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

IDENTIFICATION OF THE TRANSCRIPTION FACTOR(S) THAT DRIVE NKT10 CELL DEVELOPMENT AND FUNCTION

SEZGIN BAL

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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Supervisor: Dr. GERHARD WINGENDER

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MASTER THESIS DEFENSE REPORT

Dokuz Eylul University Izmir International Biomedicine and Genome Institute Department of Genomics and Molecular Biotechnology, Molecular Biology and Genetics graduate program Master of Science student, Sezgin Bal has successfully completed his Master of Science thesis titled '**IDENTIFICATION OF THE TRANSCRIPTION FACTOR(S) THAT DRIVE NKT10 CELL DEVELOPMENT AND FUNCTION**' on the date 29/09/2020.

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°C:	Celsius degree
µ:	Micro- (10-6)
µg:	microgram
µl:	microliter
µm:	micrometer
µM:	micromolar
aa:	Amino Acid
AF488:	Alexa Flour 488
AF647:	Alexa Flour 647
AF700:	Alexa Flour 700
APC:	Allophycocyanin
APC-Cy7:	Allophycocyanin-cyanine7
APC-eF780:	Allophycocyanin– eFlour 780
APCs:	Antigen presenting cells
Bcl6:	B cell lymphoma 6
BCR:	B cell receptor
BSA:	Bovine serum albumin
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
CEBPα:	CCAAT/enhancer-binding protein alpha
cm:	Centimeter
cRPMI:	Completed RPMI
CTL:	Cytotoxic T cell
CXCL13:	Chemokine (C-X-C Motif) ligand 13
DCs:	Dendritic cells
dH2O:	Distilled water
ddH2O:	Double-distilled water
DMSO:	Dimethylsulfoxid
DNA:	Desoxyribonucleic acid
DNase:	Desoxyribonuclease
dsDNA:	Double-stranded DNA dsRNA: Double-stranded RNA
E4BP4:	E4 promoter–binding protein 4
EDTA:	Ethylenediamine tetraacetic acid
eF660:	eFlour 660
eFluor 450:	eFlour 450
EtOH:	Ethanol
FACS:	Fluorescent activated cell sorting
FasL:	Fas ligand
FBS:	Fetal Bovin serum
Fc:	Fragment crystallizable
FCS:	Foetal calf serum
FITC:	Fluorescein isothiocyanate
FITC:	Fluorescein isothiocyanate
FoxP3:	Forkhead box P3
FR4:	Folate receptor 4

FSC-A:	Forward scatter area
FSC-H:	Forward scatter height
FSC-W:	Forward scatter width
g:	gram; acceleration of gravity (9.81 m/s2)
GC:	Germinal centers (GCs)
h:	hour
IFN:	Interferon
Ig:	Immunoglobulin
IL:	Interleukin
IL-10:	Interleukin 10
IL-12:	Interleukin 12
IL-13:	Interleukin 13
IL-17A:	Interleukin 17A
IL-21:	Interleukin 21
IL-4:	Interleukin 4
ILCs:	Innate lymphoid cells
iNKT:	Invariant Natural Killer T
Kb:	Kilobase
kD:	Kilodalton
L:	Liter
LN:	Lymph node
m:	Meter; milli- (10-3)
M:	Molar
MACS:	Magnetic activated cell sorting
MAF:	MAF BZIP Transcription Factor
mg:	Milligram
MHC:	Major histocompatibility complex
Min:	Minute

ml:	Milliliter
mm:	Millimeter
mRNA:	messenger RNA
MW:	Molecular weight
n:	nano- (10-9)
NaN3:	Sodium azide
NK1.1:	Natural Killer 1.1
NKT_{FH}:	Follicular helper NKT
OD:	Optical density
PB:	Pacific blue
PBS:	Phosphate buffered saline
PE:	Phycoerythrin
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
PMA:	Phorbol 12-myristat 13-acetat
qPCR:	Quantitative PCR
rcf:	Relative centrifugal force
RNA:	Ribonucleic acid
RORγt:	RAR-related orphan receptor gamma T
rpm:	Revolutions per minute
RT:	Room temperature
RT-PCR:	Reverse-Transcriptase Polymerase Chain Reaction
s:	Second

scWAT:	Subcutaneous white adipose tissue
SDS:	Sodium dodecyl sulphate
Slamf6:	Signaling lymphocyte activation molecule 6
SSC-A:	Side scatter area
SSC-H:	Side scatter height
SSC-W:	Side scatter width
Tbet:	T-box transcription factor
TCR:	T cell receptor
TFH:	T follicular helper
Th:	T helper
TNF:	Tumor necrosis factor
TRAIL:	Tumor necrosis factor-related apoptosis inducing ligand
Tris:	Tris- (hydoxymethyl) -aminomethan
U:	Unit
UV:	Ultraviolet light

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It's easy in the day but harder in the night
I thought I felt the pain but hadn't fought this fight
You woke up next to me and then we said goodbye
And now it's settled in that something wasn't right
Oh, but we're a long way, long way from there
And it's a cold case, a cold case to bear
Now I can only see you in my rear view
So where did you go, where did you go
I ruined our sweet tune
But how could I know, how could I know
Once we were two dancing souls
Now all that's left is skulls and bones
But I can only see my future
In my rear view
And don't we owe us more from all that in the past
We knew before them all that what we had would last
Stayed up for days so long and all into the night
Our final morning dawned and you had taken flight
Oh but we're a long way, long way from there
And it's a cold case, a cold case to bear...
Now I can only see you in my rear view
So where did you go, where did you go
I ruined our sweet tune
But how could I know, how could I know
Once we were two dancing souls
Now all that's left is skulls and bones
But I can only see my future
In my rear view
Now I can only see you in my rear view
So where did you go, where did you go
I ruined our sweet tune
But how could I know, how could I know
Once we were two dancing souls
Now all that's left is skulls and bones
But I can only see my future
I can still see my future
in my rear view

ABSTRACT

IDENTIFICATION OF THE TRANSCRIPTION FACTOR(S) THAT DRIVE NKT10 CELL DEVELOPMENT AND FUNCTION

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Invariant Natural Killer T (*i*NKT) cells are a distinct population immune cells that share the characteristics of both adaptive T cells and innate Natural Killer (NK) cells. They express semi-invariant $\alpha\beta$ T-cell receptor (TCR) and several NK cell receptors. Unlike conventional T cells that recognise protein fragments, *i*NKT cells recognise glycolipid antigens presented by the non-polymorphic MHC class I-like molecule, CD1d. There are five main *i*NKT cell subsets: NKT1, NKT2, NKT17, NKTFH, and NKT10 cells. These subsets express unique set of transcription factors and produce a distinct combination of cytokines. Of these subsets, NKT10 cells are characterized by the production of the anti-inflammatory cytokine IL-10, as well as several proteins expressed by T_{regs}. However, the master transcription factor for NKT10 cells is unknown. To better understand of how NKT10 cells are regulated, we studied the expression of the transcription factors CEBPα, E4BP4 and c-MAF in NKT10 cells at both protein and mRNA level. Our data indicate that c-MAF is the only one of these transcription factors that is specifically expressed in NKT10 cells. Moreover, we studied the TCR-dependent activation of *i*NKT cells by the model antigen α-GalCer by measuring the expression of Nur77 and CD3ε. NKT1, NKT2, and NKT17 cells were activated within 2 hours of α-GalCer injection and the route of the injection (intra-venous vs. intra-peritoneal) had no impact on the strength of the activation. These findings provide a better understanding of the functionality of NKT10 cells and *i*NKT cell activation. This will likely facilitate subsequent research and the development of NKT10 cell-based therapeutic approaches, for example for the treatment of autoimmune diseases.

Keywords: *i*NKT cells, NKT10 cells, c-MAF, *i*NKT cell activation, Nur77, autoimmune diseases

ÖZET

NKT10 HÜCRE GELİŞİMİNİ VE FONKSİYONUNU DÜZENLEYEN TRANSKRİPSİYON FAKTÖRLERİNİN BELİRLENMESİ

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Doğala yakın öldürücü T (*i*NKT) hücreleri, hem adaptif T hücrelerinin hem de doğal öldürücü (NK) hücrelerinin özelliklerini paylaşan farklı türdeki bağışıklık hücreleridir. Bu hücreler hem yarı-değişmeyen bir TCR hem de bazı NK hücrelerindeki hücre yüzeyi reseptörlerini ekprese eder. Protein parçalarını tanıyan geleneksel T hücrelerinin aksine, *i*NKT hücreleri, polimorfik olmayan MHC sınıf I benzeri molekülü olan CD1d tarafından sunulan glikolipid antijenleri tanır. Beş ana *i*NKT hücre alt tipi bulunmaktadır: NKT1, NKT2, NKT17, $NKT_{FH,}$ ve NKT10 hücreleri. Bu alt tip hücreler, kendilerini özgü transkripsiyon faktörleri üretir ve farklı sitokin kombinasyonlarına sahiptir. Bu hücre tipleri arasından, NKT10 hücreleri, anti-enflamatuar olan IL-10 sitokinin yanı sıra, T_{regs} hücreleri tarafından eksprese edilen çeşitli proteinlerin üretimi ile karakterize edilir. Ancak, NKT10 hücrelerini düzenleyen transkripsiyon faktörü henüz bilinmemektedir. NKT10 hücrelerinin nasıl düzenlendiğini daha iyi anlamak için, NKT10 hücrelerinde, hem protein hemde mRNA düzeyinde, CEBPα, E4BP4, ve c-MAF transkripsiyon faktörlerinin ekspresyonunu inceledik. Verilerimiz, NKT10 hücrelerinde, bu transkripsiyon faktörleri arasından sadece c-MAF'in spesifik olarak eksprese edildiğini göstermektedir. Ayrıca, Nur77 ve CD3ε ekspresyonlarını ölçerek, model antijen α-GalCer tarafından uyarılan *i*NKT hücrelerinin, TCR'ye bağlı aktivasyonunu inceledik. NKT1, NKT2 ve NKT17 hücreleri, 2 saat süresince α-GalCer ile aktive edildi ve enjeksiyon rotasının (intra-venöz vs. intra-peritoneal) aktivasyon gücü üzerinde hiçbir etkisi olmadığı gözlemlendi. Bu bulgular, NKT10 hücrelerinin ve *i*NKT hücre aktivasyonunun işlevselliğinin daha iyi anlaşılmasını sağlamıştır ve muhtemelen otoimmün hastalıkların tedavisi için, NKT10 hücre bazlı terapötik yaklaşımların sonraki araştırmalarını ve geliştirilmesini kolaylaştıracaktır.

Anahtar Kelimeler: *i*NKT hücreleri, NKT10 hücreleri, c-MAF, *i*NKT hücre aktivasyonu, Nur77, otoimmün hastalıklar

1. INTRODUCTION AND OBJECTIVES

Invariant Natural killer T (iNKT) cells are a subset of αβ T cells that recognize glycolipid antigens presented on the MHC class I-like molecule CD1d and share characteristics of both T cells and NK cells. Five main iNKT cell subsets are currently recognized: NKT1, NKT2, NKT17, NKT_{FH}, and NKT10 cells. These iNKT cell subsets are analogous to the conventional $CD4^+$ T cell subsets Th1, Th2, Th17, T_{FH} cells, and Tregs. However, unlike conventional $CD4^+$ T helper subsets, which differentiate into effector cells following activation, most iNKT cell subsets develop already in the thymus. iNKT cell subsets are characterized by the expression of specific transcription and the cytokines they can secrete. However, the defining transcription factor for NKT10 cells is still unknown. Additionally, the *in vivo* distribution of NKT10 cells is not very well established. Therefore, this thesis had two main aims:

1) Determining the transcription factor that regulates the IL-10 secretion by NKT10 cells. To address this, we investigated the expression of the transcription factors CEBPα, E4BP4, and c-MAF in NKT10 cells at both protein and mRNA level. It was previously claimed that E4BP4 is the defining transcription factor of NKT10 cells. However, this suggestion is based on unphysiologically overexpression of E4BP4 in iNKT cell lines *in vitro*. Based on our preliminary data, we rather hypothesize that c-MAF is the transcription factor specific for NKT10 cells.

2) Examining how the routes of antigen injections influences the activation of iNKT cells *in vivo*. To address this, we injected mice with α-GalCer either i.v. or i.p. and monitor the expression of the activation marker Nur77 in iNKT cells. Furthermore, we hypothesized that the route of injections impacts the *in vivo* distribution of iNKT cell subsets.

2. LITERATURE REVIEW

2.1. The immune system

The main task of the numerous cells that comprise the immune system is to protect the host against diseases and pathogens, including bacteria, fungi, and viruses. An effective immune response is achieved by the collective, complex, and coordinated actions of the immune cells. These cells maintain protection by identifying, targeting, and eliminating pathogens and cancer cells that threaten host viability. The successful immune response depends on the discrimination of molecular signatures of invading pathogens and cellular constituents that pose no risk to the host.

