Investigating the immunosuppressive microenvironment created by Acute Myeloid Leukaemia using invariant Natural Killer T cells as a novel therapeutic approach.

by VICTORIA STAVROU

A thesis submitted to the

University of Birmingham

for the DOCTOR OF PHILOSOPHY.

Institute of Immunology and Immunotherapy

University of Birmingham

July 2021

Acknowledgments:

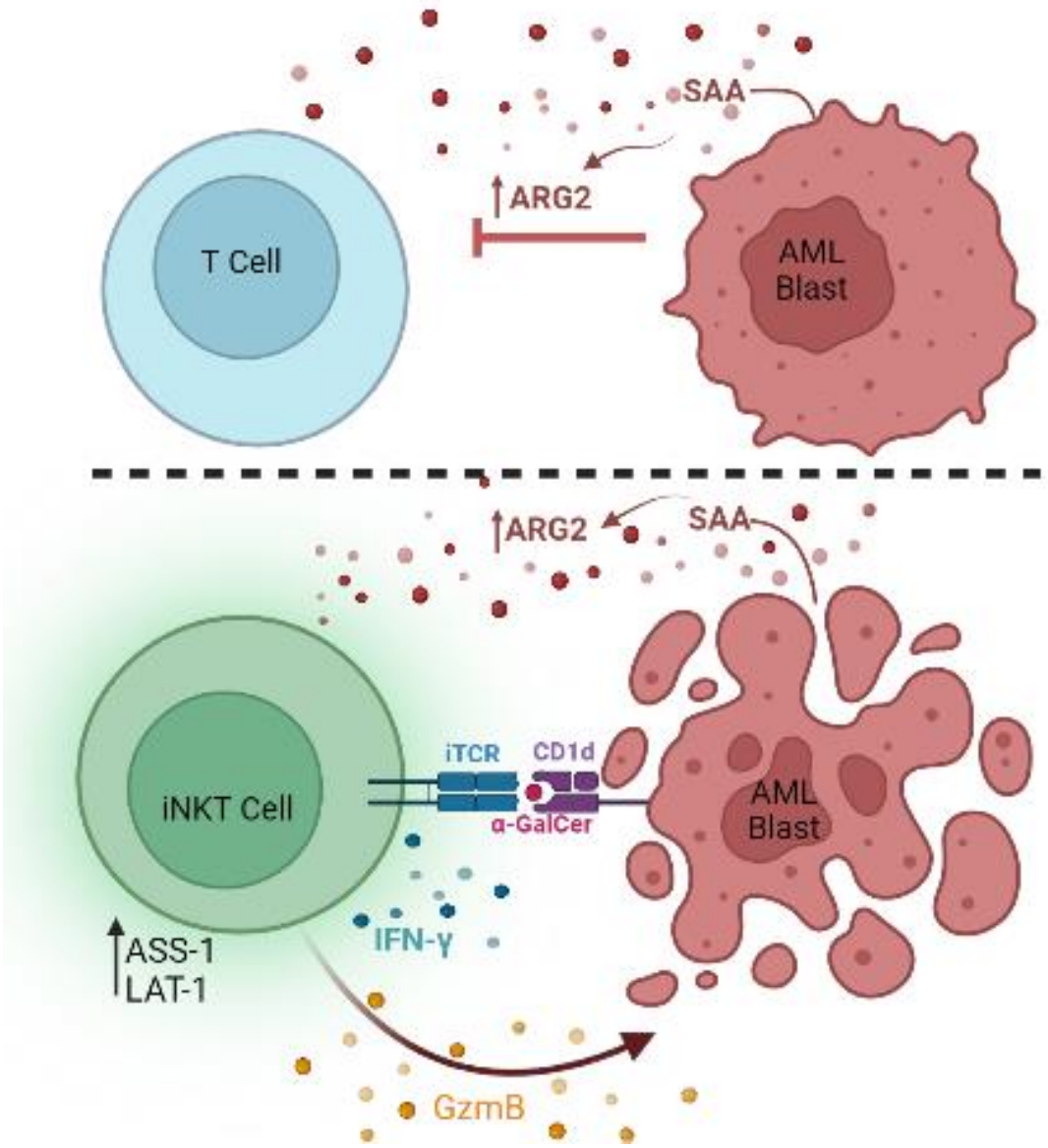
I am immensely grateful to my mentor, Dr Carmela De Santo for the academic guidance and emotional support that has upheld, powered and cheered me on through this challenging milestone. I consider myself lucky to have completed this journey under her supervision.

It is a great privilege to have been a part of the De Santo and Mussai team for the past years. A big thank you to both, for their generosity, compassion and knowledge. To Dr Francis Mussai, I express my appreciation for his diligence to continue to inspire and drive me to be my best academic self.

I also thank my colleagues at the University of Birmingham for always supporting me. They have been a source of resilience throughout this journey and I attribute my success to their academic knowledge, kindness and friendship. I will always treasure our banter and countless memories together. To Sarah, thank you for the unending motherly support. Special thanks to Stone for the earnest advice and commitment shown towards my work; his help has been immeasurable. To the endless hours spent in the lab with Luciana, I thank you for your help, the encouragement, compassion and knowledge driving me forward daily. Special thanks to Silvia for always having my back in organising and motivating me to do more. I am grateful to Dan for making my life easier; his work has been instrumental to my research. It has truly been a pleasure and honour to have worked with such amazing group; extended thanks to Ugo and Nikki. They will all be dearly missed.

Finally thank to my family, I am forever indented to the unconditional support and love you have shown me during my PhD journey and beyond. I dedicate this PhD to the loving memory of my late mother, Fodini Stavrou; you are the light guiding me forward. I thank you, for making me who I am today. To my father and my sister, thank you for being my rock. To my grandparents, I am beyond grateful for the support, love and generosity. To my friends Thekla and Ioli, I am grateful for your constant support and cheerleading me on.

Graphical abstract



Abstract

Acute myeloid leukaemia (AML) creates an immunosuppressive microenvironment to conventional T cells through Arginase 2 (ARG2) inducing arginine depletion. We identify that AML blasts release the acute phase protein serum amyloid A (SAA), which acts in an autocrine manner to upregulate ARG2 expression and activity and promote AML blast viability.

Invariant natural killer T cells (iNKT) are a distinct population of T-cells with the unique ability to bridge the innate and adaptive immune system by mediating a range of immune responses and play a key role in tumour immunosurveillance. In AML the low level of iNKT cells post stem cell transplant is associated with poor overall survival; however it is unclear if iNKT cells directly regulate the growth of malignant cells. We establish that iNKT cells can detect and interact with AML blasts in a CD1d/CD40 dependent manner. α-GalCer-loaded AML blasts induce potent iNKT cell activation resulting in release IFN-γ and initiating clonal expansion *in vivo* and *in vitro*. Activated iNKT cells can reduce tumour burden by directly killing AML blasts *via* granzyme B pathway inducing apoptosis.

Despite the low arginine AML microenvironment which is hostile to T cells, iNKT cells retain their ability to proliferate, become activated and elicit a cytotoxic response against AML due to the upregulation of LAT-1 and ASS-1 dependent amino acid pathways. Finally, activated iNKT cells can further restore T cell proliferation both *in vitro* and *in vivo*.

This illustrates that iNKT cells are key players in creating an anti-leukemic response, thus, stimulation of iNKT cell activity has the potential to be exploited as an immunotherapeutic approach against AML or as an adjunct to boost antigen-specific T cell immunotherapies in cancers.

Contents

Frequently used Abbreviations:

α-GalCer: α-Galactocylceramide

AML: Acute Myeloid Leukaemia

APCs: Antigen-presenting cells

ARG: Arginase

ASS-1: Argininosuccinate synthetase 1

ATRA: All-trans Retinoic Acid

DCs: Dendritic Cells

ECAR: Extracellular Acidification Rate

ELISA: Enzyme-linked Immunoassay

FACS: Fluorescence-activated Cell Sorting

FPR2: Formyl-peptide receptor-2

HSCT: Hematopoietic Stem Cell Transplantation

IFN-γ: Interferon-gamma

mAb: Monoclonal Antibody

MACS: Magnetic-activated Cell Sorting

MDSC: Myeloid-derived Suppressor Cells

OCR: Oxygen Consumption Rate

PI: Propidium iodide

SAA: Serum Amyloid A

E:T ratio: Effector-to-Target ratio

TCR: T-cell receptor

TLR: Toll-like Receptor

TME: Tumour microenvironment

Chapter 1-Introduction

Leukaemia

Leukaemias are a diverse and heterogenous group of haematological disorders identified by the abnormal proliferation and development of leukocytes triggered by chromosomal abnormalities or gene mutations. Leukaemias are classified as either acute or chronic depending on the level of cell differentiation and maturation from the originating haematopoietic precursor cells. They are further sub classified based on the myeloid or lymphoid lineage cell from which they arise. The precise cause for the majority of leukaemias is as of yet unknown. However, numerous genetic and environmental risk factors have been described [1-3]. Recently, there have been several advances in our understanding of the genetic aberrations in leukaemia, such as the presence of the oncogenic mixed lineage leukaemia (MLL) gene and fusion proteins which drive leukemic transformation and disease progression [4]. Despite such advances the five-year-survival rates from some leukaemias such as acute myeloid leukaemia (AML) remain poor.

