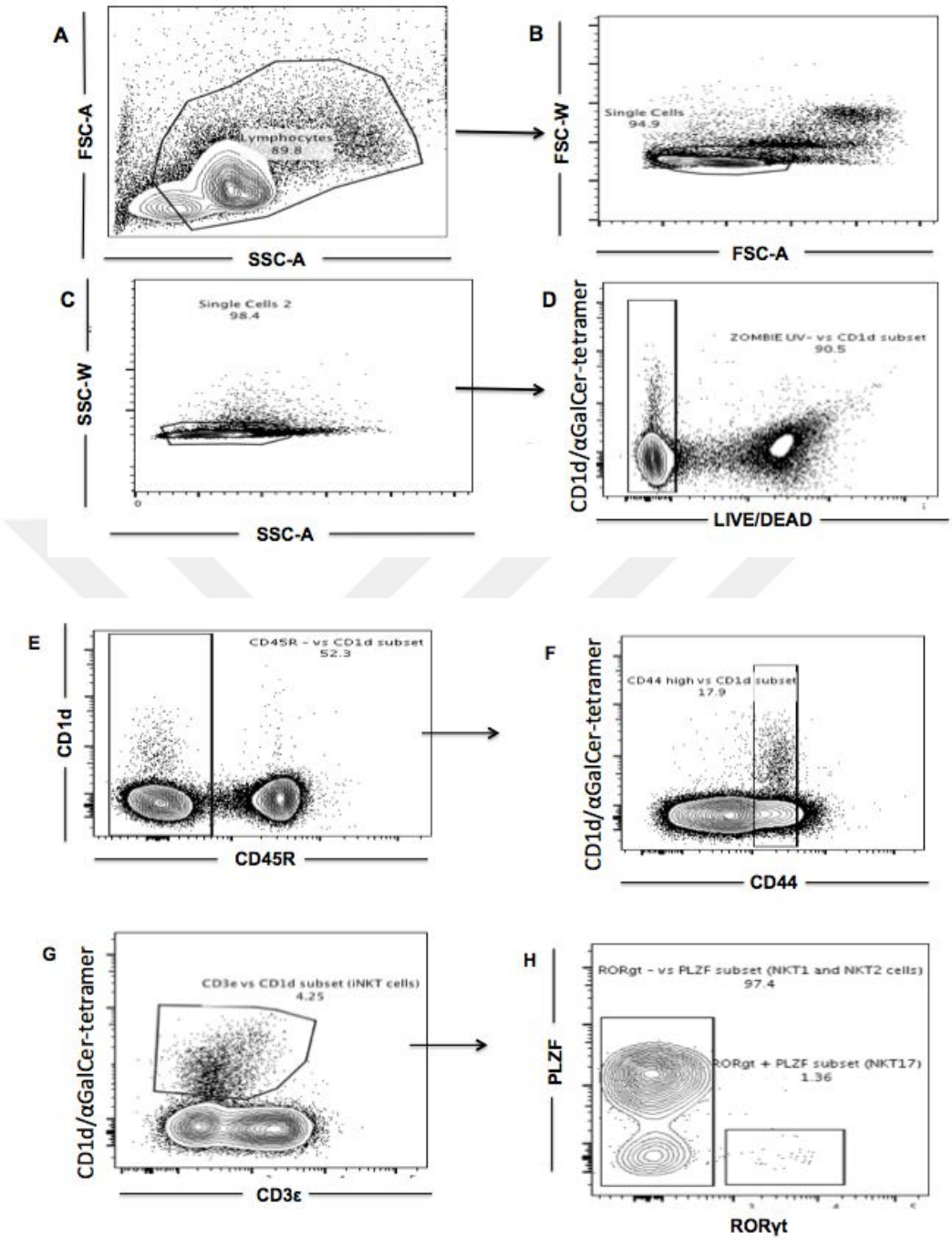


Figure 1: The gating strategy of *i*NKT cell subsets: (A) Lymphocytes were identified as SSC-A vs. FSC-A. **(B, C)** Doublets were excluded with FSC-W vs. FSC-A (B) and SSC-W vs. SSC-A (C) gating. **(D)** Live splenocytes were negative for the live/dead marker. **(E-G)** *i*NKT cells were identified as CD19/CD45R CD44^{high} CD3ε^{int} and CD1d/αGalCer-tetramer^{+} cells. **(H)** Gating of RORγt^{+} NKT17 cells vs. NKT1/2 cells (RORγt^{neg}). A representative gating tree and their subsets for splenic *i*NKT cells from BALB/c mice is shown. The numbers in the graphs represent the percentage of cells within the gate.

4.2. *In vivo* distribution of *i*NKT cell subsets via surface markers

*i*NKT cell subsets are distinguished by the transcription factors they express and the cytokines they produce. Each subset expresses the transcription factors PLZF, TBET, and RORγt at different levels. However, the cell damaging nature of the transcription factor staining does not allow further functional analysis of the *i*NKT cell subsets. To be able to do further studies on the *i*NKT cell subsets, it is necessary to identify the cells alive. Therefore, we aimed to distinguish *i*NKT cell subsets by surface markers that they express. To this end, we compared suitable surface markers with the transcription factor based sub-setting of *i*NKT cells. Splenocytes were taken from BALB/c mice and stained with various surface markers together with PLZF, TBET, and RORγt. The majority of NKT1 cells were positive for CD49a, in contrast to NKT2 and NKT17 cells **(Figure 2A, C).** To identify these two *i*NKT cell subsets, we tested antibodies against CD4, CD150, CD278, CD304, and SLAMF6. We found that NKT17 cells lack CD4 expression, while NKT2 and most of NKT1 cells are positive for CD4 **(Figure 2C)**. However, the other markers tested, CD150, CD278, CD304, and SLAMF6, did not allow us to clearly distinguish the three *i*NKT cell subsets **(Figure 2B, C)**.