During evolution, the immune system developed various types of immune responses, ranging from cellular activities to soluble factors. The differences in pathogen detection and functional capacity of the immune cells gave rise to the two major pillars of the immune system: innate and adaptive immunity. Innate immunity represents the first line of defence and is important for survival. Its cells respond immediately to challenges without lengthy differentiation. In contrast, the adaptive or acquired immunity is slower as it requires initial education. However, unlike innate immunity, the adaptive immune system can impart long-lasting protection by providing memory of the previously encountered infections. Despite their differences, the two immune responses are highly collaborative and tightly connected.

2.2. Innate immunity

The components of the innate immune system exist in both vertebrates and invertebrates as well as plants. When the pathogens penetrate certain barriers, such as epithelial cells for instance, they are encountered by innate immune cells and the innate immune response is triggered. These cells express germline-encoded receptors that allow them to recognize specific structures of pathogens directly. In this way, innate immune cells can attempt to eliminate pathogens early, prior to the formation of infectious foci (Uthaisangsook et al., 2002).

The innate immune system is composed by an array of different cells, including, granulocytes (eosinophils, basophils, neutrophils, and mast cells), macrophages, dendritic cells (DCs), and natural killer (NK) cells. These cells can target pathogens directly in cell-cell interaction or can release soluble factors to eliminate pathogens. Granulocytes are the most numerous among white blood cells and are multi-nucleated. They contain granule enzymes that allows them to digest microbes after phagocytosis, a process by which certain cells engulfs other cells or particles. Such phagocytic cells are called phagocytes. Besides microbes, they also can also destroy tumour cells via phagocytosis, which is a crucial part of the anti-tumour immunity (Savina and Amigorena 2007). Similar to granulocytes, macrophages are professional phagocytes and are the 'big eaters' of the immune system. In addition to phagocytosis, they can release toxic molecules, like oxygen-reactive species, to kill target cells. Macrophages have a variety of other important tasks, ranging from tissue homeostasis to wound healing. They can release cytokines, soluble factors that provide intercellular communication (Do Vale et al., 2002). Cytokines have a significant role in an effective immune response as they allow the immune cells to function cooperative. Dendritic cells are an important part of the innate immune response as they can act as 'messengers' between innate and adaptive immunity. They have a tremendous impact on adaptive immune responses and are also considered as professional phagocytes (Banchereau et al., 2000). Lastly, the real 'killers' of the innate immune system, are Natural Killer (NK) cells, named due to their ability to rapidly destroy target cells. This killing or cytotoxicity, is achieved either by the release of the content of cytotoxic granules or by surface molecules binding to death receptors. These receptors or the granule molecules, mainly perforin and granzymes, then either trigger apoptosis, a programmed cell death programme, or directly lyse the target cells. Healthy host cells are protected from NK cells by the expression of certain receptors that inhibit NKT cells. When a physiologically stressed cell, such as virally infected cells or tumour cells, fail to express these receptors, they can be destroyed by NK cells in a cytotoxic manner. Similar to macrophages, NK cells can also secrete cytokines to enhance the immune response (Zamai et al., 1998).

Although innate immunity is essential for a proper immune response, it is sometimes insufficient to provide long-lasting protection to the host. Here, adaptive immunity comes into play.

2.3. Adaptive immunity

Although innate immunity is present in each living species, vertebrates developed a more sophisticated, robust, and finely tuned immune response, called adaptive immunity. The adaptive immune system is not fully mature at birth, but rather develops and changes throughout the lifespan. The adaptive immune cells can react to small components parts of the invading pathogens, called antigens. They can also develop immunological memory against previously encountered antigens and can thereby elicit a faster response upon re-stimulation (Campos, 1998). The adaptive immune system is composed of B and T lymphocytes. Unlike innate cells, which have germ-line encoded receptors, the antigen-specific receptors of B and T cells, are built from a small number of highly diverse building blocks through genetic recombination (Chaplin, 2003). This and other processes lead to an extraordinary diversity in the antigen-specific receptors of the adaptive immune cells. Although T cells and B cells share the same bone marrow progenitor, they display many differences.

2.3.1. B cells

B cells originate and develop in the bone marrow. B cells are unique due to their ability to express proteins called antibodies (Ab), also known as immunoglobulins (Ig), which are soluble versions of their antigen-specific receptor, called B cell receptor (BCR) (Loder et al., 1999). The BCR is composed of two transmembrane protein, the heavy chains, and two smaller two light chains. The transmembrane domain mediates the BCR signalling. Both heavy and light chain contain a constant and a variable region. The variable regions, as the name implies, are highly variable and is the part that interact with the antigen. The extraordinary diversity of the BCR is mediated through a recombination process called V(D)J rearrangements. The BCR locus consists of many genes for the variable (V), diversity (D), and joining (J) region. During B cell development, these genes are randomly assembled and together they form a mature BCR. Fully developed B cells, now called naïve B cells, are found in the secondary lymphoid (Van Noesel and Van Lier, 1993).

After naïve B cells are activated through their BCR complex following antigen recognition, they either differentiate into plasma cells or memory B cells. Plasma cells secrete large amounts of antibodies to fight infections via several mechanisms. For example, antibodies can neutralize pathogens by blocking essential parts on the pathogen’s surface (Kindler and Zubler, 1997). Furthermore, following antigen binding, the constant region of the antibody can be recognized by Fc receptors on immune cells to facilitate phagocytosis or cytotoxicity. Some of the plasma cells become long-lived plasma cells and migrate back to the bone marrow and continue to produce antibodies. Memory B cells can also home to the bone marrow, where they remain and defend the host against future infections upon reencounter of their antigen (Teichgräber 2004). Apart from antibody-mediated immune responses, B cells can also secrete immunomodulatory cytokines (Blair et al., 2000).

2.3.2. T cells

The adaptive immune response also relies on T cell, as they are directly involved in the adaptive immune responses and can also control B cell immune responses. T cells develop in the thymus from immature, bone marrow-derived thymocytes. Similar to the B cells, the antigen-specific receptor of T cells, called T cell receptor (TCR), also undergoes V(D)B rearrangement. The TCR is composed of two chains; either α and β chains or γ and δ chains (Engel and Hedrick, 1988). Approximately, 95% of T cells use α and β chains; whereas the remaining 5% have TCRs built from γ and δ chains (Boismenu and Havran, 1994). The rearrangement process involves V and J segments at the α locus; V, D and J segments at the β locus; and variable nucleotide sequence changes at the gene junctions. The resulting protein chains are linked through disulphide bonds. The transmembrane domains of the TCR interact with four different CD3 molecules, which together form the TCR complex. Similar to the BCR, the part of the TCR that interacts with the antigen displays the highest diversity. The human genome is capable of producing approximately 10^{15} different TCRs, allowing T cells to recognize wide range of antigens (Wack et al., 1998).

After the successful expression of the TCR complex, the development of most abT cells branches into two functionally divergent lineages, which can be distinguished based on the expression of either the CD4 or CD8 co-receptor. Importantly and in contrast to B cells, $\alpha\beta$T cells do not directly recognise their antigens, usually small protein fragments called peptides. Rather, these peptides are presented to the TCR via specific antigen-presenting molecules, called major histocompatibility (MHC) molecules (Guillet et al., 1986). Due to structural features, two main types of classical MHC molecules are distinguished. MHC class I and, MHC class II. MHC class I molecules are expressed on all nucleated cells and present peptides of cytosolic origin of approximately 8-10 amino acids in lengths. In contrast, MHC class II molecules are expressed mainly by specialist antigen presenting cells (APCs) such as DCs, macrophages, and B cells and present peptides of approximately 13-18 amino acids that derive from proteins the cell has taken up e.g. by phagocytosis (Rammensee, 1995). The thymocytes strongly interact with either MHC class I or MHC class II molecules and terminally differentiate into $CD8^{+}$ T cells or $CD4^{+}$ T cells, respectively. These naïve T cells then exit the thymus into the periphery (König et al., 1995).

For their activation, naïve T cells interact with APCs and receive initially two kind of signals. The first one requires the interaction of the TCR and the MHC/antigen peptide complex; the second involves the interaction of co-receptors, like CD28 on T cells with CD80/CD86 on the APCs (Greene et al., 1996). At the contact point, the TCRs and co-receptors aggregate, into an organized structure, termed the immunological synapse. This leads to recruitment of signalling molecules. The co-receptor complex recruits Lck, a tyrosine kinase, which activates the tyrosine kinase ZAP-70, leading to the phosphorylation of other downstream molecules (Robey and Allison, 1995). Although the activation is rapid, the phosphorylation requires sustained signalling, which is influenced by the half-life and affinity of the TCR-MHC interaction (Kalergis et al., 2001). The TCR signalling ultimately leads to the expression of transcription factors, which regulate aspects like cytokine production, proliferation, and further functional differentiation.

T cell subsets

The lineage commitment of $CD4^+$ or $CD8^+$ $\alpha\beta$T cells is maintained by the expression of distinct transcription factors. For example, CD8+ T cells depend on the activity of the Runx family of transcription factors; whereas $CD4^+$ T cells require the functions of GATA3 and Th-POK (He et al., 2005). Both $CD4^+$ or $CD8^+$ T cells can form memory cells as an important feature of adaptive immunity (Sun and Bevan, 2003). Nevertheless, they show significant differences in their functionality and roles during an immune response.

CD8⁺ T cells

Activated naïve $CD8^+$ T cells differentiate into cytotoxic T cells (CTLs). CTLs can induce apoptosis in target cells by either releasing perforins and granzymes or by the use of death receptors, such as CD178 (Fasl) or the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL/CD253) (Mirandola et al., 2004). In addition to their capacity to destroy target cells through direct cellular cytotoxicity, CTLs can also secrete important cytokines such as interferon-gamme (IFN-γ) or tumour necrosis factor (TNF). Both cytokines display pro-inflammatory properties and play a central role in inflammation (Barth Jr et al., 1991).

CD4+ T cells

In contrast, activated $CD4^+$ T cells differentiate into T helper (T_h) cells. Th cells are called 'helper cells' as they support the activation of other immune cells and have a strong immunomodulatory role. For instance, they can enhance $CD8^+$ T cell or B cell responses (Husmann et al., 1988; Flynn et al., 1998). Naïve $CD4^+$ T cells can differentiate into several subsets of T_h cells, including T_h1, T_h2, T_h17, T_h19, T follicular helper (T_{FH}), or regulatory T (T_{reg}) cells (Araujo-Pires et al., 2014). The subsets are determined by 'master' transcription factors and each subset has a unique cytokine profile. These cytokines are the critical regulators of inflammation and can act pro-inflammatory and anti-inflammatory to induce or reduce inflammation, respectively.

T_h1 cells are characterized by their expression of the T-box transcription factor TBX21 (T-bet) and the secretion of the cytokines IFN-γ and IL-2 (Bao et al., 2011). T_h1 can support the activation of $CD8^+$ T cells and macrophages (Hsieh et al., 1993). T_h2 cells express GATA-3 as a mastery transcription factor and can modulate B cell responses by releasing IL-4 and IL-13 cytokines (Liang et al., 2012). T_h17 cells are usually defined by their capacity to release IL-17, as well as TNF and IL-22 cytokines. Both the 'signal transducer and activator of transcription 3' (STAT3) and 'retinoic-acid-receptor-related orphan nuclear receptor gamma T' (RORγt) are required for the differentiation into T_h17 cells (Chen and O'Shea, 2008). These cells can induce inflammation and are also important in host defence against pathogens. T_h9 cells mainly secrete IL-9 but also IL-10 (Pang et al., 2014). Bcl6 is the transcription factor that drives differentiation of $CD4^+$ T cells into T_{FH} cells. These cells mainly produce IL-21 and have a significant role in the selection and survival of B cells (Choi et al., 2011). Lastly, Tregs express FoxP3 as a master transcription factor and mainly secrete IL-10 and TGF-β (Dardalhon et al., 2008). They act as immunosuppressive cells and can inhibit immune activation by either cell-contact dependent or independent mechanisms.

Having a unique transcriptional and cytokine profile, T_h cells have been initially considered as 'terminally' differentiated cells. However, this paradigm has been challenged by recent studies that revealed flexibility between $CD4^+$ T cell subsets. For example, it has been shown that Tregs under certain conditions can express other master transcriptional factors, including T-bet, GATA-3, or RORγt. This subset plasticity allows T helper cells to quickly adapt to different immune responses (Hirahara et al., 2013).

Innate-like T cells: bridging innate and adaptive immunity

An effective immune response requires a 'cross-talk' between innate and adaptive immunity. Unlike conventional T cells, innate-like T cells share features of both innate and adaptive immune cells, hence their name. For example, these cells can respond to infections rapidly but also express a TCR. The best-characterized innate-like T cells are $\gamma\delta$-T cells, mucosal-associated invariant T (MAIT) cells, and Natural Killer T (NKT) cells (Gao and Williams, 2015). The antigens that activate $\gamma\delta$-T cells are still poorly defined. MAIT and NKT cells have a limited TCR repertoire and can recognize a limited number of conserved non-peptide antigens presented by non-classical MHC class molecules (Godfrey et al., 2015). Innate-like T cells display an effector memory phenotype and are usually tissue-resident (Chou et al., 2018). They play a crucial role in bridging innate and adaptive immune responses.