Acute Myeloid Leukaemia

Tumour Biology

AML targets both children and adults. It is the most common acute types of leukaemia in adults, representing approximately 80% of the cases in the United States [5, 6]. It is an age-associated disease, with incidence rates increase gradually from age 40-44, and rises sharply from age 60-64, with the highest prevalence in the 85-89 age group in males, and ≥90 in females [7]. Elderly patients and those over 65 contribute to most newly diagnosed cases. 70% of patients aged 65 and older will succumb to their disease and die within a year of diagnosis [8]. AML accounts for ~20% of paediatric leukaemias and is the leading cause of deaths from haematological malignancies in children [5]. Weight loss, anorexia and fatigue are all common symptoms in AML patients. Additionally, anaemia, thrombocytopenia and leucocytosis are common in these patients due to bone marrow failure [9] .

AML is heterogeneous set of diseases which manifest initially in the bone marrow (BM) by the malignant accumulation and expansion of poorly differentiated haematopoietic precursors resulting in the formation of AML blasts (myeloblasts). AML can progress rapidly and if not treated it can be fatal within months or even weeks [1, 6, 10].

Aetiology

The aetiology of AML is multifactorial. AML can develop in patients with an underlying haematological disorder. Transformation to AML from a prior clonal illness such as myelodysplastic /myeloproliferative neoplasms (MDS/MPN) is described as secondary AML (sAML)[11]. AML can also occur as a consequence to prior treatment with cytotoxic agents (such as, exposure to radiation, alkylating agents or topoisomerases II) referred to as therapy-related

d AML [12]. Nevertheless, most cases appear as a *de novo* malignancy in previously healthy individuals. Congenital abnormalities and environmental factors are the main drivers in AML predisposition. Children with down syndrome have a higher probability (10-to-20-fold increase) in developing acute leukaemia [13] . Additional inherited diseases connected with AML are: Fanconi anaemia, Klinefelter syndrome, Li-Fraumeni syndrome and neurofibromatosis [14]. Environmental Factors such as ionizing radiation exposure have also been associated to AML. An increased in AML incidence was observed between the survivors following the atomic bomb explosions in Japan, with a peak after 5 to 7 years of exposure [15]. It has been observed that chronic exposure to particular chemicals such as benzene appear to have a higher risk for developing AML [16]. Also, people in contact to herbicides, embalming fluids and ethylene oxides are more susceptible to developing AML. Finally, smoking has been considered as a high-risk factor in developing AML, specifically French-American British (FAB) subtype M2 (see below for a brief review of subtypes) in people over 60 [17].

Cytogenetics

It has long been established that chromosomal abnormalities such as translocations, insertions, deletions, inversions, aneuploidies are present in 52% of AML cases and function as critical markers used in AML diagnosis and as indicators of prognosis [18, 19]. The patient cytogenetic profile can be stratified into low, intermediate, or high-risk categories. Certain cytogenetic abnormalities, including the balanced translocations between chromosomes 8 and 21 t(8;21)(q22;q22), chromosome 15 and 17 t(15;17)(q22;q12), as well as inversions such as inv(16)(p13.1;q22) are associated with longer remission and survival, while alterations of chromosomes 5, 7, complex karyotype (described as >3 chromosomal abnormalities) and 11q23 are associated with poor response to therapy and shorter overall survival [20]. Approximately 40%–50% of the AML patients diagnosed have a normal karyotype with somatic changes and/or alterations in gene expression identified as cytogenetically normal-AML (CN-AML) [21]. Even though a considerable heterogeneity is observed in CN-AML, patients are in the intermediate risk group to relapse thus molecular screening especially in CN-AML is essential for prognostic classification and treatment approach [9].

Molecular Abnormalities

Animal studies led to the development of a two-hit model of leukemogenesis, providing a conceptual outline for classifying the different mutations correlated with AML. It indicated that two separate types of concurrent genetic mutations denoted as Class I and Class II mutations were necessary for malignant transformation of a myeloid precursor [22, 23]. Class I mutations are linked with the activation of pro-proliferative pathways, allowing for uncontrolled proliferation, evasion of apoptosis [6]. They comprise mutations involved in the constitutive activation of tyrosine kinases or dysregulation of downstream signalling molecules. Examples of Class I mutations are those found in FLT3 (internal tandem duplications, ITD, and tyrosine kinase domain mutations, TKD), K/NRAS, TP53

and c-KIT genes and detected in ~28%, 12%, 8% and 4% AML cases respectively [6, 22]. Class II mutations impair normal hematopoietic differentiation for leukaemia to develop [22, 24]. For example, translocations linked with the core-binding factor (CBF) were involved with impairing differentiation of essential transcription factors, such as retinoic acid receptor alpha (RAR), and proteins that are associated in transcriptional regulation like p300, CBP and TIF2 [22, 25, 26]. An additional class of epigenetic mutations has emerged that led to downstream responses on both proliferation and differentiation contributing to more than 40% of AML cases. Such mutations include aberrant methylation and acetylation of the IDH-1, IDH-2, DNMT3A and TET2 genes [6, 25, 27].

Despite knowledge of the precise molecular and genetic abnormalities present in AML, the direct impact of individual mutations in AML disease progression has yet to be completely understood. The 'two-hit model' eludes that AML pathogenesis and behaviour greatly relies on the interactions between various somatic alterations and chromosomal rearrangements. As it is common for NMP1 a class II mutation to arise in association with FLT3-ITD a class I mutation or epigenetic modifications in DNMT3A and IDH-1 or IDH-2. Similarly, the c-KIT mutation in combination with t(8;21) or inv(16) leads to major implications on prognosis [23, 27-29].

Current studies have identified epigenetic modifications as histone modification and DNA methylation of the AML genome, proposing that the picture is more intricate with Class I and II mutations just a portion of it [25, 30, 31]. Moreover, the absence or presence distinct of gene mutations and/or modifications in gene expression can additionally classify AML cases and influence the prognosis [9, 32, 33]. This is especially significant in CN-AML. With the introduction to next generation sequences (NGS) the genetic landscape of AMLs has been extensively studied, leading to the identification of approximately 30 recurrently mutated genes. Similarly, in a study of the AML genome from blast isolated from 200 patients for the Cancer Genome Atlas, it was estimated that on average of 13 of these mutations are found in blast isolated from CN-AML patients [33]. Since major

molecular abnormalities have been determined, they are used to determine prognostic outcome and aid with AML treatment. Some of the relevant gene mutations are in: Nucleophosmin 1 (NPM1), DNA Methyltansferase 3A (DNMT3A), Fms-Like Tyrosine Kinase 3 (FLT3), Isocitrate Dehydrogenase (IDH), Ten–Eleven Translocation 2 (TET2), Runt-Related Transcription Factor (RUNX1), CCAAT Enhancer Binding Protein α (CEBPA), Additional Sex Comb-Like 1 (ASXL1) and MLL [20, 34].

Additionally, changes to actual epigenome for example global hypomethylation and localized CpG hypermethylation are being further explored [24, 25, 35]. However, several of these mutations in epigenetic modifiers and regulators are not considered as Class I or II mutations, implying that the two-hit model is an oversimplification [26] Given that some genetic aberrations in mutations in AML such as trisomy 22 (which is well recognized in inv(16) leukaemia) have an ambiguous role in leukemogenesis, it is no longer sufficient to consider the two-hit model alone [25, 36, 37]. Furthermore, evidence suggests that a sequence of events is necessary for leukemogenesis to occur. Mutations have to take place at a specific point in cell development, and in a distinct order, for leukemic transformation to occur as demonstrated in [25, 37] in acute promyelocytic leukaemia (APL). The promyelocytic locus gene (PML) - retinoic acid receptor alpha (RAR) fusion protein PML-RAR fusion protein can happen at any timepoint in myeloid cell development, however it is only related with AML if the translocation arises at an early stage while there are adequate amounts of the protease neutrophil elastase (which is elevated in promyelocytes) [30, 38]. Therefore, it is probable that new revised models for AML pathogenesis will be highly complex as new mutations are identified and their role in leukemogenesis is assessed.