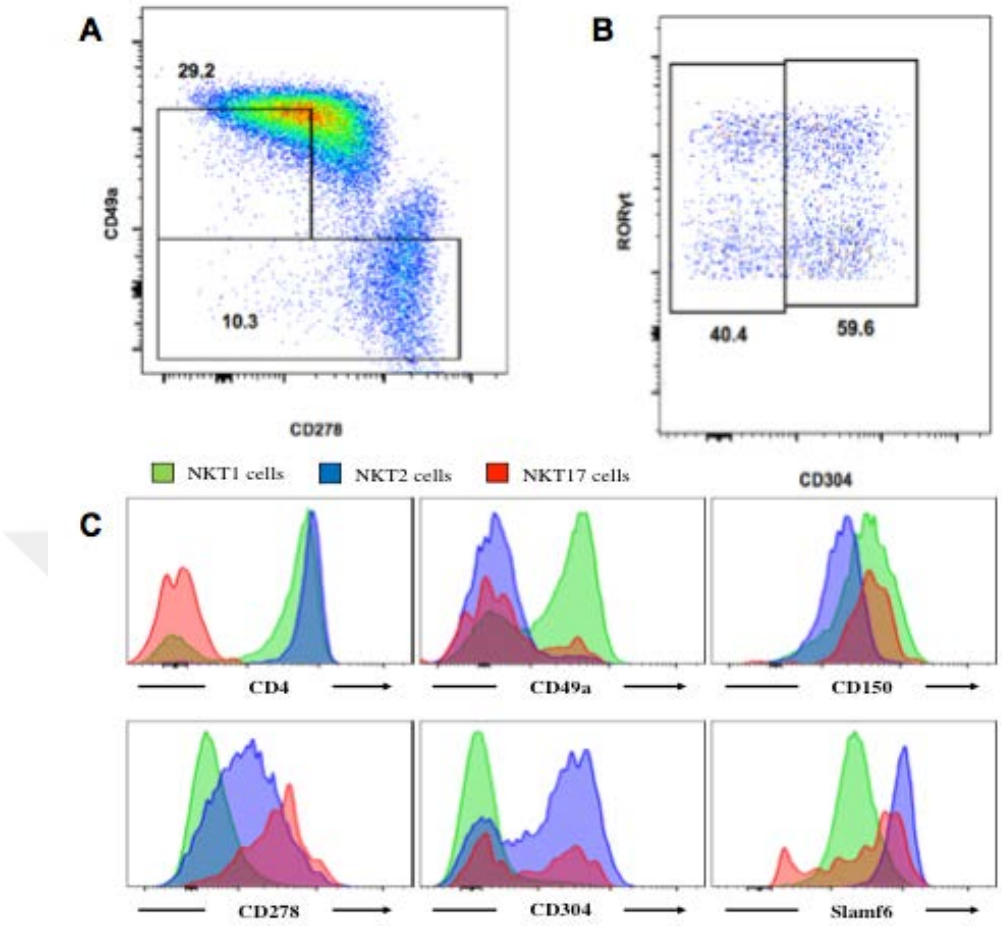


Figure 2: Expression of indicated markers on *i*NKT cell subsets: (A) Representative dot plots of the CD49a and CD278 expression on splenic NKT1 and NKT2 cells from BALB/c mice, (B) Representative dot plots of the RORγt and CD304 expression on NKT17 cells. **(C)** NKT1 (green), NKT2 (blue), and NKT17 (red) cells from BALB/c splenocytes were stained for indicated markers. *i*NKT cells (live CD3ε$^+$ CD8α$^-$ CD19/CD45R$^-$ CD1d/αGalCer-tetramer$^+$ cells) were stained and representative data from one of at least three independent experiments are shown.

We also noticed some RORγt$^+$ CD49a$^+$ *i*NKT cells **(Figure 3).** To clarify, if these RORγt$^+$ CD49a$^+$ *i*NKT cells were indeed NKT17 cells or contamination, we measured their IL-17A production. We observed that all CD49a$^+$ cells were IL-17A negative **(Figure 3).** As only RORγt$^+$ cells can produce IL-17A, we concluded that the RORγt$^+$ CD49a$^+$ *i*NKT cells were not NKT17 cells.