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

In 1987, researchers discovered a small distinct subset of $CD4^-$ $CD8^-$ T cells, expressing TCRs of limited diversity (Lanier et al., 1987). Subsequent studies discovered that these cells can bear certain NK cell markers, like NK1.1 in mice and CD161 in humans (Arase et al., 1997). As they share characteristics of both T cells and NK cells, they were named as Natural Killer T (NKT) cells. NKT cells are classically divided into two main subpopulations: Type I or 'invariant' NKT (*i*NKT) cells and Type II or 'non-classical' NKT cells. Among NKT cells (Terabe et al., 2005), *i*NKT cells are the better characterized cell type both in humans and mice. The term 'invariant' of *i*NKT cells is given due to their expression of a less diverse TCR. In contrast to conventional T cells, the TCR rearrangement of *i*NKT cells is restricted to the combination of the gene segments Vα14-Jα18 with a limited set of Vβ8.2, Vβ7, or Vβ2 in mice and with Vβ11 in humans (Ronet et al., 2001). Studies show that the successful generation of the semi-invariant TCR is essential for *i*NKT cell development.

2.4.1. iNKT cell antigen recognition and activation

*i*NKT cell development is also thymus-dependent and they develop from the same precursor as conventional T cells. Unlike T cells, which recognize peptide antigens presented by classical MHC class molecules, *i*NKT cells recognize lipid antigens presented by CD1d molecules. CD1d (see **Figure 1.**). is a highly conserved, non-polymorphic MHC class I-like molecule and is constitutively expressed by most cells. CD1d is initially loaded with self-lipids in the endoplasmic reticulum and then transported to the cell surface. Later, it is endocytosed and fuses with both endosome and lysosome so that the self-lipids, with the help of accessory proteins, are exchanged with lipids of extracellular origin (Jayawardena-Wolf et al., 2001). Self-lipids or endogenous lipids, such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) are naturally present in tissues (Gumperz et al., 2000); whereas the mycobacterial lipids PIM4 and α-galactosyl-diacylglycerol are examples of lipids derived from microbes (Fischer et al., 2004). Lipid presentation by CD1d is a finely-tuned system and each presented lipid contributes to the quality of the response. Among *i*NKT cell ligands, alpha-Galactosylceramide (α-GalCer) was the first discovered and is one of the strongest agonists for *i*NKT cells (Kawano et al., 1997; Motoki et al., 1995). It is a glycosphingolipid and was originally isolated from the marine sponge *Agelas mauritianus* (Natori et al., 1994). Having a high affinity for CD1d and being a strong stimulant, α-GalCer undoubtedly facilitated *i*NKT cell research and is broadly used in immunoassays.

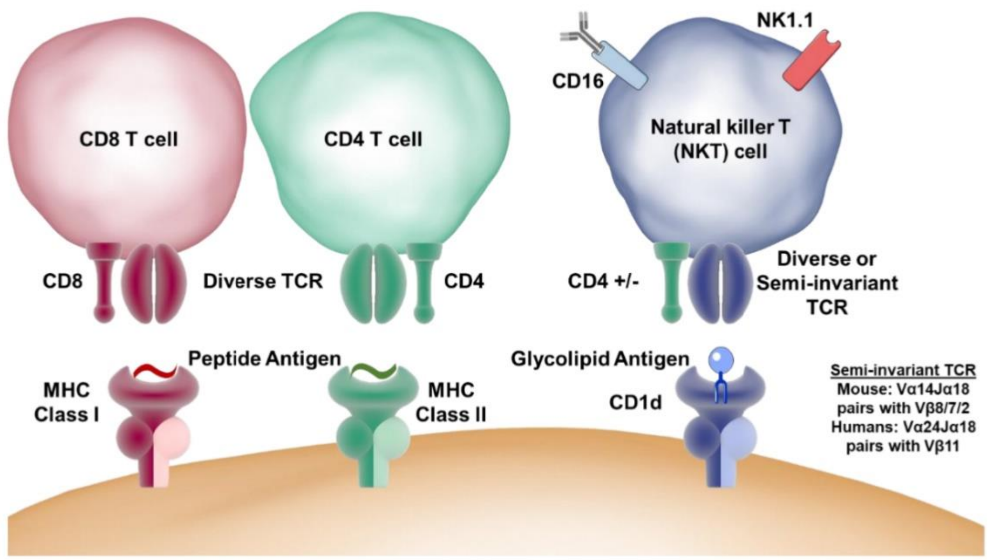

Figure 1. The recognition of glycolipid antigens by *i*NKT cells. Unlike conventional T cells [they are CD4+/Cd8+], which recognize peptides in the context of MHC class molecules, *i*NKT cells recognize lipids bound to CD1d. Furthermore, *i*NKT cells express a semi-invariant TCR and NK cell markers.

There are two modes of *i*NKT cell activation, either TCR-dependent or TCR-independent way (Holzapfel et al., 2014). TCR-dependent activation refers to the activation of *i*NKT cells through the TCR and CD1d/lipid complex interaction. Nonetheless, TCR and CD1d interaction by itself is not always sufficient to induce activation. For example, *i*NKT cells bind to the CD1d/beta-galactosylceramide (β-GalCer) complex but are not activated (Kawano et al., 1997). Structural differences in ligands and the kinetics of lipid loading has a significant impact on *i*NKT cell activation. The TCR-independent activation of *i*NKT cells is usually mediated by pro-inflammatory cytokines, similar to memory T cells. Activated APCs, can produce pro-inflammatory cytokines such as IL-12 and IL-18 and these cytokines can induce *i*NKT cell activation (Fujiki et al., 2008).

Another possible, albeit less efficient, way to activate *i*NKT cell is through NK receptor cross-linking. *i*NKT cells can be activated via NK1.1 in mice (or NK2GD in humans) in the absence of lipid antigens (Nagarajan and Kronenberg, 2007). This kind of activation allows *i*NKT cells to be involved in immune response in a CD1d-independent manner.

2.4.2. iNKT cell effector functions

In addition to sharing both innate and adaptive immune cell phenotypes, *i*NKT cells also display both innate and adaptive functions. The ability to rapidly respond to challenges by producing large amounts of cytokines upon activation is an important characteristic of *i*NKT cells. The rapid response is partially due to the constitutive expression of the relevant cytokine mRNAs in *i*NKT cells. Unlike conventional T cells, which have a defined T_h1 or T_h2 cytokine profile, *i*NKT cells can produce both T_h1 and T_h2 cytokines, such as IL-4 and IFNγ (Naka et al., 2001). Apart from cytokine productions, *i*NKT cells can also be cytotoxic, similar to $CD8^+$ T or NK cells and via similar mechanisms (Kawano et al., 1999).

Due to their diverse effector functions, *i*NKT cells can act like 'a double-edged sword', as the can either ameliorate or exacerbate various immune responses, including microbial infections, autoimmune disease, and anti-cancer responses.

2.4.3. iNKT cell subsets

The diversity impact of *i*NKT cells on immune responses is thought to be due to the existence of functional *i*NKT cell subsets, analogous to $CD4^+$ T cells. However, unlike conventional $CD4^+$ T cells, which differentiate into effector subsets following activation, most *i*NKT cell subsets develop already in the thymus. There are five main *i*NKT cell subsets: **NKT1**, **NKT2**, **NKT17**, **NKT_{FH}**, and **NKT10** cells (see **Figure 2.**). Differentiation into NKT1, NKT2, NKT17, and NKT10 cells occurs in the thymus (Buechel et al., 2015); whereas *i*NKT cells can differentiate into NKT_{FH} cells in the periphery (Rampuria et al., 2015).

*i*NKT cell differentiation requires the expression of certain transcription factors such as 'promyelocytic leukaemia zinc finger protein' (PLZF) (**Figure 2.**). PLZF drives *i*NKT cell lineage commitment (Kovalovsky et al., 2008). While its expression in conventional T cells is low or absent, there is a variance in PLZF expression among different *i*NKT cell subsets (Savage et al., 2008). These subsets also express a unique master transcription factors and produce a distinct combination of cytokines.

NKT1 cells are characterized by their expression of the master transcription factor T-bet and low-levels of PLZF. They can produce IFNγ and IL-4 upon stimulation and can participate in the elimination of target cells through cytotoxicity. NKT2 cells express GATA-3 as a regulatory transcription factor and high levels of PLZF. They can produce IL-4, which can affect, for example, T and B cell differentiation (Yasuoka et al., 2016). RORγt is the master transcription factor of NK17 cells. These cells express intermediate levels of PLZF and can secrete high amounts of IL-17 when stimulated (Constantinides and Bendelac, 2013). IL-17 is an important pro-inflammatory cytokine involved in neutrophil recruitment and activation. Similar to conventional T_{FH} cells, NKT_{FH} cells express Bcl6 as a transcriptional regulator. They produce IL-21 and play an important role in B activation, proliferation, and antibody secretion (Tonti et al., 2012). Lastly, NKT10 cells are the most recently identified subset of *i*NKT cells (Sag et al., 2014). NKT10 cells are characterized by the production of the anti-inflammatory cytokine IL-10, as well as several proteins expressed by $T_{regs.}$ However, the master transcription factor of NKT10 cells remains to be elucidated.

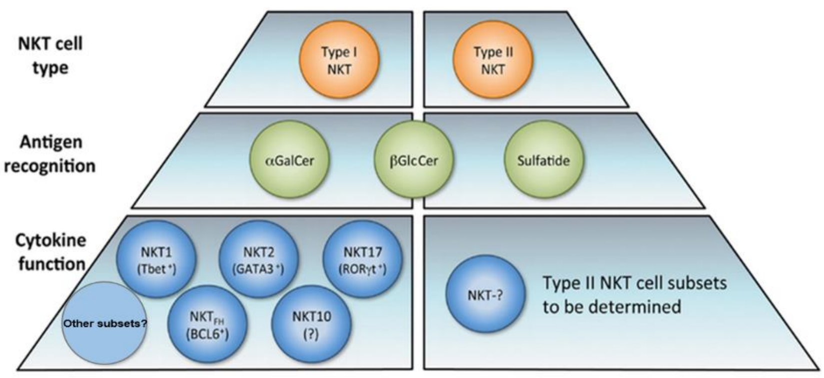

Figure 2. CD1d-dependent NKT cell subtypes. Only type I NKT cells bind to α-GalCer, while some type II NKT cells bind to sulfatides. Where known, the master regulatory transcription factor for each NKT cell subset is indicated in brackets

*i*NKT cells are usually tissue-resident similar to memory T cells. Mice and humans show significant differences in *i*NKT cell frequency and subset distribution. In most mouse strains, *i*NKT cells are more frequent than in humans. NKT1 cells are the predominant *i*NKT cell subset in mice (Singh et al., 2018). However, BALB/c mice have higher frequencies of NKT2 and NKT17 cells than C57BL/6 mice. *i*NKT subsets are also enriched differently in distinct mouse tissues. NKT1 cells preferentially home to the spleen and the liver; NKT2 cells are enriched in the lungs; NKT17 and NKT$_{FH}$ cells are mainly present in the lymph nodes (Lee et al., 2015); and NKT10 cells are usually enriched in the adipose tissue (Sag et al., 2014).