Classification of the disease

In 1976 the first effort was made to distinguish AML between different types. FAB classification depends on the morphology and cyto-chemical characteristics of the malignant cells and defines eight distinct immunotypes (FAB M0 to M7). Subtypes M0 to M5 all start in an immature state of

white blood cells, while M6 begins from a very immature form of red blood cells (erythroblasts) and M7 from immature state of cells which can form platelets (megakaryoblasts) [19]. However, the FAB classification did not consider factors involving prognosis such as genetic abnormalities. In 2002 a new classification system was developed by the World Health Organisation (WHO) attempting to merge new developments made in the diagnosis and management of AML [39]. This was revised in 2008 and later in 2016 [40, 41]. The WHO classification system of AML combines genetic information with morphology, immunophenotype and clinical presentation to outline six major disease entities: AML with myelodysplasia-related features; therapy associated AML; myeloid sarcoma; AML not otherwise specified; myeloid proliferation related to Down syndrome and AML with recurrent genetic abnormalities (Table 2). Within the group of AML with recurrent genetic abnormalities, there are 11 subtypes which are further outlined according to specific chromosomal translocations. Genetic abnormalities also inform the diagnosis of AML with myelodysplasia-related changes: along with a history of MDS or morphological evidence of dysplasia in two or more myeloid cell lineages, the presence of myelodysplasia-related cytogenetic abnormalities such as monosomy 5 or 7, and deletion 5q or 7q identify cases of AML with myelodysplasia-related features [6]. AML was classified by the WHO in 2016 as at least 20% or greater myeloblasts in the bone marrow or peripheral blood with myeloid lineage [40]. However, the ≥20% criterion is negated in NMP1 mutated AML or cases with CBF AML (cytogenetic abnormalities t[8;21], t[16;16], inv [16]) or acute promyelocytic leukaemia. Diagnosis of AML in each of these is independent of blast percentage [42]. The 20% myeloblast limit is used to distinguish AML from MDS. Monoclonal antibodies against CD33, CD14, CD13 and CD11b are useful markers in identifying AML blasts [43, 44]. They are regarded to be restricted to cells committed to myeloid differentiation, however they are not specific to leukaemia as they can also be present on normal hematopoietic elements and usually do not correlate to prognosis with the exception of CD34. CD34 is present on undifferentiated hematopoietic progenitors but can also be detected on blasts of patients with either AML or ALL [6]. Studies have shown that AML patients

whose blasts strongly express CD34 have an inferior outcome due to chemotherapy-resistant leukaemia. This is particularly true for those with less morphologically differentiated leukaemia in which other myeloid-associated antigens are not strongly expressed [43].

Table 1: WHO classification of AML. (*Taken from:* De Kouchkovsky, I. and M. Abdul-Hay, ***Acute myeloid leukemia: a comprehensive review and 2016 update***. 2016)

Types	Genetic abnormalites
AML with recurrent genetic abnormalities	AML with t(8:21)(q22;q22); RUNX1-RUNX1T1
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11
	APL with PML-RARA
	AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A
	ML with t(6;9)(p23;q34.1); DEK-NUP214
	AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
	AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1
	AML with BCR-ABL1 (provisional entity)
	AML with mutated NPM1
	AML with biallelic mutations of CEBPA
	AML with mutated RUNX1 (provisional entity)
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
	AML with minimal differentiation
	AML without maturation
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Acute erythroid leukemia
	Pure erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis
	ML associated with Down syndrome

Mechanisms of immune evasion

A functional T cell population is essential for leukaemia surveillance and control. Suppression of T cell function in the bone marrow microenvironment is a common characteristic of AML [10]. The tumour microenvironment (TME) can be characterised as the cellular and non-cellular environment of the tumour. This environment is comprised of immune, endothelial and stromal cells and are critical for the development, proliferation, and survival of cancer, but also soluble factors [45]. Characteristics of the TME differ between different cancer types but also between patients with the same cancer. For instance, the TME in solid cancers such as renal cell carcinoma, is comprised of various of functionally distinct suppressive myeloid and regulatory or effector T cells populations [46]. This complex combination of cells has prognostic ability on the outcome of disease. Interestingly even within the same patient significant variations can also be detected in the make of the TME of various metastatic masses, with each individual mass having a unique environment and immune cell infiltration [47]. This diversity among and within patients contributes to the varying treatment outcomes.

Expectedly, the TME in haematologic malignancies varies significantly to solid tumours. For leukaemia, the BM is the sanctuary for most of leukemic stem cells, but blood and secondary lymphoid organs, including the spleen and lymph nodes, are also regarded to be elements of the TME [10]. The immune microenvironment has been well defined in several hematologic malignancies, such as acute lymphoblastic leukaemia (ALL), Hodgkin lymphoma (HL), chronic myeloid leukaemia (CML), and chronic lymphocytic leukaemia (CLL)[48-52], but less is known about the microenvironment in AML [53].

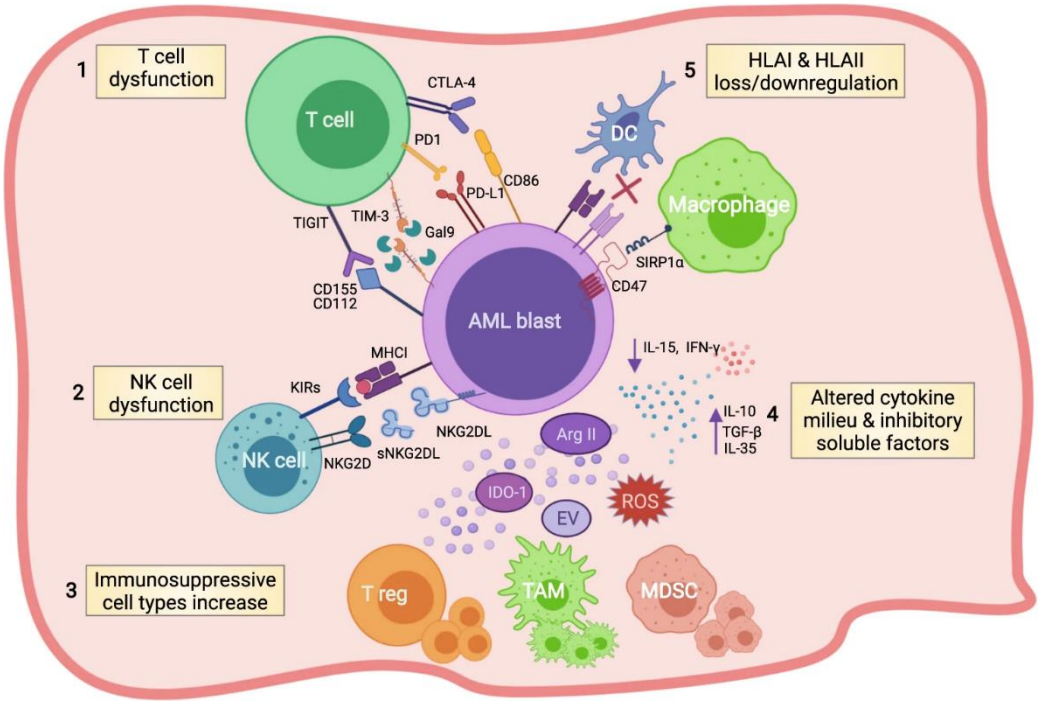


Figure 1.1: Mechanisms of immune evasion by AML blasts [53]. (Taken from: Sarah Tettamanti, et al., ***Catch me if you can: How AML and its nice escape immunotherapy***. Leukemia, 2022**.** AML blasts use a range of mechanisms to avoid immune response. They supress T- effector functions by overexpressing inhibitory T-cell ligands or drive T-cell exhaustion and apoptosis. The induce expansion of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) and induce the switch of macrophages to tumor-associated macrophages (TAMs). Moreover, they can shift the cytokine milieu and release of soluble factors such as ARG2, reactive oxygen species (ROS), indoleamine 2,3-dioxygenase-1 (IDO1).

One mechanism by which AML blasts evade immune surveillance is by the downregulation in the expression of the major histocompatibility complex (MHC) class I and II on blasts [54], and the upregulation in the expression of inhibitory ligands like Galectin 9 (Gal-9), programmed death-ligand 1(PDL-1) and B7-H3 (CD276), and Inhibitory checkpoints are molecules within the immune system that can modify or switch off an immune response [54-56]. These molecules induce intracellular signalling events that disrupt activation cascades, thus suppressing T cell clonal expansion and cytokine release, and can also lead to T cell exhaustion. Exhausted T cells can be phenotypically identified by increased expression of several inhibitory receptors CCD244, PD-1, CD160, T cell immunoglobulin domain and mucin domain 3 (TIM-3) and lymphocyte-activation gene 3 (LAG-3) [54, 57, 58]. This process is essential for generating and maintaining peripheral tolerance through normal

immune responses. AML blasts take advantage of this system by expressing inhibitory ligands for these checkpoint receptors, thus supressing the immune system and avoiding destruction. By blocking interactions between the ligands and the checkpoint molecules could possibly reverse tumour evasive effect. Nonetheless, immune checkpoint inhibitors (ICPIs) used as monotherapy to target solid tumours, showed very modest clinical responses in early AML clinical trials. Although the mechanism for reduced efficacy of ICPIs as monotherapy in AML is not completely understood, disease burden is one of the most probable reasons. Therefore, with combination therapies, it is still possible for ICI to be used. [59, 60].