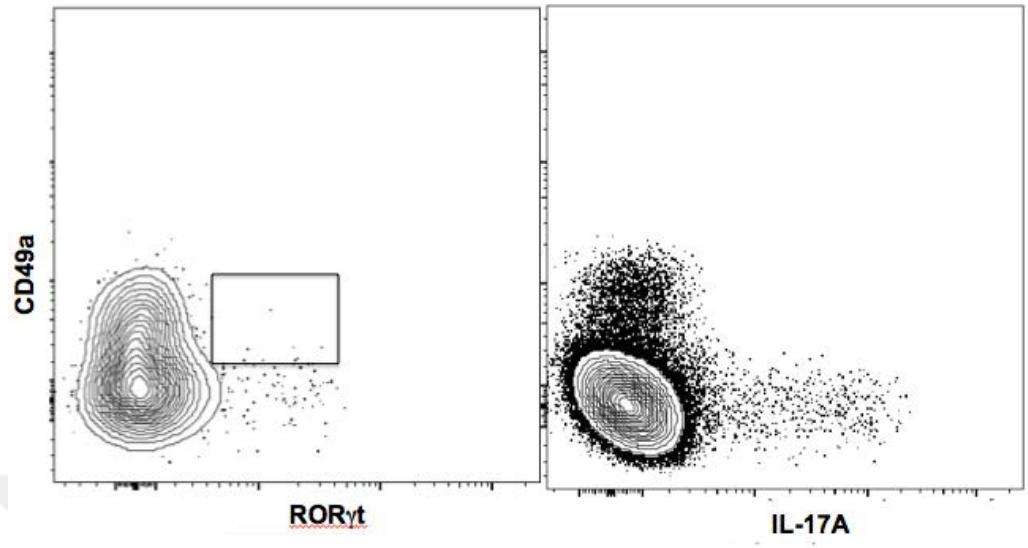


Figure 3: NKT17 cells are CD49a negative. Splenocytes from BALB/c mice were left unstimulated or were stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) for 4 hours in the presence of Golgi-transport inhibitors (n = 3). *i*NKT cells (live CD3ε^{+} CD8α^{neg} CD19/CD45R^{neg} CD1d/αGalCer-tetramer^{+} cells) were stained and their cytokine production was determined. Representative data shows (left panel) CD49a^{+} vs. RORγt^{+} and (right panel) CD49a^{+} vs. IL-17A expression of *i*NKT cells. Representative data from one of at least three independent experiments are shown.

4.3. The cytokine production by *i*NKT cells

*i*NKT cells produce large amounts of cytokines rapidly after stimulation and it is currently assumed that the kind of cytokines produced differs among *i*NKT cell subsets. For example, it is thought that NKT2 cells are the main producers of *i*NKT cell-derived Th2 cytokines, like IL-4 and IL-13 (Lee et al., 2013). The frequency of NKT2 cells is higher in BALB/c mice than C57BL/6 mice (Lee *et al.,* 2015) and, consequently, one would expect more IL-4 production by *i*NKT cells from BALB/c than C57BL/6 mice. This has indeed been suggested for the thymus, using an IL-4 reporter mouse (Lee et. al., 2013). However, no intracellular cytokine staining directly for IL-4 in NKT2 cells has been reported to date. To do this, we stimulated single cell suspensions of thymus and spleen from BALB/c and C57BL/6 mice were for four hours with PMA and ionomycin in the presence of Golgi-transport inhibitors. After the

stimulation, the samples were prepared for flow cytometric analysis of intracellular cytokines. We observed that in most of the experiment the cytokine production of *i*NKT cells did not differ between BALB/c and C57BL/6 mice **(Figure 4A, B)**, even though BALB/c mice have more NKT2 cells (Lee et al., 2015). Even when directly comparing the ratio of IFNγ to IL-4 produced by iNKT cells from BALB/c and C57BL/6 mice, no difference was observed **(Figure 4C)**. Thus, our data are not consistent with the assumption that NKT2 cells are the predominant source of *i*NKT cell-derived Th2 cytokines.