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. General laboratory equipment

3.1.1.1. Laboratory

Autoclave (Systec, VX-150, 166L, SN:8373)
Biological safety cabinet (ThermoFisher Scientific, class II type A2- SN:41806507)
Cell analyzer LSR FortessaX20 (BD Biosciences, #647780S1, SN:H647780S1001, model no:N/A)
Cell sorter FACSAria III (BD Biosciences)
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)

3.1.1.2. Vivarium

Anesthesia 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)

3.1.2. Consumables

Aluminum foil 30m, Migros
Cell culture multi-well plate 24 well (Greiner Bio-One (#662160))
Cell culture multi-well plate 48 well (Greiner Bio-One (#677180))
Cell culture multi-well plate 6 well (Greiner Bio-One (#657160))
Filter syringe (33mm x 0.22mm, CHROMFILTER (#S33-CA22-S))
Flask, cell culture 250mL (Greiner Bio-One (#658175))
Flask, cell culture 50mL (Greiner Bio-One (#690175))
Gloves examination (Small, Medium, Large) (MyGlove, Labmarker Dis Ticaret Ltd. Sti. (#9604 0203))
Microplate (96-well F- bottom, Greiner Bio-One (#655081))

Multi-well plate (cell culture 96 well, Greiner Bio-One (#655180))

Parafilm: Wrap (4" Wide; 125 Ft/Roll (#PM-996))

Pasteur pipette (glass, 225mm, ISOLAB (#084.01.002))

Pasteur pipette (polyethylene, 3mL, non-sterilized, ISOLAB (#084.02.001))

Petri dish 10 cm (ISOLAB, (#081.01.100))

Petri dish 5 cm (ISOLAB, (#081.01.060))

Pipet tips, 10mL (Rainin, (#17004280))

Pipet tips, 1mL (Rainin (#17001977))

Pipet tips, 200mL (Corning Inc. (#4845))

Pipet tips, 250mL (LTS for multichannel, Raining (#17000506))

Pipettes, serological, 10mL (sterile, Greiner Bio-One (#607180) and SPL Lifesciences (#91010))

Pipettes, serological, 25mL, sterile (Greiner Bio-One (#760180) and sterile, SPL Lifesciences (#91025))

Pipettes, serological, 5mL sterile, (Greiner Bio-One (#606180) and SPL Lifesciences (#91005))

Reaction tube, 0.5mL with attached cap (Greiner Bio-One (#667201))

Reaction tube, 1.5mL with attached cap (Greiner Bio-One (#616201))

Reaction tube, 2mL with attached cap (Greiner Bio-One (#623201))

Reservoirs (reagent – disposable, 55mL, non-sterilized, ISOLAB (#006.03.055))

Syringe (single use with needle, 5mL, Genject)

Tube 15mL (polypropylene), (canonical bottom, Greiner Bio-One (#188271))

Tube 50mL (polypropylene), (canonical bottom, Greiner Bio-One (#227261), Sarstedt (#62.547.254) and Orange Scientific (#4440100N))

Tube 5mL (polypropylene), (round bottom, Stem Cell Technologies (#352063))

3.1.3. Reagents

3.1.3.1. Media and Buffers

α-Galactosylceramide (αGalCer, $C_{50}H_{99}NO_9$, Avanti Polar Lipids, #867000P, [1 mg/mL] in DMSO, stored at -20°C)

Ethyl alcohol (70%)

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 ($C_{41}H_{72}O_9$, 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, $C_{36}H_{56}O_8$, 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)

3.1.3.2. Antibodies

Specificity	Clone	Species	Isotype	Conjugate	Ordering number	Company
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	6D5	Rat	IgG2a, κ	BV510	115546	BioLegend
CD19	6D5	Rat	IgG2a, κ	BV570	115535	BioLegend
CD19	6D5	Rat	IgG2a, κ	Pacific Blue	115523	BioLegend
CD1d/α-GalCer tetramer	N/A	Mouse	N/A	BV421	N/A	NIH tetramer core facility
CD1d/α-GalCer tetramer	N/A	Mouse	N/A	PE	N/A	NIH tetramer core facility
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
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

CD150/SLAM	TC15-12F12.2	Rat	Rat IgG2a, alpha	BV785	115937	Biolegend
CD278	15F9	Armenian Hamster	IgG	PE	2-9940-81	ThermoFisher/eBio
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
GATA3	L50-823	Mouse	IgG1, κ	BV711	565449	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
IL-10	JES5-16E3	Rat	IgG2b, κ	APC	505010	BioLegend
IL-10	JES5-16E3	Rat	IgG2b, κ	PE-Dazzle 594	505034	BioLegend
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
RORγt	Q31-378	Mouse	IgG2a, k	PE-CF594	562684	BD Biosciences
RORγt	Q31-378	Mouse	IgG2a, κ	BV650	564722	BD Biosciences

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
TNF	MP6-XT22	Rat	IgG1, κ	FITC	554418	BD Biosciences
TNF	MP6-XT22	Rat	IgG1, κ	BV711	506349	BioLegend

Table 1. The list of the antibodies used in this study.

3.1.3.3. Probes

Gene	Gene ID	Catalogue Number	Company
β-Actin	Mm00607939_s1	4448484	Thermo Fisher Scientific
CEBPα	Mm00514283_s1	4453320	Thermo Fisher Scientific
E4BP4	Mm00600292_s1	4453320	Thermo Fisher Scientific
c-MAF	Mm02581355_s1	4453320	Thermo Fisher Scientific
PLZF	Mm01176868_m1	4453320	Thermo Fisher Scientific
IL-21	Mm00517640_m1	4453320	Thermo Fisher Scientific

Table 2. The list of probes used for this study. TaqMan Gene Expression Cells-to-CT™ (catalogue number (AM1728) was used for the analysis of the expression of the indicated genes.

3.2. Methods

3.2.1. Mouse procedures

Materials

- 1x PBS
- Adipose tissue buffer: 1x PBS, 10μM P2x7 receptor inhibitor, 2%BSA
- Centrifuge
- CO2 gas chamber
- Collagenase II
- Dissection stage
- Eppendorf tubes
- $EtOH_2$ (70%)
- Injection needles (1mL)
- Isoflurane chamber
- Laminar flow
- Mice (C57BL/6J: WT and CD45.1)
- Operating scissors
- Operating tweezers
- Petri dish
- Shaker
- Sterile cotton
- α–GalCer

3.2.1.1. Mouse handling and euthanasia

Method

All the mice used in this study were bred in the vivarium of the Izmir Biomedicine and Genome Center. The mice were housed in single-ventialted cages under alternating 12 hours (h) light/dark cycles at 24ºC. Mice were sacrificed by either cervical dislocation or CO_2 asphyxiation and all organs and tissues were removed under sterile conditions. All procedures were performed according to the institutional animal ethical rules and with approval by the animal ethics committee.

For the euthanasia, the surface of the cages was sterilized with 70% EtOH. The cages and the CO_2 gas chamber were placed inside the laminar flow. The mice were gently put into the CO_2 gas chamber and were exposed to CO_2 for a few minutes. Euthanasia was verified by lack of reflexes following a toe pinch.

3.2.1.2. Tissue removal

Method

i. Spleen isolation

In order to isolate the spleen, the mice were placed onto a dissection stage. The fur of the mice was sprayed with 70% EtOH. With the previously sterilized and auto-cleaved operating scissors, the fur of the mice was cut open vertical along the center from the hip to the chest. The body cavity was opened to expose the spleen. Then, the spleen was removed from the connective and fat tissue. The spleen was placed into a 15 mL falcon tubes containing 5 mL of 1x PBS and stored on ice.

ii. Thymus isolation

In order to isolate the thymus, the mice were placed onto a dissection stage. The fur of the mice was sprayed with 70% EtOH. With the previously sterilized and auto-cleaved operating scissors, the fur of the mice was cut open vertical along the center from the hip to the chest. Then the ribs were cut left and right from the sternum. The cut sternum was removed and, with the help of tweezers, the thymus was exposed. The white coloured thymus with two lobes located just behind the heart and beneath the opened rib, was removed using scissors. The thymus was placed into a 15 mL falcon tubes containing 5 mL of 1x PBS and stored on ice.

iii. Bone marrow isolation

In order to isolate the bone marrow, the mice were placed onto a dissection stage. The fur of the mice was sprayed with 70% EtOH. With the previously sterilized and auto-cleaved operating scissors, the hind legs were cut off just above the pelvic-hip join. Then the femur was exposed by cutting away the knee, the tibia, and by removing the muscles and residual tissues surrounding the femur. The femur was placed into a 15 mL falcon tubes containing 5 mL of 1x PBS and stored on ice.

iv. Adipose tissue isolation

In order to isolate the adipose tissues, the mice were placed onto a dissection stage. The fur of the mice was sprayed with 70% EtOH. There were two types of adipose tissues isolated for this study: subcutaneous white adipose tissue (scWAT) and visceral white adipose tissue (vWAT). When isolating the scWAT and vWAT, the bottom half of the mice was de-gloved using forceps. For the subcutaneous white adipose tissue, the lymph node present in the middle of the fat tissue was removed using tweezers and the scWAT was removed from the underlying skin. For the visceral white adipose tissue, the peritoneum was cut transversely using scissors. The visceral white adipose attached to either testes or ovarium was then removed using tweezers. The adipose tissues then were placed into a 15 mL of a falcon tube filled with 5 mL of 1x PBS containing 10 μM of P2X7 receptor inhibitor and 2% BSA and stored on ice.

3.2.1.3. In vivo injections

Method

Depending on the experiment, the mice were injected either intraperitoneally (i.p.) or intravenously (i.v.). For intravenous injection, the retro-orbital route was used as an alternative to the tail vein route. In all of the injections, α-GalCer was used as a stimulant and PBS for control groups. A 1 mL syringe was used to administer the solutions. The solutions inside the needles were warmed to room temperature to prevent injury to the animals. In order to remove the bubbles inside the solution, the syringe was held upright, tapped gently, and the air was slowly pushed out of the syringe until liquid was visible. The experimental and the control groups were marked by either using a marker on the tail or gently cutting an ear of the animal.

Intraperitoneal injection (i.p.)

The cages were initially sprayed with EtOH. The mice were placed on top of a cage gently inside the laminar flow. Then, from the back, the neck of the mouse was held tight between index finger and thumb. The feet of the mice were pushed to the lower right quadrant of the abdomen and the point where the feet touched the abdomen was determined as the injection point. The needle was inserted upwards pointing at a 30-40^{o} angle. The solution then was administered and the needle was pulled straight out. After the i.p. injection, the mice were put back into the cages inside the laminar flow.

Intravenous injection (i.v.)

Similar to the i.p. injections, the laminar flow and the cages were sprayed with 70% EtOH for i.v. injections. Unlike i.p injection, retro-orbital injection requires anaesthesia. The mice were transferred from the cages into the anaesthesia chamber. Anaesthesia was induced with 5% isoflurane in O_2, followed by 2-3% isoflurane to maintained anaesthesia. Anaesthesia was confirmed by a lack of reflexes following a toe pinch. By gently pressuring the top and the bottom areas covering the eye, the eye ball of the animal was exposed for the injection. The needle was carefully introduced to the medial canthus at an angle of 30^{o}. The solution was administered and the needle removed. In case of a small drop of blood observed upon injection, it was cleaned gently with a sterile cotton. The mice then were placed back to the cages inside the laminar flow.

3.2.2. Cells procedures

Materials

- 1x PBS
- 40μM cell strainer
- 70μM cell strainer
- 96V-bottom well plate
- Adipose tissue buffer: 1x PBS, 10μM P2x7 receptor inhibitor, 2%BSA
- Antibodies
- Cells
- Centrifuge
- Collagenase II
- Complete medium: (RPMI-1640 medium, 5% (vol/vol) heat-inactivated FBS, 100 U/mL penicillin-streptomycin, 0.05% (vol/vol) 2-Mercaptoethanol, 4 μM L-glutamine)
- FACS buffer: (PBS, 5% (vol/vol) heat-inactivated, 0.1% NaN_3)
- FACS tubes
- Forceps
- Glass Pasteur pipettes
- Lymphoprep solution
- Scissors
- Shaker
- Tweezers
- GolgiPlug (Brefeldin A)
- GolgiStop (monensin)

3.2.2.1. Generation of single cell suspensions

Method

i. Spleen

For spleen homogenization, a 70 µM cell strainer was placed on the top of an open 50 mL falcon tube. The spleen was placed onto the strainer with the help of tweezers. Using a syringe plumber, the spleen was meshed through the strainer along with 1x PBS. Then 1x PBS was added up to 20 mL. The solution was centrifuged at 400 rcf at 4°C for 6 minutes and the single cell suspension was obtained by re-suspending the pellets in 5 mL of 1x PBS.

ii. Thymus

For thymus homogenization, 70 µM cell strainer was placed on the top of an open 50 mL falcon tube. Then the thymus was placed onto the strainer with the help of tweezers. Using a syringe plumber, the thymus was meshed through the strainer along with 1x PBS. Then 1x PBS was added up to 20 mL. The solution was centrifuged at 400 rcf at 4°C for 6 minutes and the single cell suspension was obtained by re-suspending the pellets in 5 mL of 1x PBS.

iii. Bone marrow

The femur was placed into a petri dish containing 3 mL of 1x PBS and was cut open at both ends using scissors. Then using a 5 mL syringe with a XXX needle, 5 mL of ice-cold 1x PBS was used to flush the bone marrow out into a 70 µM cell strainer that was placed on an open 50 mL falcon tube. Using a syringe plumber, the bone marrow was meshed through the strainer along with 1x PBS. Then 1x PBS was added up to 20 mL. The solution was centrifuged at 400 rcf at 4°C for 6 minutes and the single cell suspension was obtained by re-suspending the pellets in 5 mL of 1x PBS.

iv. Adipose tissues

The adipose tissue was placed onto a petri dish and cut into small pieces with scissors. The fine pieces of the adipose tissue were then placed into a 15 mL falcon tube containing 1x PBS and 1 mg/mL collagenase II. The adipose tissue was incubated at 37°C at 200 rpm using shaker for 40 minutes. After the incubation the solution was passed through a 70 µM cell strainer into a 50 mL falcon tube using a syringe plumber. Then 1x PBS was added up to 20 mL. The solution was centrifuged at 400 rcf at 4°C for 6 minutes and the single cell suspension was obtained by re-suspending the pellets in 5 mL of 1x PBS.

3.2.2.2. Antibody staining for flow cytometry

Method

Cells were either stained with antibodies against surface or intracellular antigens. Lymphoprep separation was used to eliminate dead cells as it was shown that it improves cytokine detection (Sag, et al., 2017). All of the washing steps were performed by centrifugation at 400 rcf at 4°C for either falcon tubes or 96V-bottom well plate, at 5 minutes or 4 minutes, respectively.