Moreover, AML blasts release reactive oxygen species and indolamine 2, 3 dioxygenase (IDO) which are potent suppressors of T cell function [62]. IDO catabolizes the degradation of tryptophan, which is required for T cell proliferation and effector function. AML blasts also recruit other immunosuppressive cells such as Tregs and myeloid derived suppressor cells (MDSCs) to the tumour microenvironment [60] all of which support AML immune escape and survival.

Tregs restrict proliferation and activation of cytotoxic T cells, acting both directly and indirectly to elicit an immunosuppressive response. Directly *via* the release of anti-inflammatory cytokines and contact dependent suppression, and indirectly, by obstructing the activation of antigen presenting cells (APCs). AML blasts express inducible T cell co-stimulator ligand (ICOSL) which stimulates T cells through co-stimulatory receptor inducible T cell co-stimulator (ICOS), promoting the differentiation and expansion of the Treg subset [63]. Additionally, IDO which is overexpressed by AML blasts and bone marrow mesenchymal cells drives the development of a Treg phenotype and restricts T cell proliferation [60].

Studies in murine model of AML show an upregulation of Tregs *in vivo* and as a result suppression of the effector function of T cells *in vitro*. Conversely, Treg depletion led to improvements in the function of the effector T cells and treatment outcomes. Indicating that Treg recruitment could be a way to

induce AML immune escape. These findings reflect several human studies demonstrating that not only are Tregs upregulated at the point of diagnosis of AML but are linked with a worse prognosis [10, 63].

However, another study exploring the levels of Tregs in AML during chemotherapy induction showed that in early phases of AML a better complete remission and overall survival rate was observed in the presence of Tregs. Similarly, elevated numbers of Tregs after hematopoietic stem cell transplantation (HSCT) for AML were associated with a beneficial response [65]. This beneficial response observed in this study could however be a consequence of the efficiency of the transplant and the fact that the immunosuppressive feature of Tregs can contribute to minimizing graft-versus-host disease (GvHD) [10, 65].

T cells have also been shown to be inhibited by various myeloid cells within the tumours such as macrophages and immature myeloid subsets MDSC. Signals within the TME can polarize macrophages into functionally distinct subsets [10]. One of the major subsets described are M2 macrophages and functionally similar to macrophages involved in wound healing, tissue repair and chronic inflammation. M2 macrophages release a range of soluble factors for example: the arginine catabolic enzyme arginase, IL-10, and transforming growth factor beta (TGF-β) in order to modify the local matrix, enhance vasculature, and suppress T cell function [10, 66]. However, there are limited studies exploring the role of macrophage subsets in AML. A confounding element is the difficulty in identifying and determining these macrophage subsets as AML blasts express and share several of these markers therefore making it challenging to conclusively distinguish macrophages from AML blasts [10].

Nonetheless, it has been shown that AML blasts in trans-well co-culture assays can differentiate healthy monocytes into an M2 like phenotype suggesting soluble factors alone can induce this polarization [67]. Higher number of M2 macrophages (defined as CD14+CD163+CD206+) were

observed in the bone marrow of patients with AML compared to healthy donors. In murine models where AML is induced by of retroviral oncogene-induced, an increase in macrophages count was observed, and shown to enhance the proliferation of the tumour cell line compared to the non-leukemic mice [68].

MDSCs are characterised by a universal ability to inhibit the immune responses, they expand at various stages during cancer, inflammatory diseases, and infection. They are broadly divided into monocytic MDSCs (M-MDSC) and polymorphonuclear MDSC (PMN-MDSC), which phenotypically resemble monocytes or granulocytes, respectively [70]. MDSCs use a range of mechanisms to suppress not only the innate but also the adaptive responses of anti-tumour immunity. They directly suppress T-cell activation and expansion and produce high level of arginase, inducible nitric oxide synthase (iNOS), reactive oxygen species (ROS) [70] and IDO [71]. Additionally, MDSCs upregulate Tregs and immune-suppressive cytokines such as IL10 and TGF-β [71]. AML patients have elevated numbers of M-MDSCs (CD11b+CD14+/− HLA-DR−CD15−) and PMN-MDSCs (CD11b+CD14−HLA-DR−CD15+) in their blood [70, 72] .

AML cells co-cultured with healthy PBMC can mediate the increase of immature myeloid cells with an MDSC like phenotype which subsequently inhibit T cell proliferation and cytokine release. There is a link between MDSC levels and Tregs in myelodysplastic syndrome (MDS) which associates with a high risk of AML transformation, suggesting a possible role for MDSC in AML progression [73]. Although studies have shown the suppressive role of MDSCs in AML, their presence may be insignificant to disease presentation. For instance, T cell function was not restored in a cell line transfer-based AML when MDSCs were depleted [58].

It is essential to consider the role of soluble factors, such as enzymes and cytokines within the TME as they can skew it from hostile to supportive towards malignant cells. AML blasts control cells in the microenvironment to produce anti-inflammatory cytokines and modify chemokine-mediated immune cell trafficking as well as changing metabolite availability. AML blasts induced alterations to the availability amino acids such as arginine in the TME which skew it towards enhancing AML blast growth and survival whilst restricting immune responses [60].

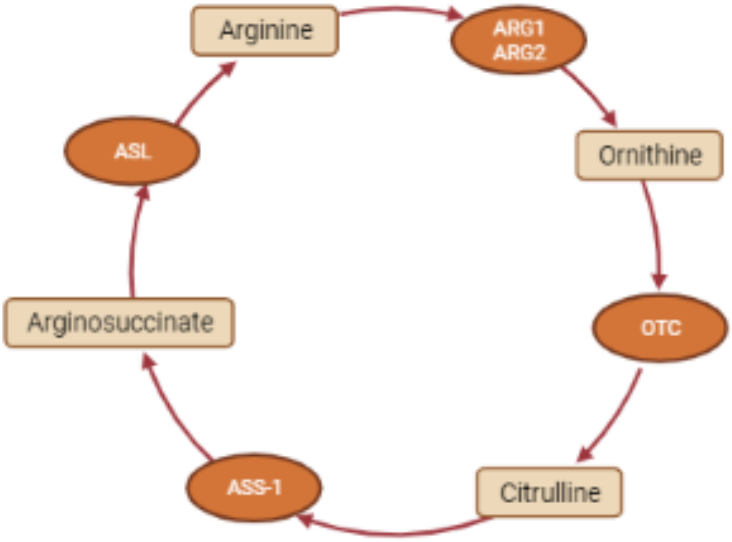


Figure 1.2: Arginine metabolism (created in BioRender)
Brief overview of arginine metabolism. Arginase 1 (ARG1) in the cytoplasm and/or ARG2) in the mitochondria catabolize arginine to urea and ornithine. Ornithine transcarbamylase (OTC) converts of ornithine to citrulline. Citrulline is produced by the catalytic activity of nitric oxide synthases (NOS) on arginine. Arginine can be resynthesized from citrulline by cells expressing the enzyme arginosuccinate synthase (ASS-1) and arginosuccinate lyase (ASL).

Mussai et al. demonstrated that secretion and release of ARG2 [75]. Arginases are manganese containing urea cycle enzymes that are responsible for hydrolysing arginine to urea and ornithine (Fig.1.2) [76]. ARG2 release acts as a distinct mechanism in which AML blasts create an immunosuppressive microenvironment and no correlation was observed between suppressive activity and clinical characteristics of patients, including FAB classification, patient age, or immunophenotype [75]. Analysis of the plasma of AML patients had a significantly higher ARG2 activity compared to blood plasma from healthy donors (9.9 versus 1.1 µmol; $p = 0.0001$).

Additionally, they showed a decrease in T cell proliferation when cultured *in vitro* in the presence of plasma from AML patients, which was relieved by replacing arginine. Added to their direct immunosuppressive effect on T cells, AML blasts can also polarize monocytes to an M2-like phenotype directly, further enhancing an immunosuppressive microenvironment [75].