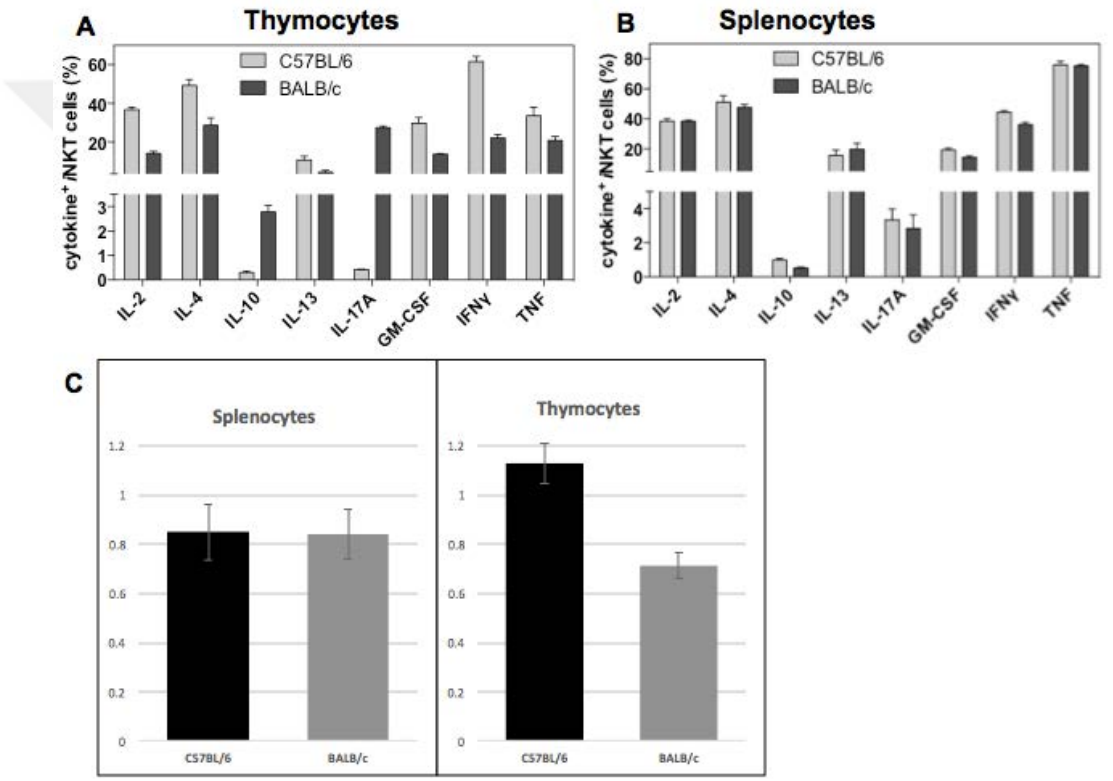


Figure 4: No difference was observed in terms of cytokine production by *i*NKT cells from BALB/c or C57BL/6 mice. Splenocytes and thymocytes from BALB/c and C57BL/6 mice were left unstimulated or stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) for 4 hours in the presence of Golgi-transport inhibitors (n = 3). *i*NKT cells (live CD3ε+ CD8α^{neg} CD19^{neg} CD1d/αGalCer-tetramer^{+} cells) were stained and their cytokine production of indicated cytokines was determined. Summary graphs of the cytokine production by **(A)** thymic *i*NKT cells and by **(B)** splenic *i*NKT cells are shown. Representative data from three independent experiments are shown. Although, some differences were statistically significant, this was not consistent over different experiments.

(C) The INFγ:IL-4 ratio for the data shown in (A) and (B) for the indicated organs is shown in BALB/c and C57BL/6 mice. INFγ:IL-4 >1 = i.e. more IFNγ than IL-4 and INFγ:IL-4 <1 = i.e less IFNγ than IL-4.

4.4. *i*NKT subset staining and enumeration

4.4.1. Peripheral NKT2 cells are hypo-responsive to antigenic stimulation

So far, we showed that there is no difference in terms of cytokine production by *i*NKT cells from BALB/c and C57BL/6 mice even though BALB/c mice have more NKT2 cells. These results led us to check on the *i*NKT cell subsets-level which contribution NKT2 cells make to the cytokine production. Cells were stimulated with three different agents to see how different pathways effect cytokine production. Therefore, BALB/c splenocytes were stimulated either with PMA/ionomycin, αGalCer, or with plate-bound αCD3ε antibodies. We observed that the splenic NKT2 cells produced less IL-4 than splenic NKT1 cells in BALB/c mice **(Figure 5)**. This supports our conclusion that NKT2 cells are not the main producer of *i*NKT cell-derived Th2 cytokines, in contrast with the literature. Rather our data suggest that NKT2 cells are hypo-responsive.