Lymphoprep density gradient

After single cell suspensions were obtained, the cells were re-suspended in 2 mL of complete medium. The cells then were transferred into 5 mL round bottom FACS tube. Using a glass Pasteur pipette, 1.5mL of Lymphoprep solution was slowly added to the bottom of the FACS tube to generate a density gradient with the cell solution at the top and the Lymphoprep solution at the bottom. The tubes were then centrifuged at 500 rcf and RT for 30 minutes without acceleration or break. The interphase containing lymphocytes were gently recovered using a 1 mL pipette. The cells were washed twice with 1x PBS and were transferred to 96-well V-bottom plate to be stained.

Surface staining

After the cells were isolated via the Lymphoprep density gradient and transferred into 96-well V-bottom plates, the plates were centrifuged at 400 rcf, 4^{o}C for four minutes. The pellets were re-suspended in 50 μL of 1x PBS containing Zombie-reagent for live and dead discrimination and incubated for 10 minutes on ice in the dark. The cells were washed twice with 1x PBS. The pellets were then re-suspended in 50 μl of master mix containing FACS buffer, unconjugated IgGs (1:1 mix of mouse IgG and rat IgG), and FcR-block as well as relevant antibodies for surface staining. The cells were incubated for 30 minutes on ice in the dark. The cells were then washed once with FACS buffer and 1x PBS. Finally, 200 μL of the stained cell solutions were added to the FACS tubes through 40 μM strainers to remove clumps and measured by flow cytometry.

Intracellular staining

If the experiment involved an analysis of a cytokine or transcription factor, a second staining step was included to access the intracellular targets. After the surface staining, the cells were pelleted and 150 μL of Cytofix/CytoPerm solution was added to the cells on ice in the dark and incubated for 10 minutes. The cells were washed twice with 1xPBS.

To stain cytokines, the cells were washed with Perm/Wash buffer and centrifuged at 500 rcf at 4^{o}C for 4 minutes. Then intracellular cytokine staining (ICCS) cocktail containing unconjugated IgGs (1:1 mix of 10μg/mL mouse and rat IgGs) the target antibodies for cytokine of interest, was added to the cells and incubated for 30 minutes on ice and dark. Then, the cells were washed with Perm/Wash buffer and re-suspended in 150 μL of Perm/Wash buffer. The cells then were incubated for 5 minutes on ice to allow antibodies to diffuse out of the cells. The cells were washed once with Perm/Wash buffer and with FACS buffer at 500 rcf at 4^{o}C for 4 minutes and 200 μL of the stained cell solutions were added to the FACS tubes through 40μM strainers to remove clumps and measured by flow cytometry.

To stain transcription factors, 150 μL of transcription factor fix/perm buffer was added to the cell pellet. The cells were incubated at 4°C in the dark for 75 minutes and then washed twice with TF Perm/Wash buffer. The cells were stained with ICCS master mix containing TF Perm/Wash buffer, unconjugated IgGs (1:1 mix of 10μg/mL mouse and rat IgGs), and the relevant intracellular antibodies. After the ICS master mix was added, the cells were incubated overnight at 4°C in the dark. After the intracellular staining, the cells were washed once with TF Perm/Wash and they were re-suspended in 150 μL of TF Perm/Wash and incubated on ice in the dark for 5 minutes to allow unbound antibodies to diffuse out of the cells. Eventually, the cells were washed once with TF Perm/Wash buffer and FACS buffer and re-suspended in 200μL FACS buffer. Then, the stained cell solutions were added to the FACS tubes through 40μM strainers to remove clumps and measured by flow cytometry.

3.2.2.3. In vitro stimulation of iNKT cells

Method

After the single cell suspensions were obtained, the cells were re-suspended in 500 μL of complete medium and transferred to a flat-bottom 24-well plate. GolgiPlug (containing Brefeldin A, final concentration 0.5 μL/mL) and GolgiStop (containing monensin, final concentration 0.33 μL/mL) were added to the medium to accumulate cytokine containing vesicles. The cells were either stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) or with αGalCer (100 ng/mL) for four or five hours at 37°C.

3.2.3. Fluorescence activated cell sorting (FACS)

Materials

- 1x PBS
- 40 μM cell strainer
- 5mL polystyrene round-bottom tube
- Ack lysis buffer
- Antibodies
- Cells
- Centrifuge
- EasySep™ Mouse PE Positive Selection Kit II: (RapidSpheres™, selection cocktail)
- FACS AriaIII
- FACS tubes
- Magnets
- Recommended medium: (PBS, 2% FBS, 1mM EDTA)
- RMPI-1640 medium
- Turk solution

iNKT cell sorting

Method

Single cell suspensions were generated from the acquired tissues. To eliminate erythrocytes, cells were incubated for 5 minutes on ice using 1 mL of Ack lysis buffer. Cells were washed with ice cold 1x PBS. Single cell suspensions were then enriched using EasySep Mouse PE Positive Selection Kit II.

Magnetic enrichment of iNKT cells

After cell suspensions were obtained, cells were counted using a hemocytometer. 10 μL of Turk solution and 10 μL of the cell suspension were mixed, added to the hemocytometer and the cells were counted with a light microscope. Cells were re-suspended at a concentration of $2x10^8$ cells/mL in complete medium and transferred into 5 mL polystyrene round-bottom tube. To prevent unspecific binding of the antibodies, FcR block was added at 10 μL/mL. As a PE-conjugated reagent, CD1d/PBS57-tetramers were used at 1:800 concentration. Cells were then incubated for 30 minutes on ice in the dark. After incubation, PBS was added as 10-fold excess and the cells suspension was centrifuged. Cells were re-suspended with the initial starting volume. Selection cocktail was added at 100 μL/mL concentration and the cells were incubated on ice in the dark for 15 minutes. To evenly disperse the RapidSpheres, they were vortexed for 30 seconds and then added to the sample at 50 μL/mL concentration. Cells were incubated on ice in the dark for 10 minutes. Recommended medium was added to the sample to reach a final volume of 2.5 mL. Then the tube was placed into the magnet without lids and were incubated for 5 minutes on ice. Afterwards, the supernatant was discarded without taking the tubes out of the magnet and 2.5 mL of recommended medium was added to the samples again for further incubation. This step was repeated three times. Then the cells were washed once with the recommended medium and once with 1x PBS.

After the *i*NKT cells were enriched via this magnetic enrichment, the cells were stained using surface antibodies including Zombie, CD45R, CD8α, CD4, and CD3ε. After surface staining the cells were re-suspended in 200 μL of complete RMPI-1640 medium and collection tubes containing 500 μL of RPMI-1640 were prepared to collect the cells. Then the cells were sorted using a FACSAria III.

3.2.4. Quantitative PCR (qPCR)

C57BL/6J background mice were used for all the qPCR experiments. As the relative frequency of *i*NKT cells is low, the TaqMan Gene Expression Cells-to-CT™ Kit was used to obtain cDNA directly from the cell lysates, instead of the regular RNA isolation. An ABI 7500 FAST machine was used to run the qPCR reactions.

Materials

- 1mL Eppendorf tubes
- 1x PBS
- 20x RT enzyme mix
- 20x TaqMan Gene Expression Assay
- 2x RT buffer
- 2x TaqMan Gene Expression Master Mix
- 96-well qPCR plates
- Applied Biosystems 7500 Real-Time PCR System
- cDNAs
- Cells
- Centrifuge
- DNase I
- Eppendorf Centrifuge
- FACS tubes
- Lysis solution
- Nuclease-free water
- PCR tubes
- Stop solution
- Thermal cycler

3.2.4.1. Reverse transcription (RT) reaction

Method

After the cells were sorted via FACS, the cells were re-suspended in 200 μL of PBS and centrifuged at 400 rcf for 5 minutes. The remaining pellet was re-suspended in 100 μL PBS and transferred to 1 mL Eppendorf tube. After centrifugation and discarding the supernatant, the cells were lysed with 50 μL of Lysis solution containing 100x DNase I. The cells were pipetted for five times and incubated for 5 minutes at RT. Then, 5 μL of Stop solution was added to the lysis mixture and incubated for 2 minutes at RT. After the lysate was obtained, 10 μL of it was taken into separate PCR tubes that contained 40 μL of RT master mix (see **Table 3** for details). The final solution was then subjected to RT thermal cycler program. Using a thermal cycler, the mixture was incubated at 37°C for 60 minutes, then at 95°C for 5 minutes to inactivate the RT enzyme (see **Table 4** for details). After the cDNAs were obtained, the cDNAs were diluted in 1:2 ratio with nuclease free water and stored at -20°C.

Component	**Volume for each reaction**
2x RT Buffer	25 μL
20x RT Enzyme Mix	2.5 μL
Nuclease-free Water	12.5 μL
Final Volume RT Master Mix	40 μL

Table 3. RT mixture components. The components of the RT master mix containing 2x RT buffer, 20x RT enzyme mix, and nuclease-free water are indicated in volumes on the table. RT master mix was prepared and used to apply reverse transcription reaction for the cells to obtain cDNAs.

	Stage	**Reps**	**Temp**	**Time**
Reverse Transcription (hold)	1	1	37°C	60 min
RT reaction (hold)	2	1	95°C	5 min
Hold	3	1	4°C	indefinite

Table 4. Thermal cycler setup. Using a thermal cycler, the lysates were incubated at 37°C for one hour, then the enzymes for reverse transcription reaction were inactivated at 95°C for 5 minutes. After the reaction was complete, the cDNAs were waited at 4°C on hold until they were stored at -20°C. Each reaction was repeated for one time.

3.2.4.2. qPCR reaction

Method

Previously obtained cDNAs and the reagents including the aliquots of TaqMan probes were taken out of -20°C and unfrozen at RT, and covered with aluminium foil to avoid light exposure. Meanwhile, the PCR cocktail for the qPCR reaction was prepared for each reaction. Based on the number of the samples, the master mix was prepared, containing 2x TaqMan Gene Expression Master Mix and nuclease free water (see **Table 5** for details). The mixture was added into 96-well PCR plates on ice and 1 μL of the desired TaqMan probes was added to the mixture. Finally, 4 μL of the cDNA solutuion was added. The 96-well plate was centrifuged at 200 rcf for 30 seconds and placed into a Applied Biosystems 7500 Real-Time PCR System to run the qPCR reaction (see **Table 6** for details).

Component	**Volume for each reaction**
2x TaqMan Gene Expression Master Mix	10 μL
20x TaqMan Gene Expression Assay (Target gene)	1 μL
20x TaqMan Gene Expression Assay (Endogenous control gene)	1 μL
Nuclease-free water	4 μL
cDNA	4 μL
Final volume PCR cocktail	20 μL

Table 5. qPCR cocktail components. The final volume of PCR cocktails contains 2x TaqMan gene expression master mix, 20x TaqMan gene expression assay for both the target gene and endogenous control gene, nuclease-free water, and cDNA. The preparations were made on ice to keep the enzymes inactivate, and cDNAs were added the last. TaqMan Gene Expression Master Mix contains ROX™ passive reference dye. FAM or VIC were used for the target gene and endogenous control gene, respectively.

	Stage	Reps	Temp	Time
UDG incubation (hold)	1	1	50°C	2 min
Enzyme activation	2	1	95°C	10 min
PCR (cycle)	3	40	95°C	15 sec
			60°C	1 min

Table 6. qPCR setup. The qPCR reaction was divided into three stages. To eliminate any previous PCR amplifications, mis-priming, or nonspecific products, the cDNAs were first incubated at 50°C for 2 minutes. Then the enzyme for qPCR reaction was activated by incubation at 95°C for 10 minutes. For gene amplifications, the cDNAs then cycled for 40 times by incubating the cDNAs at 95°C for 15 seconds and at 60°C for a minute, repeatedly.

3.2.5. Statistical analysis

Materials

- Microsoft Office Professional Plus 2016
- GraphPad Prism 8.0.1

Method

For the *in vitro* experiments, paired-t test was used to statistically compare the control and the stimulated *i*NKT cells. For the *in vivo* experiments, unpaired-t test was used to statistically compare the control and the stimulated *i*NKT cells. If there were more than two groups to compare, ANOVA, post-doc test was applied. Statistical significance was established by considering the *P* values less than 0.05. At least three biological replicates were used for statistical analysis.

4. RESULTS

4.1. The general gating strategy to identify *i*NKT cells

In order to analyse *i*NKT cells by flow cytometry, the following strategy was applied for the experiments performed in this thesis (see **Figure 3**). Using FlowJo v.10, the lymphocytes were identified via FSC-A and SSC-A to eliminate debris. Doublets were eliminated by excluding cells high for FSC-W (FSC-W vs. FSC-H) and SSC-W (SSC-W vs. SSC-H). After the compensation between the fluorescent channels was adjusted, dead cells were eliminated by gating of live cells, i.e. negative for the live/dead-marker Zombie. CD8α^+ and CD45R$^+$ cells were excluded to eliminate CD8α^+ T cells and B cells. To improve the gating and exclude cells with unspecific antibody-binding, cells with an unnatural high binding of the CD4 antibody were gated out. As *i*NKT cells express the memory marker CD44, this was used to further enrich for *i*NKT cells. Finally, CD1d/PBS57-tetramer$^+$-CD3ε^+ cells were gated and defined as *i*NKT cells.