Current therapy and recent developments

Over the past 40 years, a plethora of clinical trials have been carried out aiming to improve therapeutic outcomes of AML patients. However, little has changed in the standard of care or mortality rates, despite advances in our understanding of disease progression. Standard treatment for managing AML involves two phases: remission induction therapy followed by consolidation therapy for those fit enough to tolerate it. Induction therapy entails standard '7 + 3' chemotherapy regimen, which involves 7 days of continuous infusion of cytarabine in conjunction with 3 consecutive days of anthracycline (usually daunorubicin) [77]. Once initial remission is attained from induction therapy, patients then undertake consolidation therapy to eradicate residual AML blasts and this program usually involved high doses of cytarabine with or without hematopoietic stem cell transplantation. AML patients younger than 60 years old could reach complete remission with this cytotoxic induction regimen. Even so, regardless of the potential of achieving initial remission, AML is challenging to eradicate, especially in older patients or when patients relapse or becomes refractory [78].

Nonetheless, since 2017 the therapeutic landscape for AML has changed with eight new therapeutic agents approved by the Food and Drug Administration (FDA) [77]. Midostaurin is used to treat adults with newly diagnosed FLT3-mutations as it appears a promising inhibitor which directly targets FLT3-ITD and FLT3-TKD mutations [80]. Enasidenib is an IDH2 inhibitor used as therapy for adults with relapsed/refractory AML with IDH2 mutations [81]. CPX-351, is a liposomal formulation of cytarabine and daunorubicin at a ratio 5:1 enhancing the circulation period within blood targeting adults with therapy-related AML [82]. Therapies targeted at AMLs in over 75 year olds include venetoclax a BCL-

2 inhibitor, in combination with low-dose cytarabine (LDAC) or hypomethylating agents (such asazacitidine or decitabine) and Glasdegib – a sonic hedgehog pathway inhibitor in combination with LDAC. While these are exciting advances in managing AML, targeted therapies are advantageous only for the subset of patients with the distinct mutations and are rarely if ever therapeutic as monotherapy [77].

The potential for targeting AML with immunotherapy could be traced back to the graft-*versus*-leukaemia response observed after allogeneic HSCT. This encouraged the development of immunotherapies [83]. Major developments in cancer immunotherapy over the past years have indisputably revolutionized therapeutic approaches for both solid cancers and haematologic malignancies. Most prominent immunotherapeutic concepts include chimeric antigen receptor T (CAR-T) cells, antibody-drug conjugates, ICPIs, T cell-recruiting antibody constructs and vaccination [77, 82]. Since 2011, at least six ICPIs have been approved by the FDA to treat different malignancies such as non-small cell lung cancer and metastatic melanoma: diseases which were regarded untreatable until recently but yet none for AML [85]. Immunotherapy ultimately works by manipulating the immune system to reactivate an antitumor immune response, allowing it to recognize and kill cancer cells. Thus, overcoming immune escape. Targeted immunotherapy depends on recognising suitable target antigens expressed on malignant or normal cells to prevent undesirable on-target off-tumour toxicity. In AML, it is challenging to choose an appropriate target antigen due to a more ubiquitous expression pattern overlapping with healthy haematopoiesis. Examples of membrane-expressed antigens in AML explored are: CD33, CD123, FLT3 and can be used for targeting with antibody-based approaches including CAR-T cells and bispecific T cell engagers [60]. Other immunotherapeutic concepts depend on the augmentation of endogenous or the priming of new immune responses, for examples using ICPIs and therapeutic vaccines. Only in recent years have novel immunotherapeutic strategies have been used clinically to target AML. This is slightly surprising, taking into account that allogeneic HSCT is one of the oldest immunotherapeutic

approaches used to manage post-remission in AML [83]. Despite HSCT being the most effective therapy to prevent relapse in poor risk AML patients, of the patients treated develop GvHD and patients invariably relapse. In addition, only small proportion AML patients are eligible for HSCT treatment [42, 76].

One of the most explored therapeutic targets for AML is CD33 (Siglec-3), an antigen that is highly expressed on 85-95% of AML blasts. Expression of CD33 is highly variable with about half of AML patients expressing CD33 on >75% of leukemic blast cells. Although anti-CD33 monoclonal antibodies (mAbs) such as lintuzumab are tolerated in human clinical trials, they failed to provide meaningful clinical benefits [82, 84]. The only approved CD33-targeting agent is gemtuzumab ozogamicin (GO; Pfizer), a humanized CD33 mAb conjugated to calicheamicin- a DNA damaging toxin. GO was approved by the FDA in 2000 for CD33 positive AML patients which were 60 years old or older at first relapse. However, a phase III trial for newly diagnosed AML patients involving GO with standard chemotherapy not only failed to improve complete remission but increased fatal toxicity so Pfizer voluntarily withdrew it. In 2017 it was re-approved for newly diagnosed CD33 positive AML patients and for r/r CD33 positive adults and paediatric patients and administered in combination with cytarabine or daunorubicin [82, 85, 86].

CAR-T cell therapy has had remarkable response in managing haematological malignancies. Great successes in treating ALL, CLL and non-Hodgkin's lymphoma justifies the benefit in developing CAR-T cells tailored to the treatment of AML. However, this has yet to be accomplished. CAR-T cells are engineered T cells recognising target antigens expressed on the surface of malignant cells. Developing CAR-T cells to treat AML proved to be challenging due to the lack of specific targets for AML risking on-target off-leukaemia toxicities. Nonetheless, some targets such as: LeY, CD33, CD123, FLT3 and NKG2D are being explored. CAR Natural Killer (NK) cells are also being studied for AML as reduced risks like cytokine release syndrome may be possible [89].

In recent years there has been significant interest in the ability of ICPIs to successfully target various malignancies. Despite over 30 clinical trials exploring the efficacy of ICPIs as monotherapy or in combination with existing drugs for treating newly diagnosed AML or post-remission and r/r AML patients, none have yet been approved [77]. ICPIs eliminate immunosuppressive signals frequently used as a mechanism by tumour cells to evade immune detection. Some therapeutic targets explored for AML and MDS are: programmed cell death protein 1 (PD-1) and cytotoxic T-lymphocyte associated protein 4 (CTLA-4). T-cell immunoglobulin mucin-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3) as well as macrophage checkpoint CD47 [87, 88]. ICPIs are more effective in cancers with a high mutational burden, probably because the higher frequency of neoantigens to present to polyclonal T cells, whereas AML has a low mutational burden which could explain the limited success [77, 87].

Vaccination strategies aim to prime new or boost pre-existing antigen-specific immune responses. This is especially important for targeting cancers with low endogenous immune responses, such as AML. AML blasts are weak stimulators of T cells and frequently acquire mechanisms that inhibit T cell responses and resist cytotoxicity. Vaccinations have been used to trigger an immune response to AML-specific antigens using peptide vaccinations, or via fusion with dendritic cells that present antigen in the suitable context could drive a primary immune response, or AML blasts that were fused with or differentiated into antigen-presenting cells (APC) [89, 90]. Vaccinations of irradiated AML blast with different cytokines or BCG improved remission but did not enhance survival. An example of the numerous dendritic cell-based vaccines developed for AML is DCP-001 is a unique DC vaccine which combines both the positive elements of allogeneic DC vaccines and multi-antigen-expressing cancer cell vaccines. DCP-001 comprises of mature DCs established through the differentiation and maturation of DCOne an AML cell line, providing AML-associated antigens. Phase-I clinical trial results demonstrated that DCP-001 vaccination was safe for elderly AML patients which induced or enhanced multifunctional antitumor immunity [91].

Unconventional T Cells

More recently, it has emerged that in addition to "conventional" peptide-specific T cells, subsets of T cells which are not restricted by MHC exist. These, cells are referred to as unconventional T cells and have functional T cell receptors (TCRs) capable of recognising non-peptide antigens such as: lipids and vitamin B metabolites and phospho-antigens by nonclassical MHC molecules including: CD1, HLA-E (HLA-E dependent presentation of Mtb-derived antigen to human CD8+ T cells) and MR1. Examples of unconventional T cells include natural killer T (NKT) cells, mucosal-associated invariant T cells (MAIT) and γδ T cells [92-94].

iNKT cells

NKT cells were first observed in mice in 1987 by three independent research groups, however, it was not until 1995 that the name "NKT cell" was given. They are a distinct population of T cells bridging the innate and adaptive system as they can be rapidly activated without the need of co-stimulation.[95]. Upon activation, they respond rapidly (within hours) producing a broad range of cytokines. In addition to these innate characteristics, and their TCR can also undergo somatic rearrangement which are adaptive features [98]. NKT cells are divided into two main groups based on their TCR chains. Type I and Type II NKT cells. Type I NKT cells are also known as invariant NKT cells (iNKT) given that they express a semi-invariant TCR arising from the binding of Vα24 segment in humans or Vα14 in mice to Jα18 in a germ-line rearrangement resulting in the invariant CD3 loop. The α-chain binds to a distinct range of Vβ chains: Vβ11 in humans and Vβ8.2, Vβ7 and Vβ2 in mice [95, 97]. In humans, iNKT cells are either double negative CD4- CD8-, CD4+ or CD8+, in contrast to mice that do not express the CD8+ subset. [100]. iNKT cells are also defined by their ability to respond to the exogenous glycolipid α-Galactocylceramide (α-GalCer) presented by CD1d [101]. Type II NKT cells like iNKT cells recognise CD1d bound lipids. However, they do not have the semi-invariant TCR α-chain as they express diverse and broader TCR repertoire. Type II NKT cells do not recognise α-

GalCer, however they do recognise sulfatide – a glycolipid believed to be abundant in several membranous proteins including myelin in the central nervous system [101]. Although type II NKT cells express a broader TCR repertoire, they have been challenging to study due to the lack of isolation techniques and a reliable specific antigen [100, 101] . Both iNKT cells Type I and II NKT cells alter the immune response during tumour development and progression. In general, iNKT cells are believed to drive an anti-tumour immune response while type II NKT cells function in an opposing manner [102].