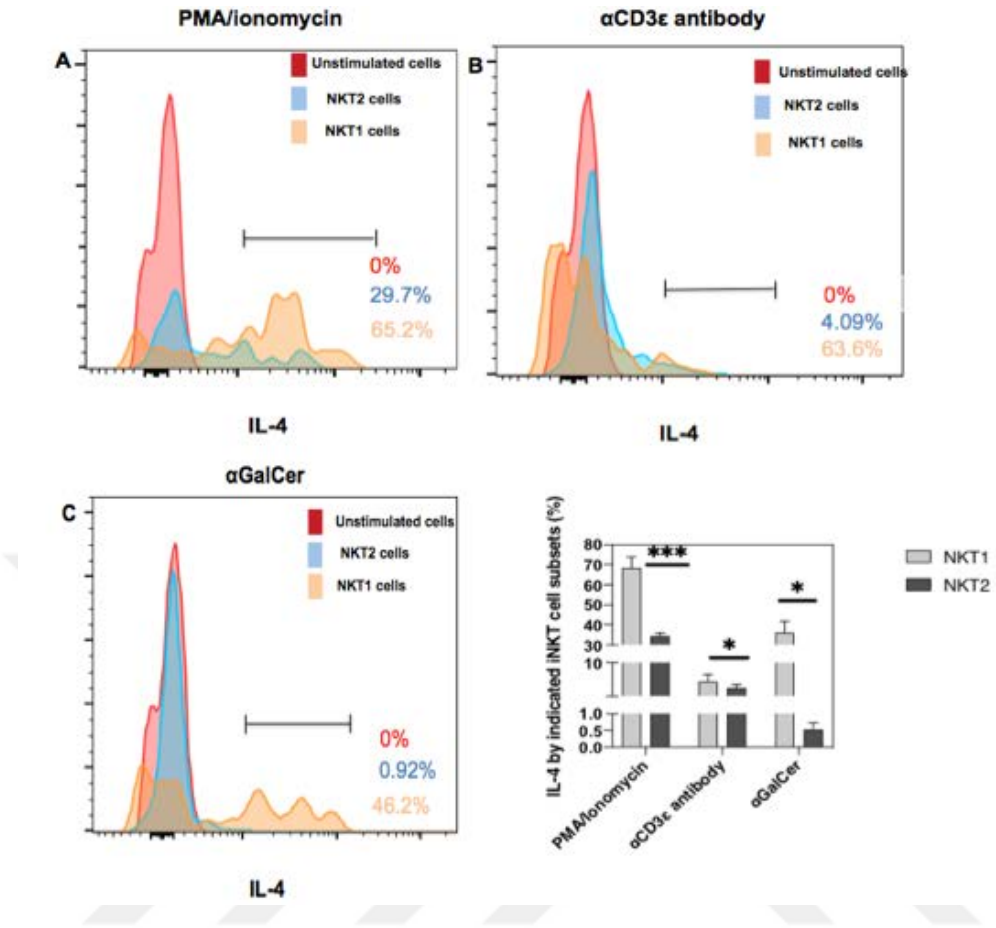


Figure 5: NKT2 cells are not the main producer of *i*NKT cell-derived IL-4. Splenocytes from BALB/c mice were stimulated either with PMA (50 ng/mL) and ionomycin (500 ng/mL), αGalCer (100 ng/mL), or αCD3ε antibodies coated plates (5 ug/ml, 5h). Cells were stained for *i*NKT cells and were analysed for the expression of IL-4. Representative data for the expression of IL-4 cytokine in NKT2 and NKT1 cells stimulated with **(A)** PMA/ionomycin, **(B)** stimulated with αCD3ε antibody, **(C)** stimulated with αGalCer. **(D)** Summary graph for the expression of IL-4 in NKT1 and NKT2 cells after stimulation (n=3).

4.4.2 Optimization of the NUR77 staining for the detecting of TCR signalling in *i*NKT cells

To directly assess whether NKT2 cells are hypo-responsive, we next measured the expression of NUR77 expression, which is a marker rapidly upregulated in T and *i*NKT cells following TCR-mediated-activation (Ashouri and Weiss, 2016).

4.4.3. Fluorochrome selection for the NUR77 staining

To optimize the NUR77 detection, we first compared the staining we could obtain with the αNUR77 antibody conjugated to two different fluorochromes (AF488 and PE). Splenocytes from BALB/c mice were stimulated either with PMA/ionomycin or α GalCer for five hours. The αNUR77 antibody conjugated with PE **(Figure 6B, D)** gave a stronger signal for NUR77 than the αNUR77 antibody conjugated with AF488 **(Figure 6A, C)**. Therefore, it was decided to use the PE conjugated αNUR77 antibody for further experiments.

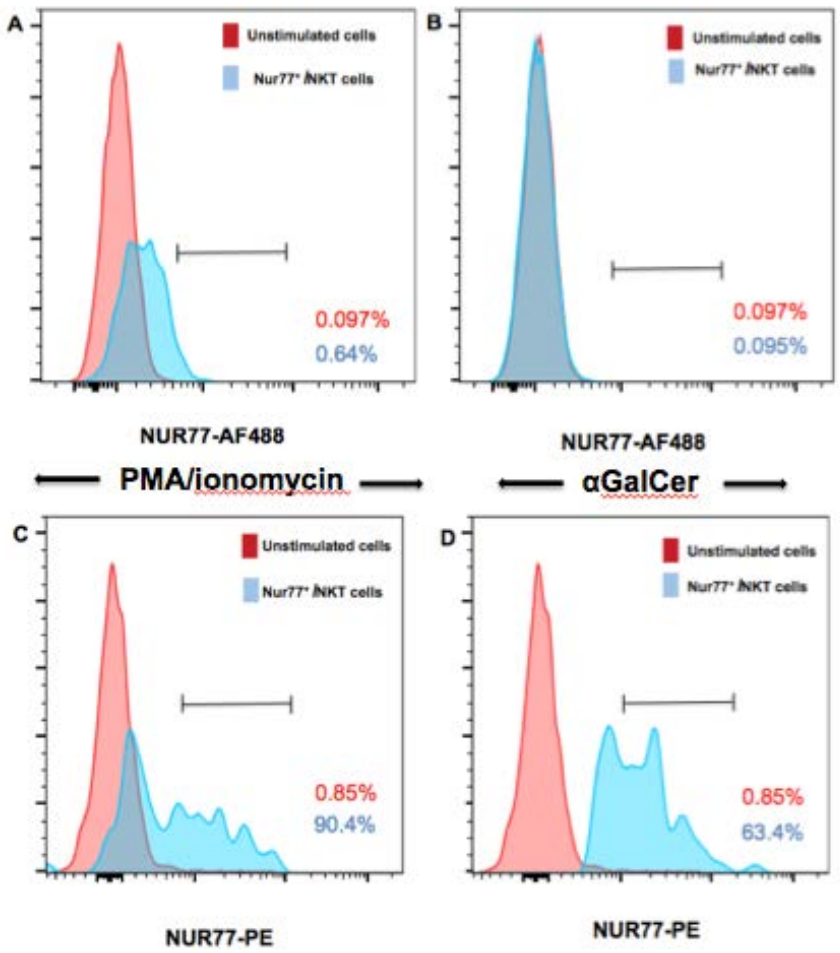


Figure 6: Two different NUR77 antibody fluorochromes were tested to detect NUR77$^+$ cells in splenic *i*NKT cells. Splenic *i*NKT cells from BALB/c mice left unstimulated (red) or stimulated (blue) with either **(A, B)** PMA (50 ng/mL) and ionomycin (500 ng/mL) or **(C, D)** αGalCer (100 ng/mL). Representative data from one of three experiments show the transcription factor staining with **(A, C)** the NUR77 antibody conjugated to AF488 and with **(B, D)** the NUR77 antibody conjugated to PE (n = 3).