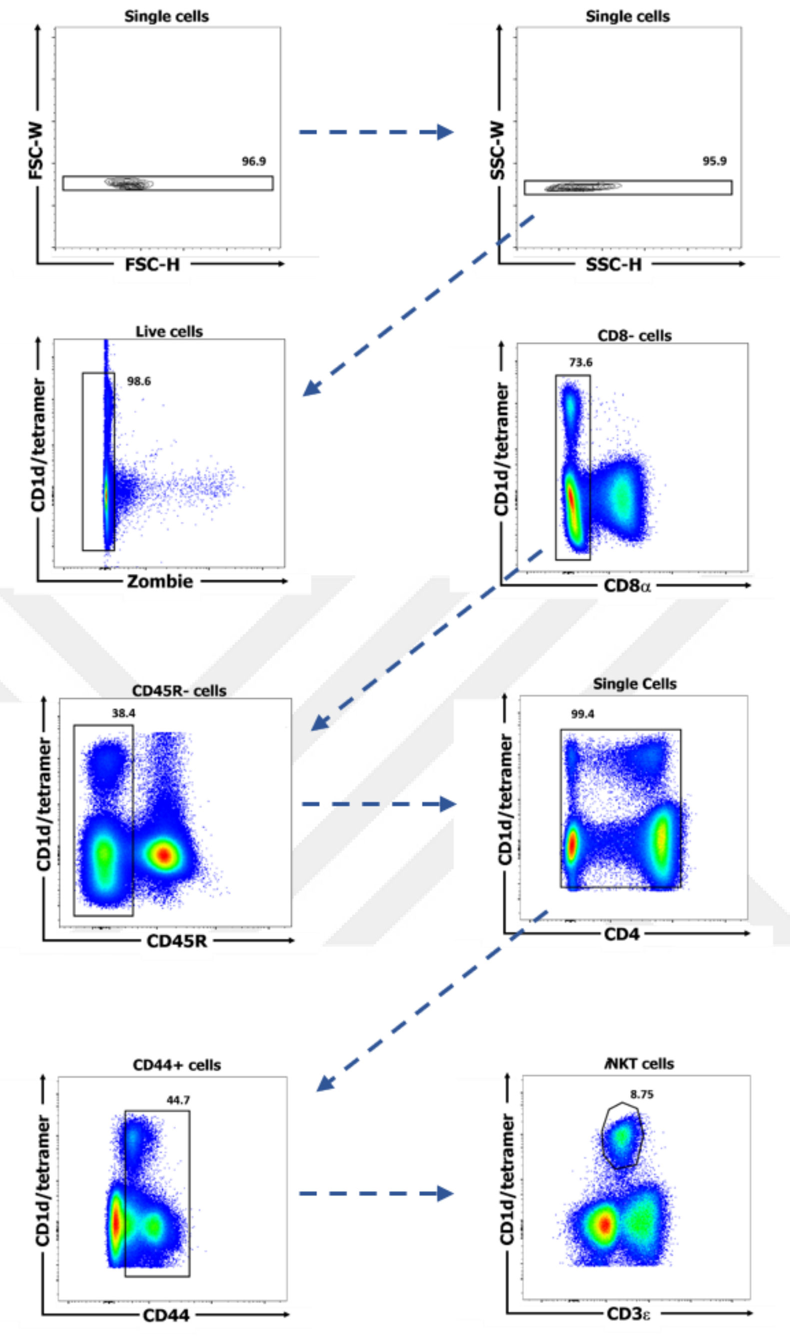

Figure 3. Representative gating strategy for identification of iNKT cells. Single cells and lymphocytes were identified based on forward scatter (FSC) and side scatter (SSC) features. Live cells were selected as Zombie negative, whilst CD8α negative and CD45R- negative populations were selected to eliminate CD8α + T cells and B cells, respectively. CD4 gating was used to eliminate cells binding antibodies unspecifically. CD44+ cells were selected prior to iNKT cell gating. Finally, CD1d/PBS57-tetramer^{+} and CD3ε+ cells were selected and identified as iNKT cells.

4.2. The transcription factors expressed by NKT10 cells

NKT10 cells are a recently identified subset of *i*NKT cells (Sag et al. 2014). Upon activation, they secrete copious amounts of IL-10, a strong anti-inflammatory cytokine. However, the transcription factor driving NKT10 cell functionality has not been defined yet. There are several transcription factors that are involved in the regulation of IL-10 production, such as CEBPA, E4BP4, and c-MAF (Hutchins, et al., 2013; Motomura, et al., 2011; Xu, et al. 2009).

NKT10 cells express surface proteins such as CD150/SLAM, CD278/ICOS, and SLAMf6 (Sag, et al., 2014). In order to understand if the pre-injected mice were actually NKT10 cells, we measured the expression of these markers in control and α-GalCer treated *i*NKT cells. As expected, all three surface markers were increased in NKT10 cells, confirming that NKT10 cells expanded in the mice injected i.v. four weeks earlier with a-GalCer (**Figure 4**).

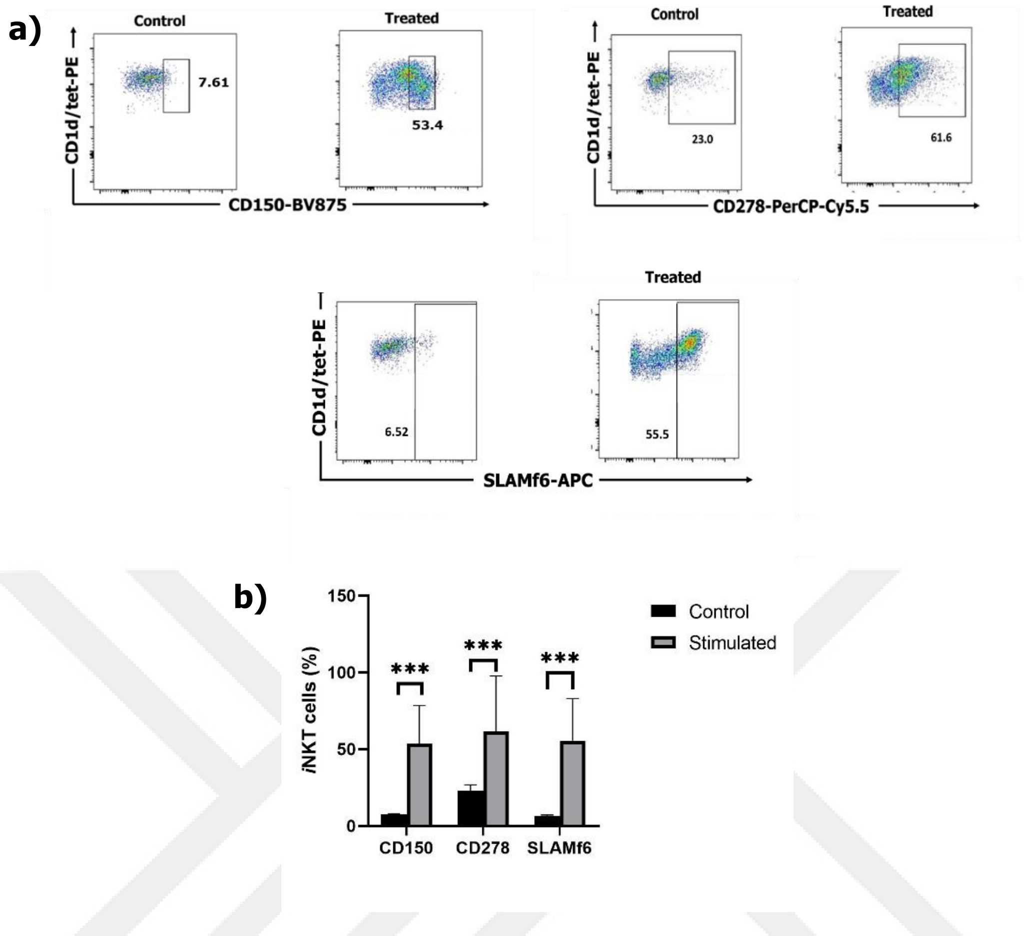

Figure 4. Flow cytometric analysis of CD150, CD278, and SLAMf6 expressions in splenic *i*NKT cells. Splenic *i*NKT cells from control and α-GalCer-pretreated (4μg four weeks earlier). **a)** Representative data for the expression of CD150, CD278, and SLAMf6 on *i*NKT cells from control (control) and αGalCer-pretreated (treated) mice. **b)** αGalCer-pretreated (grey) mice expressing the indicated marker. Representative data from 2 independent experiments are shown.

As the main cellular characteristics of NKT10 cells is the rapid secretion of high amounts of IL-10, we looked at the protein expression of CEBPA, E4BP4, and c-MAF by flow cytometry. We observed that the expression of CEBPα and E4BP4 was comparable high or low, respectively, in NKT10 and control *i*NKT cells (**Figure 5**). c-MAF expression, however, was increased in NKT10 cells compared to control *i*NKT cells (**Figure 5**).

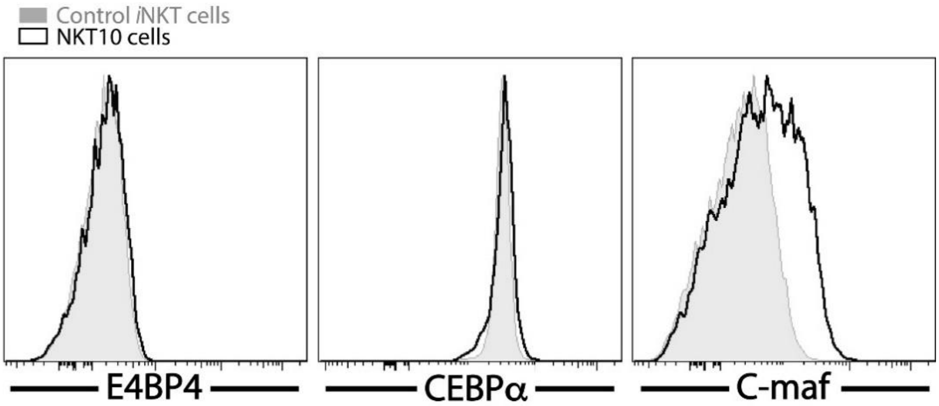

Figure 5. Flow cytometric analysis of E4BP4, CEBPα, and c-MAF expression in *i*NKT and NKT10 cells. C57BL/6 mice were i.v. injected with either PBS (200 μL) or α-GalCer (4 μg, 200 μL). 4 weeks later, *i*NKT cells were isolated from both control and preinjected mice and stained and identified as shown in **Figure 3**. Splenic control *i*NKT cells (tinted) and NKT10 cells (IL-10^{+} *i*NKT cells from α-GalCer-pretreated mice, black) were compared for the expression of the transcription factors E4BP4, CEBPα, and c-MAF as indicated. Representative data from 3 independent experiments are shown.

After the protein expression analysis of NKT10 cells, we next investigated the expression of CEBPα, E4BP4, c-MAF, and PLZF, as well as of IL-21 at mRNA level. To this end, the genes were analysed by quantitative PCR (qPCR) in splenic *i*NKTs cells from control mice and from mice pre-injected with a-GalCer to increase the frequency of NKT10 cells. Similar to our protein data, CEBPα was expressed in both NKT10 cells and control *i*NKT cells, whereas E4BP4 was either very low or absent in both groups. c-MAF expression increased in NKT10 cells, whereas it was either very low or absent in control *i*NKT cells (**Figure 6a**). We did not observe a change in the PLZF expression. There was a slight increase in the IL-21 gene expression in *i*NKT cells (**Figure 6b**) Sag, et al., previously showed that NKT10 cells are distinct from NKT_{FH} cells (Sag, et al., 2017), and the slight increase in the IL-21 expression likely indicates the presence of low numbers of NKT_{FH} cells remaining in the spleen 4 weeks after the initial αGalCer injection. Our results suggest that c-MAF is specifically expressed in NKT10 cells, but not in control *i*NKT cells, both protein and mRNA level.