Unlike conventional T cells, where their development relies on MHC class I or II, iNKT cells development is dependent on β2-microglobulin [105], suggesting the ability of iNKT cells to respond to CD1d protein. iNKT cells recognise lipid and glycolipid antigens presented by a non-polymorphic transmembrane protein CD1d [106]. This was also established *in vivo* were $CD1d^{-/-}$ knockout mice lacked any iNKT implying its involvement in the positive selection for the development of iNKT cells [107].

CD1d

CD1d is an MHC class I like molecule and a member of the CD1 family. The CD1 family is made up of group 1 proteins: CD1a, CD1b, Cd1c; group 2 proteins CD1d and the group 3 which includes CD1e – an intermediate member [106, 107]. CD1a, CD1b, CD1c, and CD1d are all cell surface proteins; whereas CD1e is an intracellular molecule is not involved in antigen presentation but enables glycolipid processing (particularly CD1b antigens) in late endosomes [107, 108]. Group 1 and group 2 CD1 proteins have been shown to present lipid antigens and not protein antigens to non-canonical T cells. CD1d is a highly conserved in mammals; sharing both structural and functional similarities between the TCRs in mice and humans [100]. Unlike humans, the mouse genome does not consist of any group1 CD1 proteins and only two copies of the CD1d gene [111].

Similar to MHC class I, the CD1d forms a heterodimeric complex, comprising of a heavy chain with three extracellular domains which non-covalently associates with a conserved β2-microglobulin (β2m). The antigen binding site is formed at the heavy chain by the α1 and α2 domains at the N-terminal, while the third α3-domain binds with β2m to sustain the α1–2 platform. Lipid presentation to iNKT cells depends on the binding groove which consists of two deep and narrow: A' and F' hydrophobic channels at the CD1d antigen binding site, allowing the projecting polar heads (e.g., a galactose sugar) of the lipid antigens to be recognised by the invariant TCR [110-112]. This is unlike MHC class I, where in the binding grooves are shallower allowing for peptides to bind which are usually nine amino acids long. Whilst the A' pocket in CD1d is bigger, deeply buried and adopts a donut shape, the F' pocket is more open and available to the antigens at the C-terminal end of the α1 helix [115]. Generally, both A' and F' pockets bind to one of the alkyl chains of a dual alkyl chain glycolipid, whereas the carbohydrate segment is positioned at the CD1d surface for TCR binding. A proper presentation of the glycolipid antigens by CD1d and TCR interaction of the visible carbohydrate epitope in association with CD1d are necessary for TCR recognition [116]. The key role of the lipid backbone is to secure and orient the carbohydrate for iNKT cell recognition, though, it can also be in contact with the iTCR at different levels; however, its main role is to anchor and orient the carbohydrate for iNKT cell recognition. It was believed that the CD1d-binding groove was rigid, and lipids would have to find an appropriate way to fit in both grooves. However, it has been discovered that upon lipid and/or TCR binding small structural changes take place particularly at the surfaces over both A' and F' grooves [115, 116]. Only synthetic glycolipids have been shown to induce structural differences in mouse CD1d, while the existence of natural lipids that have similar effects are currently undetermined. Even so, structural changes driven by lipids particularly across the F' pocket significantly impact iNKT cell activation [119] as the principal binding site for the TCR [120].

Specific mechanisms are required for lipid antigens to be presented by CD1 molecules. This includes mechanism mediating the uptake of these molecules by antigen-presenting cells (APCs) and their

loading onto CD1 proteins. Due to the hydrophobic nature of lipids, binding of proteins or lipoproteins are necessary for extracellular transport [114, 119]. Binding of apolipoprotein E to lipid complexes following internalization via the low density lipoprotein receptor (LDLR) has been demonstrated to enable the presentation of several antigens by CD1d [120, 121]. Loading of antigens can occur at the cell surface [122]. However, loading efficiency can be enhanced by certain proteins in the endosomal and lysosomal compartments which promote lipid antigen exchange. For example, saposins in endosomes [123-126] and microsomal triglyceride transfer protein [127] have been shown to enhance the antigen loading to CD1 with a certain degree of specificity [127, 128]. A range of chaperones have been shown to control lipid antigen processing and presentation, by inhibiting improper binding of lipids to the CD1d molecule. Thus, CD1d is internalised and undergoes vesicular trafficking to lysosomal and endosomal compartments, it then switches its ligand with lipid antigens which can be endogenous to the cell, or exogenous (natural or synthetic) obtained through endocytosis [131]. Finally, lipid presentation is achieved by CD1d being recycled back to the surface of the cell to present the antigen to the iTCR of the iNKT cells. Despite the CD1d- lipid antigen presentation mediated pathway being well established, disorders connected to lysosomal storage are observed in mutant mouse models, suggesting there could be possible faults with the present theories [132].

Cd1d binding groove can bind to a range of different chemical moieties and through CD1d trafficking it can encounter various endogenous lipids of the secretory pathway. Mass spectrometry has allowed the detection of such endogenous lipid ligands bound to human and mouse and CD1d with two main classes been identified: sphingolipids and glycerolipids with one, two, or four acyl chains [116].

It has been shown that glycolipids derived from different microbes can activate iNKT cell after being processed by APC [131, 132]. antibody range of exogenous lipid antigens from *Borrelia burgdorferi, Sphingomonas spp.* , *Helicobacter pylori* , *Streptococcus pneumonia* and Group

B *Streptococcus* [133, 134], and even hydrophobic peptides [135-137] have been suggested to be recognised by CD1d and capable of stimulating iNKT cells.

Natural Ligands

Glycolipids derived from various microbes can activate iNKT cells after being processed by APCs [131, 132]. A range of exogenous lipid antigens have been suggested to be recognised by the invariant TCR. These include lipopeptidophosphoglycans from Entamoeba histolytica and Leishmania donovani, an ester cholesterol produced by *Helicobacter pylori* [140] and mycobacterial phosphatidylinositol mannoside [141]. However, their antigenicity has not been completely defined. *Sphingomonas* spp. are a Gram-negative α-Proteobacteria with unusually high levels of glycosphingolipids (GSL) instead of LPS. It appears that α-glucuronosylceramide and α-galacturonosylceramide have a strong structural resemblance to α-GalCer. Despite the ability of monosaccharide containing GSL antigens to stimulate iNKT cells to some degree *in vitro* and *in vivo,* they are all weaker antigens than α-GalCer. For instance, the iNKT cell TCR affinity for αGalA-GSL/CD1d complex is 50-fold weaker than for the αGalCer/CD1d[140]. GSLs with oligosaccharide headgroups are not antigenic or do not activate as well as their monosaccharide counterparts, possibly due to a failure of the APCs to reduce the structures to a monosaccharide form in lysosomal compartments.

α-linked galactosyl and glucosyl diacyglycerols (DAGs) have been found to be abundant in *Borrelia burgdorferi* and *Streptococcus pneumoniae*, respectively have been involved in activating iNKT cells [133, 141]. *Borrelia burgdorferi* is a spirochete and causes Lyme disease. A mouse strain which was resistant to Lyme arthritis appears to acquire symptoms when CD1d gene was deleted, suggesting that this immune response is partly CD1d dependent. *B. burgdorferi* is the first example of a pathogenic microbe consisting of glycolipid antigens that stimulates iNKT cells, and it is also the first example of an iNKT cell antigen which is not a glycosphingolipid [144]. High levels of BbGL-2, a DAG were detected on *B*. *burgdorferi* during a lipid analysis. Both purified and synthetic BbGL-2 can

activate iNKT cells. It is composed of a d-galactose saccharide group linked with an α-anomeric glycosidic bond and is a DAG lipid, lipid chains can differ in length and degree of saturation. Synthetic versions of the DAG antigen from *B. burgdorferi*, carrying various acyl chains at both sn-1 and sn-2 position, demonstrated that they play a role of the lipid backbone in iNKT cell activation [145]. The glycolipid, BbGL-2c (sn-1, oleate, sn-2, palmitate) is most effective in activating murine iNKT cells, whereas BbGl-2f (sn-1, linoleate, sn-2, oleate) was a better antigen for human iNKT cells [146] . This data indicates that even though antigens have an identical α-anomeric galactose for TCR recognition, to αGalCer, the structure of the lipid backbone that links the glycolipid to CD1d can control iNKT cell activation.