4.4. The optimization of the incubation time for the stimulation was established

To optimize the stimulation protocol, we tested two different time periods for the stimulation. Splenocytes from BALB/c mice were stimulated either with PMA/ionomycin as a positive control or with plate-bound αCD3ε-antibodies. No difference for the frequency of NUR77-positive cells was observed between the stimulation for one or two hours **(Figure 7)**. Therefore, a stimulation time of one hour was chosen for the further experiments.

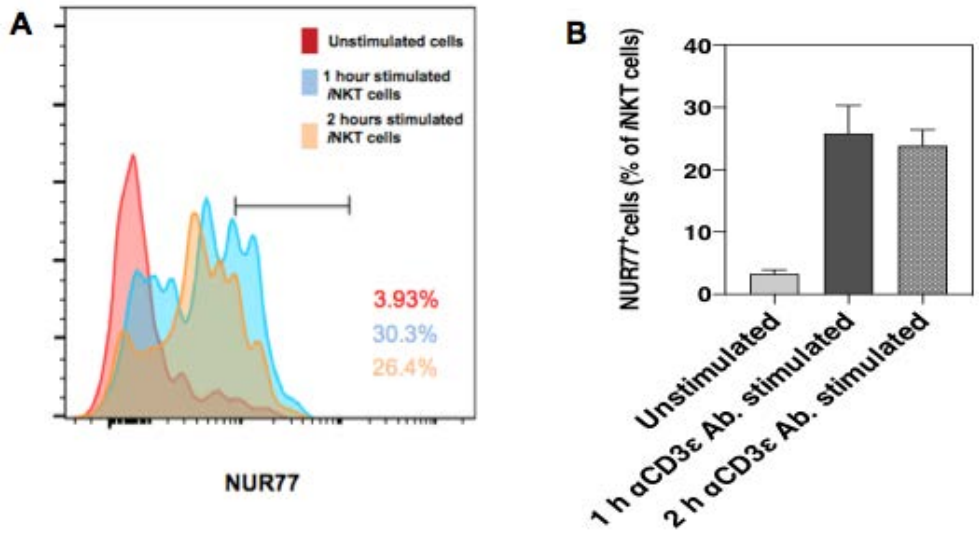


Figure 7: The frequency of splenic *i*NKT cells following one or two hours stimulation. Splenic *i*NKT cells from BALB/c mice were left unstimulated (red) or were stimulated with αCD3ε antibodies (coated at 5 µg/ml, 5h) for one hour (blue) or two hours (orange) (n = 3). Splenic *i*NKT cells were gated as live $CD3\varepsilon^{+}$ $CD8^{neg}$ $CD19^{neg}$ CD1d/αGalCer-tetramer^{+} cells. Preliminary results are shown (n = 1).

4.5. Comparison of the NUR77 expression by *i*NKT cell subsets

As shown in Figure 4**,** NKT2 cells are not the main producer of *i*NKT cell-derived Th2 cytokines. Therefore, we next measured the expression of NUR77, as indicator for TCR triggering, following stimulation on the level of *i*NKT cell subsets. Splenocytes from BALB/c mice were stimulated with either PMA or ionomycin as a positive control, or with plate-bound αCD3ε-antibodies. When comparing the resulting NUR77

expression, we observed no difference between NKT2 cells and NKT1 cells **(Figure 8)**. This indicates that the TCR signalling occurs in NKT2 cells in a similar fashion to NKT1 cells, but that this does not lead to cytokine production in NKT2 cells. Thus, in consideration of our data, we concluded that NKT2 cells are hypo-responsive to antigen stimulation.

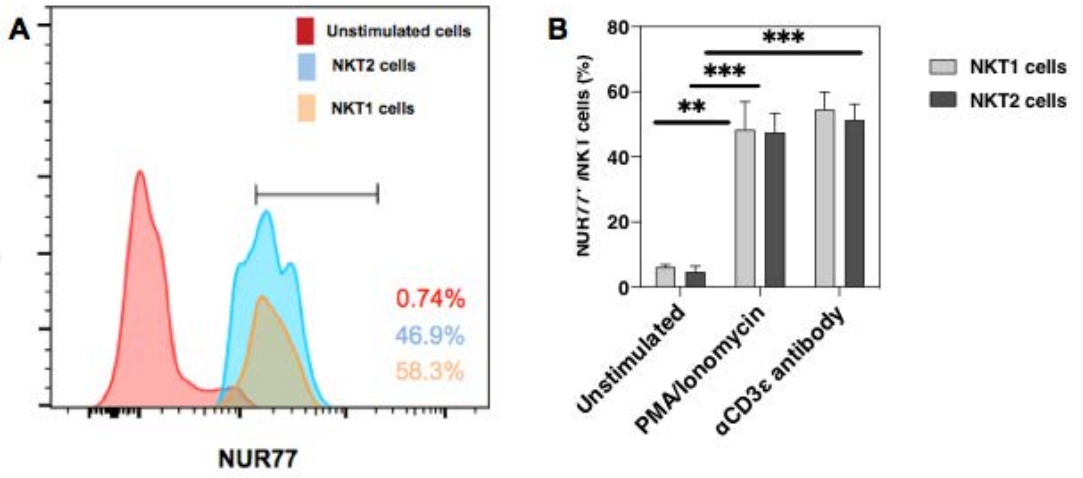


Figure 8: NKT2 cells express NUR77 marker in a similar fashion to NKT1 cells. Splenocytes from BALB/c mice were either left unstimulated or were stimulated with αCD3ε antibodies (coated with 5 µg/ml,1h) (n = 3). After stimulation, splenocytes were stained for *i*NKT cells and gated as live CD8α^{-} CD19^{-} CD44^{high} CD3ε^{int} CD1d/αGalCer-tetramer^{+} cells. Representative data of three independent experiments are shown.