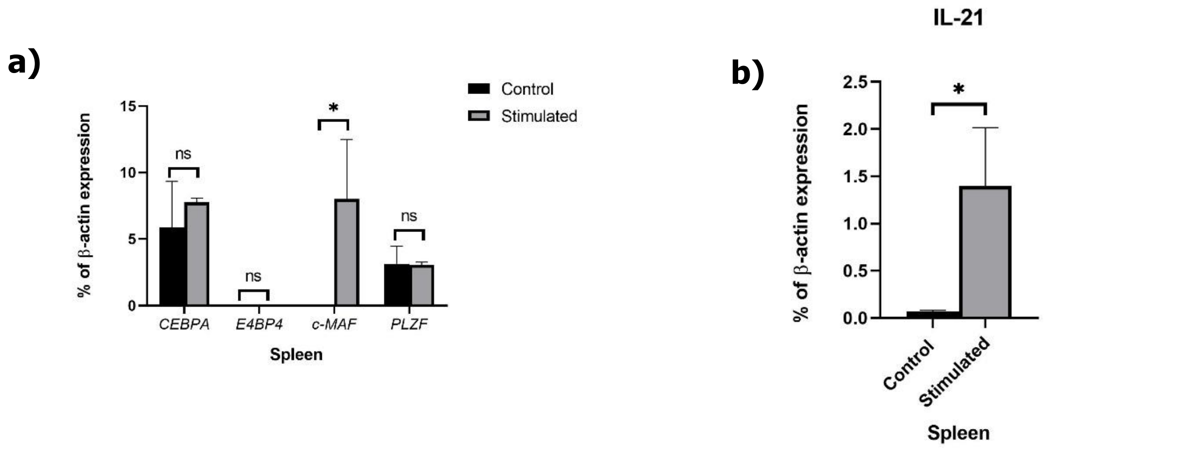

Figure 6. qPCR analysis of splenic iNKT cells. iNKT cells from control (black) and α-GalCer-pretreated (1 month earlier, grey). **a)** CEBPα, E4BP4, c-Maf and PLZF expression in iNKT cells: C57BL/6 mice were i.v. injected with either PBS (Control) or with 4 μg/mouse α-Galcer. 4 weeks later, iNKT cells (IL-10$^+$ iNKT cells) were purified via FACS and their cDNA was analysed by qPCR for the expression of CEBPα, E4BP4, c-Maf, PLZF and **b)** IL-21 as indicated (n=3 for both **(a)** and **(b)**, $p < 0.05$). Representative data at least from 3 independent experiments.

After monitoring the expressions of the transcription factors, CEBPα, E4BP4, and, c-MAF at both protein and mRNA level in NKT10 cells, we next measured in vivo distribution of NKT10 cells. To this end, single cell suspension from bone marrow, liver, lung, spleen, thymus, subcutaneous white adipose tissue (scWAT), and visceral white adipose tissue (vWAT) from C57BL/6 mice were stimulated *in vitro* to induce IL-10 production. Although we could identify IL-10$^+$ iNKT cells in all organs analysed, their frequency was particular high in the vWAT and scWAT (**Figure 7**).

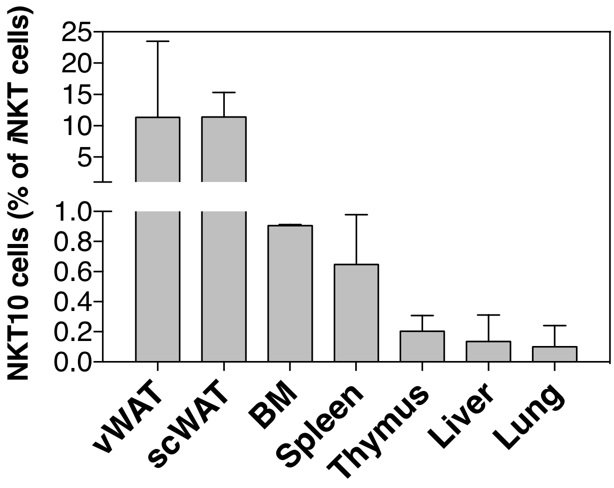

Figure 7. *In vivo* distribution of NKT10 cells. The cells from bone marrow, liver, lung, spleen, thymus, scWAT, and vWAT were isolated from C57BL/6 mice and they were stimulated *in vitro* for 4h with PMA (50 ng/mL) and ionomycin (500 ng/mL) in the presence of the Golgi-transport inhibitors Brefeldin A and monensin. The cells were stained for iNKT cells as shown in **Figure 3**. The summary graph shows he percentage NKT10 cells (IL-10$^+$ iNKT cells) within all iNKT cells in the indicated tissues (pooled data from 2-5 experiments per organ).

As NKT10 cells were the most frequent in vWAT and scWAT compared to other, we next monitored the mRNA expression of CEBPα, E4BP4, c-MAF, PLZF, and IL-21 in the adipose tissues of C57BL/6 control and aGalCer-pretreated (four weeks) mice. Similar to our qPCR results for splenic iNKT cells (Figure 6), CEBPα expression similar in both control and NKT10 cells (**Figure 8a**). E4BP4 expression was either low or absent in both groups, c-MAF expression increased in NKT10 cells whereas it was absent in the control iNKT cells, and PLZF expression was similar in both groups (**Figure 8a**). Unlike splenic iNKT cells, we did not observe any expression of IL-21 in the adipose tissues (**Figure 8b**) and this suggests that no NKT$_{FH}$ cells are present in the adipose tissues.

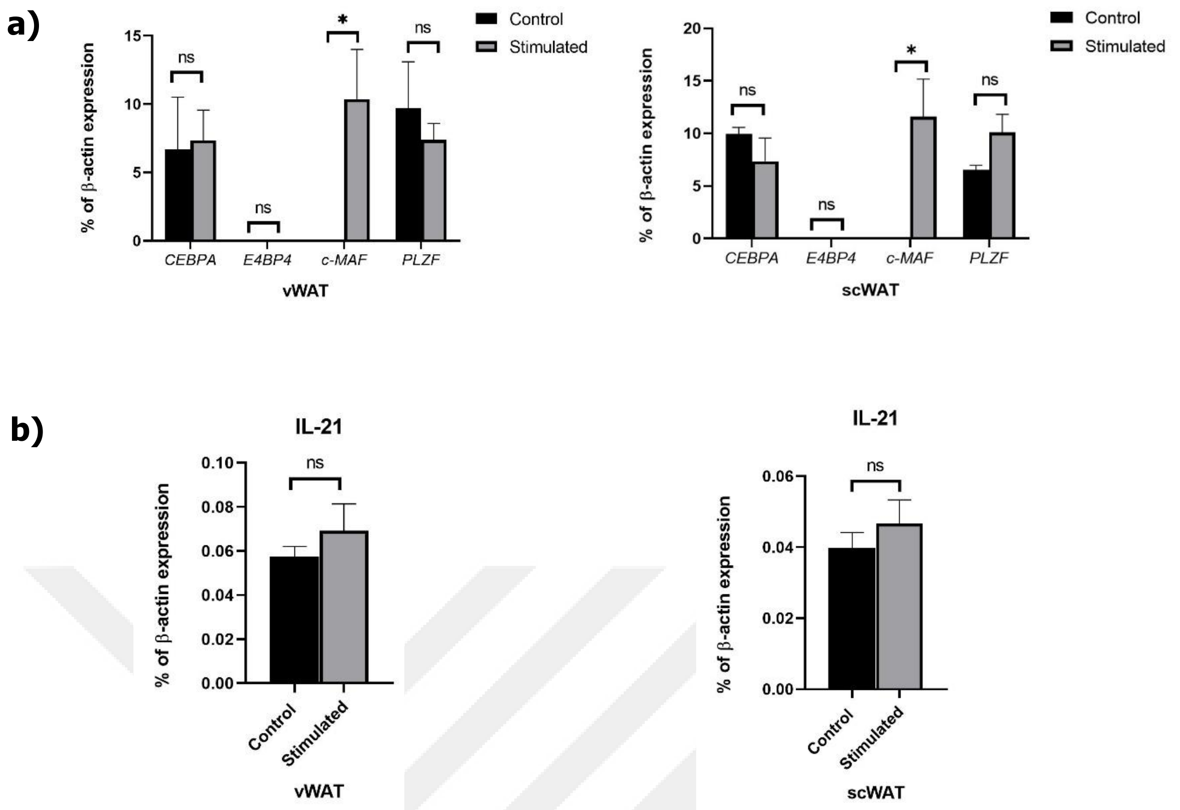

Figure 8. qPCR analysis of *i*NKT cells isolated from either vWAT or scWAT: *i*NKT cells from control (black) and α-GalCer-pretreated (1 month earlier, grey). **a)** CEBPα, E4BP4, c-Maf and PLZF expression in *i*NKT cells: C57BL/6 mice were i.v. injected with either PBS (Ctrl) or with 4 μg/mouse α-Galcer. 4 weeks later, *i*NKT cells were purified via FACS and their cDNA was analysed by qPCR for the expression of CEBPα, E4BP4, c-Maf, PLZF and **b)** IL-21 as indicated (n=3 for both **(a)** and **(b)**, $p < 0.05$). Representative data from two independent experiments.

4.3. The impact of route of injection on *i*NKT cell activation

Like conventional T cells, *i*NKT cells also express a TCR structure, however with an invariant Vα-chain (Bendelac, et al, 1995). Following activation in a TCR-dependent manner, *i*NKT express Nur77, which can, therefore, be used as an indicator for TCR-triggering (Moran, et al., 2011; Tuttle, et al., 2018). Although it was shown that the route of injection, for example i.p. or i.v., affects the in vivo distribution of the immune cells (Huck, et al., 2008), this has not been addressed for *i*NKT cells. Therefore, we monitored Nur77 expression in *i*NKT cells in the spleen of C57BL/6 mice after administrating α-GalCer by either i.p. or i.v. injections. We observed that there was an increase in Nur77 expression in splenic *i*NKT cells in both routes of injections (**Figure 9a**). Importantly, the difference between i.p. and i.v. injections, was not statistically significant (**Figure 9b**). Additionally, we also examined the surface expression of the activation marker CD69 on *i*NKT cells as an independent indication that the cells were indeed activated (**Figure 9c**).

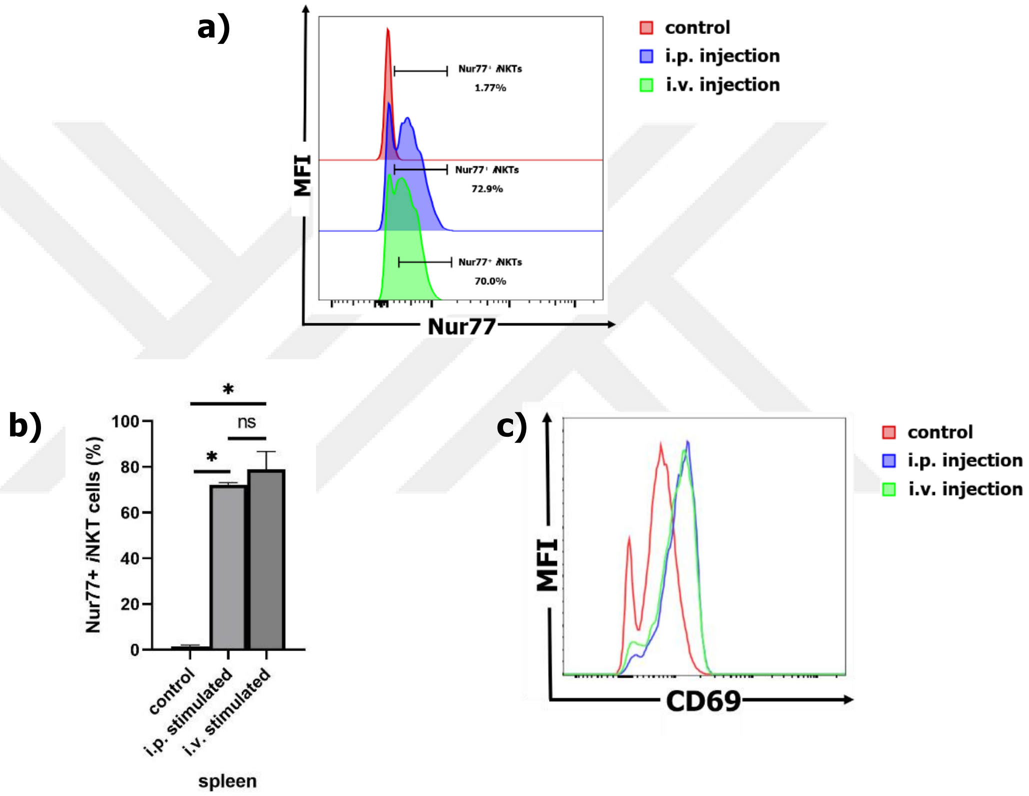

Figure 9. Flow cytometric analysis of Nur77 expression in splenic *i*NKT cells following different routes of α-GalCer injections. C57BL/6 mice (n=3) were either injected i.p. or i.v. with 2μg α-GalCer or were injected with PBS (control). Two hours later, splenocytes were isolated from the mice and *i*NKT cells were analysed as shown in Figure 3. **a)** Representative data and **b)** summary graph for the Nur77 expression in *i*NKT cells from control mice or mice with injected i.p. (i.p. stimulation) or i.v. (i.v. stimulation) with α-GalCer. **c)** Representative data for the CD69 expression on *i*NKT cells from indicated mice. Representative data from two independent experiments are shown.

After observing the expected in Nur77 expression in the splenic *i*NKT cells in the splenic *i*NKT cells, we next investigated its expression in *i*NKT cell subsets. NKT1 cells were gated as PLZF^{low}-RORγt^{-}; NKT2 cells were gated as PLZF^{high}-RORγt^{-}; and NKT17 cells were gated as PLZF^{int}-RORγt^{+} *i*NKT cells (**Figure 10a**). The frequency of Nur77^{+} NKT1 and NKT2 cells (**Figure 10b**) was comparable in mice injected with α-GalCer either i.p. or i.v. In contrast, the frequency of Nur77^{+}NKT17 cells was slightly higher in i.p. injected mice compared to i.v. injected mice. (**Figure 10b**).