Streptococcus pneumonia is a Gram-positive bacterium responsible for pneumonia. A protective role of iNKT cells was observed in infected mice with pulmonary *S. pneumonia*, due to the release of IFN-γ by the iNKT cells. α-glucosyl-containing diacylglycerol antigens (Glc-DAG)-s2, is the main iNKT antigen in these bacteria, and was the first microbial antigen detected which was not a galactosyl moiety. In addition, Glc-DAG-s2 antigen is also capable of binding human iNKT cells [147], indicating that the significance of analogous synergies between polar and lipid portion of the streptococcal iNKT antigens is also maintained in humans.

Helicobacter pylori is a Gram-negative spiral bacterium and, in some cases, can trigger gastritis, peptic ulcers, gastric cancer and lymphoma. iNKT expansion is known to be driven by some *H. pylori* lipid extracts, resulting in a demonstrable protective role of iNKTs in eliminating the bacterium. Cholesteryl phosphatidyl α-glucoside (αCPG) synthesized by *H. pylori* can bind to CD1d and stimulate iNKT cells. Levels of the cholesteryl α-glucosyltransferase – the enzyme catalysing the production of the iNKT antigen cholesteryl α-glucoside correlated with the severity of gastric atrophy induced by of H. pylori [146].

In 1997, Kawano and team discovered α-GalCer, as the first known ligand presented to iNKT cells by CD1d, potently activating them. α-GalCer isolated from a marine sponge called *Agelas mauritanus* serves as an important tool for the study and exploration of iNKT biology; by determining the response of activated iNKT cells both in vitro and *in vivo* [111]. The development and use of α-GalCer loaded CD1d tetramers vastly enhanced our understanding of iNKT research [149]. The glycosphingolipid α-GalCer is composed of an α-anomeric galactose linked to a ceramide backbone. The ceramide backbone is formed by a sphingoid base carrying an N-amide-linked saturated C26 acyl chain. CD1d binds to the C26 acyl chain of α-GalCer at the A' groove and the sphingoid base in the F' groove, exposing the galactose moiety over the CD1d-binding pocket for binding TCR and stimulating iNKT cells [148].

iNKT development

Like conventional αβ T cells, iNKT cells develop in the thymus and arise from common lymphoid progenitors. Initial studies exploring iNKT development based on cell surface molecules expressing CD44 and NK1.1, proposing that all iNKT cells carry out same development progressing program from most immature stage S0 to a terminal mature stage S3 [149, 150]. β chain rearrangement occurs at the DN3 stage and α chain rearrangement at the $CD4^+$ $CD8^+$ [double positive (DP)] stage. However, the DP selection occurs on CD1d-expressing DP cortical thymocytes and not thymic epithelial cells. According to this model, iNKT cells progress from the most immature stage S0 ($CD24^+CD44^-NK1.1^-$) where positive selection occurs to stage 2 by downregulating CD24 and subsequently upregulating first CD44 to their final mature stage S3 ($CD24^-CD44^+NK1.1^+$) acquiring natural killer NK1.1 [149, 150].

Nonetheless, this categorization is not ideal since it cannot be applied for all iNKT cells. For example, this failed to include iNKT cells producing IL-4 but do not express NK1.1 or iNKT cells producing interleukin-17 (IL-17)[151, 152]. Consequently, a new functional classification was suggested for murine iNKT cells into the three terminal differentiated subsets, dependent on their expression of

lineage-specific transcription factors and the cytokines produced: iNKT1 (PLZF^{lo}, T-bet^{+}), iNKT2 (PLZF^{hi}, GATA-3^{hi}) and iNKT17 (PLZF^{int} RORγt)(Fig1.3) [153].

It has been proposed that immediately after selection all iNKT cell have a common progenitor known as NKT0 (Egr2 ^{hi}CD24^{hi}CD44-CD69^{hi}) and subsequently differentiate into iNKT cell subsets. They then downregulate CD24 and transition into CCR7+ multi-potent NKT cell progenitors (NKTp) [154, 155]. Development occurs in the cortex of the thymus where DP NKTp are positively selected by thymocytes presenting lipid antigens by CD1d. Survival and commitment to iNKT cell lineage only occurs when the TCR of DP thymocytes has “right” specificity. A strong TCR selection (agonist selection) is necessary, in combination with secondary co-stimulatory signals induced by CD80/CD86 interactions [156, 157]. Homotypic binding between signalling lymphocyte activation molecule family (SLAMF) receptors (Slamf1 and Slamf6) upregulate early growth response protein (Egr)1 and Egr2 to induce stable expression of the NKT lineage-specific promyelocytic leukaemia zinc finger transcription factor (PLZF). PLZF is encoded by the zinc finger and BTB domain-containing protein 16 (Zbtb16) gene. Upon TCR interaction, upregulation of Egr2 and its subsequently recruited to the zbtb16 promoter is a strong determinant of iNKT cell lineage fate [160] .

Undifferentiated NKTp cells either exit the thymus or continue their differentiation in the medulla into one of the effector subsets iNKT1, iNKT2 or iNKT17. The precise signals driving their migration from cortex to medulla and factors controlling differentiation of iNKT cell subsets in the medulla are not known. Intra-thymic transfer and foetal thymic organ culture (FTOC) have established that subsets: iNKT1, iNKT2 and iNKT17 are terminally differentiated [157, 161].

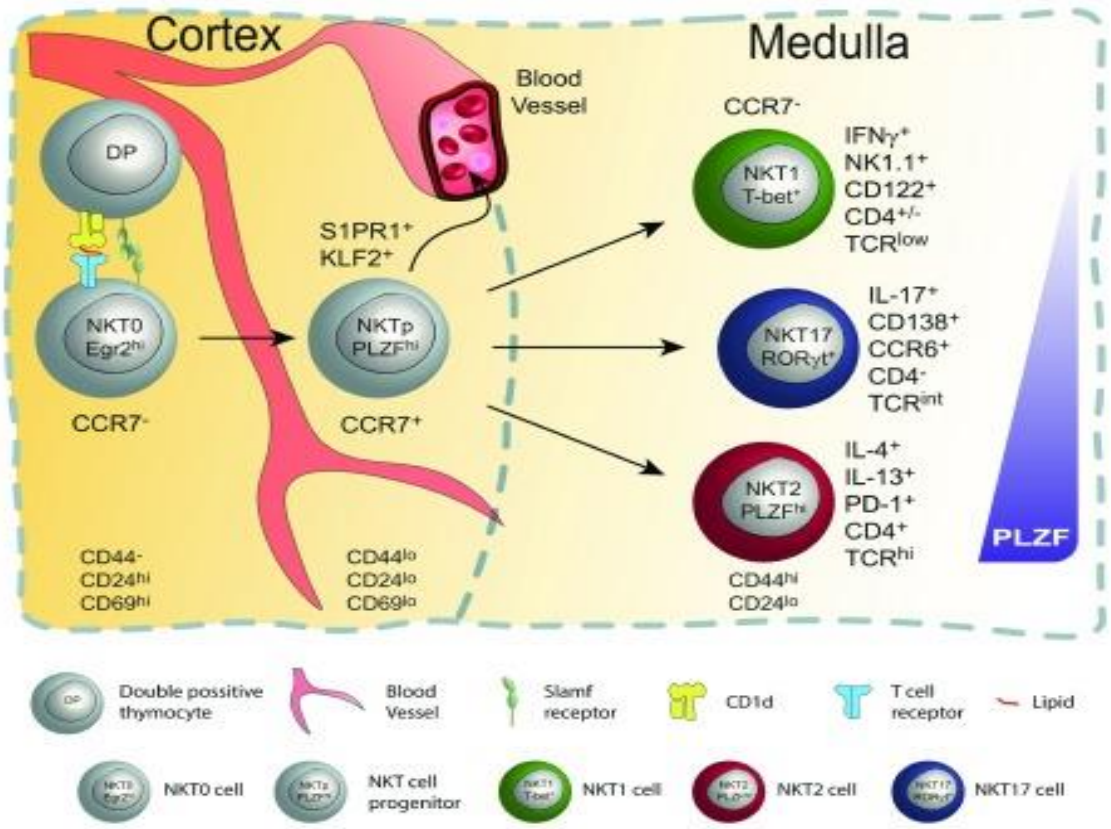


Figure 1.3: iNKT cell development in thymus [157]. (Taken from: Kristin Hogquist., et al., ***Recent advanced in iNKT cell development***. F1000Research, 2020.
Positive selection of DP iNKT progenitors by thymocytes presenting lipids by CD1d lead to survival and lineage commitment. TCRs of DP thymocytes require the right specificity for selection and commitment to the iNKT cell lineage. Both, a strong TCR signal and co-stimulatory signals are necessary. Upregulation of Egr1 and Egr2, drives PLZF induction and stable expression. Immediate post-selection iNKT cells are labelled as NKT0 cells. CD24 is downregulated by NKT0 cells and change into CCR7 $^{+}$ multi-potent NKTp. NKTp cells can leave the thymus or continue their differentiation into one of the effector subsets (iNKT1, 2, or 17).