5.DISCUSSION

In this study, we characterized iNKT cell subsets in detail phenotypical and functionally. Although *i*NKT cell subsets can be distinguished by some transcription factors they express, the cell destructive nature of the required fixation step makes the isolation of viable cell subsets impossible. Isolation of *i*NKT cell subsets has been reported using IL-4 reporter mice in a previous study (Lee et al, 2013). This technique only allows to segregate *i*NKT cell subsets by their cytokine production. According to this technique, NKT1 cell are the IFNγ-producing cells, NKT2 cells are the IL-4-producing cells, and NKT17 cells are the IL-17-producing cells. However, recent techniques showed that IL-4 production is not limited to NKT2 cells (Georgiev H, Ravens I, Benarafa C, et al, 2016 and Engel I, Seumois G, Chavez L, et al, 2016). Furthermore, thymic NKT1 and NKT17 cells, segregated by their CD43HG and ICOS expression, produced IL-4, IL-10, and IL-13 in similar fashion to NKT2 cells (Cameron and Godfrey, 2018). Here, we suggest a surface marker panel to distinguish *i*NKT cell subsets via surface markers (Figure 2). We successfully isolated NKT1 cells via αCD49a antibody-staining. Moreover, NKT17 cells are distinguished by expression of CD278 and the lack of CD4 expression. However, the other markers we tested were not suitable to improve the separation of *i*NKT cell subsets.

So far, it has been reported that NKT2 cells are the main producer of *i*NKT cell-derived Th2 cytokines (Lee et al, 2015). However, a previous study showed that there is no difference in terms of the cytokine production of splenic *i*NKT cells between BALB/c and C57BL/6 mice (Sag et al., 2017) even though BALB/c mice have more NKT2 cells (Lee et al, 2013). We confirmed this here and extended this finding to thymic *i*NKT cells (Figure 4A, B). Furthermore, *i*NKT cell-derived IL-4 production is not restricted to NKT2 cells (Cameron and Godfrey, 2018). To investigate the contribution of NKT2 cells to the *i*NKT cell-derived IL-4, we stimulated BALB/c splenocytes *in vitro* to induce cytokine production. We show that the splenic NKT2 cells produced much less IL-4 than splenic NKT1 cells in BALB/c mice (Figure 5). These data do not support the previous study on NKT2 cells as the main producers of *i*NKT cell-derived IL-4 (Lee et al, 2015). Therefore, we next, checked the TCR activity of NKT2 cells via NUR77 expression. Splenic NKT2 cells that were stimulated *in vitro* with αCD3ε

antibodies expressed NUR77 in a similar fashion to NKT1 cells (Figure 8). This demonstrates that NKT2 cells are hypo-responsive. Our data, thus, uncover an important facet of *i*NKT cell biology. Such knowledge is important to better understand the functional contribution of *i*NKT cell subsets during infections and other diseases. To test whether this hypo-responsiveness of NKT2 cells also extents to other effector functions, we will next investigate the cytotoxic potential of *i*NKT cell subsets.

6. CONCLUSION

*i*NKT cells are a unique subset of T cells. Unlike T cells, they recognize glycolipids via the MHC class 1 like molecule CD1d. After activation, they can produce both Th1 and Th2 cytokines. *i*NKT cells have several subsets, which are currently distinguished by the transcription factors they express and the cytokines they produce. NKT1, NKT2, and NKT17 cell express the transcription factor PLZF, TBET, and RORγt at different levels. NKT2 cells are known as the main producer of *i*NKT cell-derived Th2 cytokines. However, in this study, we provide new knowledge on NKT2 cells. We show that NKT2 cells are not the main producer of *i*NKT cell-derived IL-4, but rather are hypo-responsive to antigen stimulation. This knowledge is important for immunotherapeutic potential and targeted therapy of *i*NKT cell subsets.

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ETHICAL REVIEW OF ANIMAL EXPERIMENTS

Date of the Meeting	21/12/2016	Day of the Meeting	Wednesday
Number	15th	Meeting Hour	09:00

To Assist. Prof. Dr. Gerhard Wingender,

It is hereby resolved by unanimous vote that the applications of your research on "Functional characteristics and transcription factor usage of mouse NKT10 cells" are ethically appropriate.

Respectfully submitted for your attention.