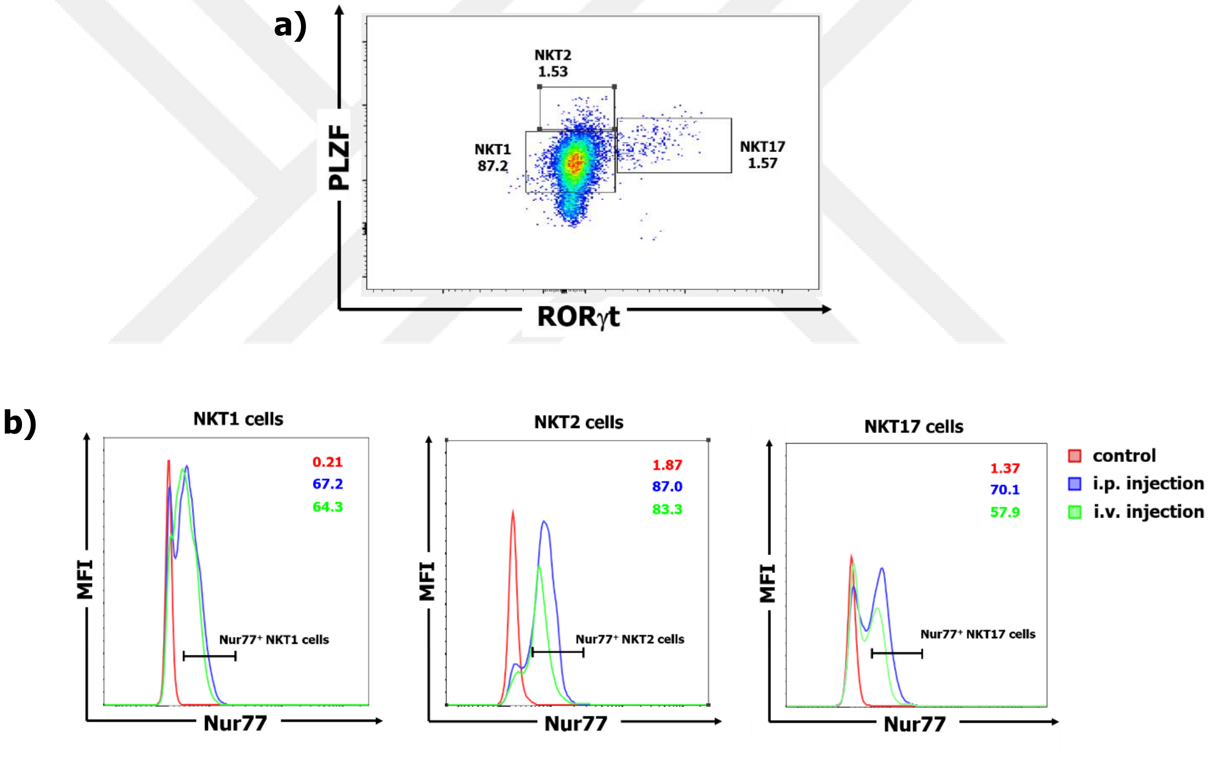

Figure 10. Flow cytometric analysis of Nur77 expression in splenic NKT1, NKT2, and NKT17 cells. C57BL/6 mice (n=3) were either injected i.p. or i.v. with 2μg α-GalCer or were injected with PBS (control). Two hours later, splenocytes were isolated from the mice and *i*NKT cells were analysed as shown in Figure 3. **a)** Representative dot plot for gating of the *i*NKT cell subsets NKT1, NKT2, and NKT17 cells. NKT1 cells were defined as PLZF^{low}-RORγt^{-}; NKT2 cells as PLZF^{low}-RORγt^{-}; and NKT17 cells as PLZF^{int}-RORγt^{+}. **b)** Representative histograms of the expression of Nur77 in NKT1, NKT2, and NKT17 cells from indicated mice. Preliminary data from one experiment are shown.

5. DISCUSSION

In this study, we investigated the transcription factors expressed by NKT10 cells at both protein and mRNA level. It was shown that NKT10 cells secrete high amounts of IL-10 upon activation (Sag et al., 2014). However, the transcription factor that drives NKT10 cell functionality remains unknown. From the transcription factors known to regulate IL-10 production in various immune cells, namely CEBPα, E4BP4, and c-MAF, we here show that c-MAF is the only one specifically expressed in NKT10 cells and not in control iNKT cells. This suggest that c-MAF could function as the potential master regulatory transcription factor of NKT10 cells.

Previously, it was suggested that E4BP4 (Nfil3) is the master regulatory transcription factor of IL-10 producing iNKT cells (Lynch et al., 2015). This conclusion was based on *in vitro* overexpression of E4BP4 in iNKT cell lines, leading increased IL-10 mRNA and protein production in these iNKT cells (Lynch et al., 2015). However, it is known that E4BP4 is involved in the regulation of IL-10 and, for example, it was shown that E4BP4 regulates the production of IL-10 in conventional $CD4^+$ T cells (Motomura et al., 2011). Therefore, the results from Lynch et al. (2015) only confirms that E4BP4 can drive IL-10 production, however, it does not demonstrate that E4BP4 is responsible for this under physiological conditions.

In contrast to Lynch et al. (2015), we did not observe E4BP4 expression in IL-10 producing iNKT cells, i.e. NKT10 cells, at either mRNA or protein level. Of the transcription factors CEBPα, E4BP4, and c-MAF, only c-MAF was increased at mRNA and protein level in NKT10 cells compared to control iNKT cells. Since it was shown that c-MAF is involved in the regulation of IL-10 production (Cao et al., 2005), we reason that c-MAF is likely be master transcription factor that drives NKT10 cell functionality.

Furthermore, our data indicate that iNKT cells in vWAT or scWAT express PLZF at both protein and mRNA level, which is also in contrast to Lynch et al. (2015). Our data are consistent with the fact that PLZF is an important transcription required for of iNKT cell survival and functionalities (Kovalovsky et al., 2008; Savage et al., 2008), as well as for lineage determination (Park, et al., 2019).

In addition to the analysis of the transcription factors in NKT10 cells, we also measured IL-21 production at mRNA level. Sag et al. (2014) previously showed that NKT10 cells are distinct from NKT_{FH} cells, which are prominent in mice six days after immunization with αGalCer (Chang et al., 2012). IL-21 is uniquely characteristic for NKT_{FH} cells among iNKT cell subsets (Chang et al., 2011). As NKT_{FH} cells largely disappear within 2-3 weeks following the αGalCer immunization (Sag et al., 2014), we expected that NKT_{FH} cells would be absent after four weeks. We observed only a small increase in IL-21 mRNA expression in splenic NKT10 cells and IL-21 expression was completely absent in iNKT cells from the adipose tissues. Our results are in line with the previous conclusion (Sag et al., 2014) that NKT10 cells and NKT_{FH} cells are functionally distinct iNKT cell subsets.

Although we showed that c-MAF might be the potential master transcription factor for NKT10 cells, there are further studies needed to strengthen this claim. For the next step, we prepared vectors for the transfection of the transcription factors CEBPα, E4BP4, and c-MAF into iNKT cell lines. We expect that all three transcription factors can drive IL-10 production when they are overexpressed. To demonstrate, however, which of the transcription factors is required for NKT10 cells under physiological conditions, we would need to analyse mice deficient for CEBPα, E4BP4, or c-MAF. We hypothesize that only the c-$MAF^{-/-}$ mice would show an impairment in IL-10 production by iNKT cells.

6. CONCLUSION

Invariant Natural Killer T (*i*NKT) cells are a distinct population of immune cells that share characteristics of both adaptive T cells and innate Natural Killer (NK) cells. They express a semi-invariant $\alpha\beta$ T-cell receptor (TCR) and several NK cell receptors. Upon activation, *i*NKT cells can rapidly secrete either T_h1 or T_h2 cytokines. There are five main *i*NKT cell subsets: NKT1, NKT2, NKT17, NKTFH, and NKT10 cells. NKT10 cells are characterized by the production of the anti-inflammatory cytokine IL-10. In this study, we showed that the transcription factor c-MAF is specifically expressed by NKT10 cells. Previous work from our laboratory showed that NKT10 cells were protective in a mouse model of multiple sclerosis, (Sag et al., 2014). Therefore, elucidating the genetic regulation of NKT10 cell function is an important step to utilize their power for immunotherapeutic interventions. Our findings point to an essential role of c-MAF for NKT10 functionality and, therefore, can lead novel solutions for the treatment of autoimmune diseases.

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8. ADDENDA

8.1. Animal Ethics Approval

T.C.
DOKUZ EYLUL UNIVERSITY

IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME INSTITUTE
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

IBG-İzmir
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T.C.
DOKUZ EYLÜL ÜNİVERSİTESİ
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.

Prof.Dr.Sedef AKGÜNGÖR
Başkan

Prof.Dr. H. Alper BAĞRIYANIK
Başkan Yardımcısı

Prof. Dr. Ensari GÜNELİ
Üye

Doç.Dr. H. Güneş ÖZHAN
Üye

Doç.Dr. Ralph Meuwissen
Üye
(Katılamamıştır)

Doç.Dr. Devrim PESEN OKVUR
Üye

Uzm. Umur KELEŞ
Üye

Uzm. Kerem ESMEN
Üye

Ecz. Ferdane KAHRAMAN
Üye
(Katılamamıştır)

8.2. Curriculum Vitae

Sezgin Bal
Izmir International Biomedicine and Genome Institute
Dokuz Eylul University Health Campus
35340 Balcova/Izmir, Turkey
Email: sezz.bal@gmail.com

Education

Izmir International Biomedicine and Genome Institute — Izmir, Turkey
Master of Science, Molecular Biology & Genetic — September 2017 – Present

Bogazici University — Istanbul, Turkey
Bachelor of Science, Chemistry — September 2011 – June 2016

Chalmers University of Technology — Gothenburg, Sweden
Erasmus, Viking History, Mathematics, Physics — January – June 2014

Research Experience

WINGENDER Group, Izmir International Biomedicine and Genome Institute, Izmir, Turkey
Master Student, September 2017 – Present

- Characterization of natural killer T 10 (NKT10) cells by identifying and investigating the transcription factors that are responsible for NKT10 cell functionality.
- Examining TCR downregulation in *i*NKT subsets upon in vitro stimulation with αGalCer.
- Application of different route of injections and monitoring its impact on *i*NKT cell subset activation.

COSBI, Koc University, Istanbul, Turkey
Visiting Student, September 2016 – February 2016

- Identification of Ras effector domains, effector domain mutations and its pathway selectivity using Pyhton.

AVIYENTE Group, Bogazici University, Istanbul, Turkey
Undergraduate Student, September 2015 – July 2016

- Modelling of organic molecules in transition states using Gaussian. Studying Diels-Alder reactions and their endo:exo ratio analysis.

DOBSON Group, University of Cambridge, Cambridge, UK
Summer Visitor, June 2013 – September 2013

- Examining human lysozyme (HuL) protein aggregation and *in vitro* amyloid formation. Seed fibril analysis and its kinetic impact on the fibril formation.

Teaching Experience

MBGE 110: Cell & Molecular Biology — February – May 2017
Koc University, Istanbul

MBGE 101: General Biology Lab — September 2016–January 2017
Koc University, Istanbul

Skills

Computer: Flowjo, Spartan, Gaussian, Python, NAMD, RNA-seq analysis (R, Galaxy)

Lab: Flow cytometry, qPCR, Cell culture, Mice Handling, Drosophila, Dynamic Light Scattering, Circular Dichroism

Language: English (Fluent), German (Upper-Intermediate), Swedish (Elementary), Ancient Greek (Written)

Scholarships

TUBITAK December 2017 – Present
Graduate Research

EMBO September 2017 – December 2017
Graduate Research

Turkish Education Foundation September 2012 – June 2015
Undergraduate Success

European Commission January 2014 – June 2014
Erasmus Program, Gothenburg, Sweden

European Commission June 2013 – September 2013
Erasmus Mobility Placement, Cambridge, UK

Memberships

Bogazici University, Erasmus Student Network February 2012 – June 2016
Guiding exchange and Erasmus students, and voluntarily working in the international relations office.

Bogazici University, Sports Committee September 2012 – June 2013
Guide at sportsfest organization

Gothenburg University, Handelshögskolan February – June 2014
HHGS

Workshops & Conferences

Uludag University April 2019
MIMIC IV, Poster Presentation

IBG, Izmir September 2018
6th International Molecular Biology Conference

Harvard University June 2018
Sabri Ulker Metabolism Conference, Travel Award

Hilton Izmir November 2017
IBG Days, Monoclonal Antibody Workshop

Hilton Izmir November 2017
IBG Days, New Frontiers in Science

References

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