Prof.Dr.Sedef AKGÜNGÖR
Chair

Prof.Dr. H. Alper BAĞRIYANIK
Vice-chair

Prof. Dr. Ensari GÜNELİ
Member

Assoc. Prof.Dr. H. Güneş ÖZHAN
Member

Assoc. Prof .Dr. Ralph Meuwissen
Member

Assoc. Prof. Dr. Devrim PESEN OKVUR
Member

Spec. Umur KELEŞ
Member

Spec. Kerem ESMEN
Member

Phrm. Ferdane KAHRAMAN

Member

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İZMİR ULUSLARARASI BİYOTIP VE GENOM ENSTİTÜSÜ
HAYVAN DENEYLERİ YEREL ETİK KURULU (İBG-HADYEK)
KARARI

İBG-Enstitü

TOPLANTI TARİHİ	21/12/2016	TOPLANTI GÜNÜ	Çarşamba
TOPLANTI SAYISI	15	TOPLANTI SAATİ	09:00

Sayın Yrd.Doç. Dr. Gerhard Wingender,

19/2016 Protokol No'lu; yürütücüsü olduğunuz "Functional characteristics and transcription factor usage of mouse NKT10 cells" isimli projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

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Başkan

Prof.Dr. H. Alper BAĞRIYANIK
Başkan Yardımcısı

Prof. Dr. Ensari GÜNELİ
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PERSONAL STATEMENT

Zeynep Gulce Tanyolac is BSc. Bioengineering graduate from Ege University in 2017 and she is MSc. Student in immunology at Izmir Biomedicine and Genome Institute since 2017. She is working on *i*NKT cells specifically, establishing the phenotypic and functional characterization of *i*NKT cell subsets. She is passionate about immunology and constantly looking ways to enhance her knowledge and skills.

EXPERIENCE

- ***Wingender group,*** Izmir Biomedicine and Genome Center, Immunology group, Izmir.
  Research assistant, August 2017- present

  Thesis Title: Phenotypic and functional characterization of *i*NKT cell subsets *in vivo*.

  - Worked under supervision of Gerhard Wingender, *PhD*
  - Responsible for planning and organizing experiments in an orderly manner, while maintaining clear and comprehensive records.
  - Investigating the *in vivo* distribution of *i*NKT cell subsets in several organs (thymus, blood, spleen, liver, lymph nodes, lung, skin, pancreas, uterus, brain, intestine, peritoneum and kidney) in C57BL/6 and BALB/c mice.
  - Measurement of *i*NKT cell activation by flow cytometry by means of Nur77 and pERK staining, as well as degranulation via CD107a/b. Sorting of *i*NKT cells in RORgt-KO-background and RORgt-reporter mice after aGalCer/DB06-1 injection. Executed multi-colour (18 colours) FACs flow cytometry on BD Fortessa.

- ***Karaman group,*** Izmir Katip Celebi University, Biomedicine group, Izmir.
  Research assistant, August 2015-May 2017

  Thesis Title; Comparative evaluation of direct and fluid mediated cold atmospheric pressure plasma treatments on wound healing.

  - Worked under supervision of Ozan Karaman, *PhD*

- Independently executed custom-made cold plasma device.

Posters;

- Kahraman E.,Demirci EA.,**Tanyolac ZG**., Karaman O. *Effect of RGD Peptide on Wound Healing Potential of Mouse Fibroblast Cells*. 20th International Biomedical Engineering Congress, 2016 (Received the third best poster award)
-**Tanyolac ZG**., Karaman O., Kursat UE., Onak G*. Comparative Evaluation of Direct and Fluid Mediated Cold Atmospheric Pressure Plasma Treatments on Wound Healing. 22*nd International Biomedicine Symposium, 2017
-**Tanyolac ZG**., Eskiocak C., Gündüz B., Wingender G. Peripheral NKT2 Cells Appear To Be Hyporesponsive In Mice. 4th International Molecular Immunology and Immunogenetics Congress. (Received the best poster award)

EDUCATION

- ***Izmir Biomedicine and Genom Center***, Izmir, Turkey
  MSc Immunology, August 2017 to present, (GPA: 3.6).
  - Extensive knowledge of *i*NKT cells.
  - Attended a therapeutic MAB engineering and production workshop.
  - Received " Laboratory Animal Handling Certificate" in 2017 given by Dokuz Eylul University ethic committee.

- ***Ege University,*** Izmir, Turkey
  Bsc, Bioengineering department with honours, September 2012- June 2017 (GPA: 3.04).
  - ***Internship***, Molecular Genetics group at Ege University, May 2014 to September 2014.
  - Executed SSR primer amplification and visualization on LiCor DNA Analyser.

ACHIEVEMENTS

- Received the third best poster award in BIOMED congress with title 'Effect of RGD Peptide on Wound Healing Potential of Mouse Fibroblast Cells' (2016).
- Earned scholarship to study abroad given by the ministry of education.
- Received registration scholarship from International Molecular Immunology and Immunogenetics Congress Committee (2019).
- Received the best poster award in International Molecular Immunology and Immunogenetics Congress (MIMIC) with title 'Peripheral NKT2

Cells Appear To Be Hyporesponsive In Mice' (2019).

SKILLS

- **Tools:** Advanced Excel, All of Microsoft Office Programmes, Xmind, Prezi, MatLab.
- **Technical:** Flowjo V10, LiCor DNA Analyser. PCR, ELISA
- **Languages:** English (advanced).

ACTIVITIES

- Member of cross-fit fellows in MACFIT sports club for 7 years.
- Trainer in MACFIT sports club during January 2017-August 2018.
- Worked for voluntary organization for underprivileged children who suffer from lymphoma during September 2015- November 2017.
- Volunteer teacher in TEGV (Turkish Education Volunteers Foundation) since 2013.