iNKT1, iNKT2 and iNKT17 cells acquire their functionality during development in the thymus [151, 160, 161], before they are distributed in the peripheral organs in a tissue specific manner. Only iNKT1 go through all the stages (S0-S3) of development described by the original four-stage model proposed and are primarily located in the red pulp of the spleen. iNKT2 and iNKT17 subsets finish their maturation as terminally differentiated effector cells at stage 2 (S2). iNKT 2 cells are mainly enriched in the T cell zone of the spleen, medullary area of the thymus and in mesenteric lymph nodes, while

iNKT17 cells are mainly located in the lung and subcapsular region of lymph nodes [157]. Given that each iNKT cell subset colonises and localises within peripheral tissues differently, thus, it is not surprising that each subset distinctively modulates immune responses. iNKT1 cells (PLZF ^{lo} T-bet^{+}) upon stimulation produce IFN-γ and low levels of IL-4. They are the only subset to display NK cell signature such as NK1.1, Nkp46, NKG2D and a soluble cytotoxic mediators [153, 162, 163]. iNKT1 cells are also divide further into CD4 $^{+}$ and CD4^{-}. CD4^{-} fraction of iNKT1 cells and have an NK-like phenotype characterised by the expression of NK cell receptors and soluble cytotoxic mediators such as: granzyme a, b, and perforin [153]. CD4^{+} iNKT1 cells fraction express higher levels of genes such as IL-4 and CD81 [153, 164]. These observations raise additional questions about iNKT cell subset development and function. For instance, are iNKT1 fractions CD4^{+} and CD4 $^{-}$ distinct, terminally differentiated cell subsets with different functions? Or do they portray intermediate and fully matured stages of the iNKT1 cell subset? An alternate iNKT cell developmental pathway was described showing that a small CD4^{-} NKT1 population can rise from CD4^{-} CD8^{-} [double negative (DN)] thymocytes [165]. Nevertheless, this pathway appeared to attribute in only a small way to the mature CD4 − iNKT1 cell pool.

iNKT2 cells express GATA binding protein (GATA)3 and highest levels of PLZF and can actively produce and secrete IL-4 under steady-state conditions. This is an essential process for CD8 innate-like T-cell generation in the thymus and periphery [163]. Mouse iNKT2 cells express the CD4 co-receptor. iNKT2 cell activation results in a Th2-like cytokine and chemokine response [168]. This subset is enriched in mouse lungs and the intestine IL-13 and IL-4 as well as C-C chemokine ligand (CCL)17, CCL22, CCL10/CCL6, and eosinophil chemotactic factor-L secreted by activated NKT2 cells may mediate airway hyperresponsiveness [168]. This Th2-type response recruits macrophages, eosinophils, neutrophils, and lymphocytes into the lungs to incite tissue damage [169].

Recently, two additionally subsets have been proposed iNKT follicular helper (iNKTFH) and iNKT10 cells, however, less is known about them. They have not yet been described in the thymus and appear

only in the periphery. Their origins and developmental cues remain uncertain. It is likely that these two subsets develop within the thymus before migrating either at minimal frequencies, or that they may represent separate states of activation. In fact, iNKTFH cells found in germinal centres have a similar phenotype to T follicular helper cells (TFH) expressing Bcl6, ICOS PD-1 and CXCR5. Bcl6 expression aids antibody class-switch and somatic hypermutation [161]. iNKTFH were originally shown at secondary lymphoid organs following antigenic immunization in the presence of α-GalCer triggering these cells to develop stable contacts with B cells and induce germinal centres through IL-21 release [155, 168]. However, iNKTFH dependent germinal centre reactions failed to generate long lived plasma cells. While, iNKT10 cells have been shown to produce IL-10 and are abundant in adipose tissues. They express low levels of PLZF and for functional competence rely on transcriptional regulator *Nfil3* (E4BP4) [155, 169].

It has been established that TCR signalling is essential for the development of iNKT cells, since positive selection relies on recognition of CD1d:lipid ligands but is also critical for subset differentiation . Moreover, TCR signal strength can bias iNKT cell progenitors towards specific subset [153, 170]. iNKT2 cells display the highest level of TCR expression whilst NKT17 expresses intermediate, and NKT1 expresses the lowest levels. This also associates with the strength of their TCR signal under steady-state conditions. Implying that different TCR signal intensities are required for the development of each subset [157]. Recent studies using SKG mouse model of arthritis originating from a spontaneous mutation in ZAP70 showed that a hypomorphic ZAP70 allele weakened TCR signalling and lead to decreased iNKT2 and iNKT17 development whereas iNKT1 cell development was relatively spared. PLZF is also an essential target gene for signalling TCR in iNKT cells [170, 171]. Mature iNKT cell subsets exhibit different signal intensities of TCR on their surface which mirror PLZF expression. A hypomorphic PLZF allele appeared to significantly reduce iNKT2 and iNKT17 frequencies, whilst iNKT1 numbers were fairly spared [157]. These findings illustrate that the quantity of TCR signal could be an essential factor contributing in iNKT cell subset differentiation.

Interestingly, thymic iNKT subsets are not equally represented in different mice strains. Certain strains, such as the B6 mice have significantly higher frequencies of iNKT1 cells and a reduced proportion of iNKT2/iNKT17 cells. In contrast, BALB/c, acquire higher proportions of iNKT2 and iNKT17 cells with a lower iNKT1 cells frequency. On the other extreme, B6 mice instead largely possess iNKT1 cells and few iNKT2/iNKT17 cells [163]. However, it is still unknown if the compositions of iNKT cells in either of these strains is a direct cause or a consequence of these phenotypes. Irrespective of their strains, iNKT cells are recognised by their capacity to interact with α-GalCer loaded CD1d tetramers and their transcription factor profile (or surface proteins known to be specifically upregulated by these transcription factors) serves as a readout of the subset proportions [168].

Mouse subsets	NKT1	NKT2	NKT17	NKT10
Subset-specific transcription factor	PLZF GATA3 T-bet	PLZF^{HI} GATA3	PLZF GATA3 RORγt	PLZF$^{LO/-}$ E4BP4 Nur77^{HI}
Surface markers	CD4$^{POS/NEG}$, IL-12R NK1.1 CXCR3, CXCR6	CD4, CD27, IL-25R NKI.1^{NEG}, IL-17RB CCR4, CXCR6	CD127^{HI}, IL-23R, NKI.1^{NEG} , CCR4, CCR6, CXCR6,	CD4, CD49a NKI.1^{NEG}, SLAMF6, PD1
Effectors	IFN-γ TNF-α, (IL-4) granzyme, perforin	IL-4, IL-13 IL-6, (Csf-2)	IL-17A IL-21, IL-22	IL-10 IL-2
Tissue location	Liver Spleen	Lungs Intestine	Lungs, skin Lymph nodes	Adipose tissue Spleen
Immunologic function(s)	Anti-tumor immunity	IL-25 induced AHR	Virus/ozone-induced AHR; tissue repair; limit systemic pathogen spread	Immune tolerance; resolve inflammation

Figure 1.4: Mouse iNKT cell subsets [168]. (Taken from: Kumar, A., et al., ***Natural Killer T Cells: An Ecological Evolutionary Developmental Biology Perspective***. Frontiers in Immunology, 2017.
Mice iNKT cells differentiate according to specific transcription factors and the cytokines produce. Each subset has a distinct location and effector function.

Self-lipid antigens

In addition to exogenous lipid antigens iNKT cells can respond to endogenous lipids, as they can also recognise self-lipids bound to CD1d. This is evident during iNKT activation mediated in the absence of infection [172]. Stimulated APCs via Toll-like receptor (TLR) agonists can activate iNKT cells in a CD1d-dependent manner by pathogens such as viruses which lack any lipids [175]. Moreover, iNKT cells activation has also been observed during cancer, autoimmune diseases and viral infections where foreign lipids are absent, and the key role of endogenous antigens in positive thymic selection further confirming the presence of self lipid activation [174]. Over the years many studies have focused on identifying potent endogenous ligands activating iNKT cells and their association in iNKT thymic selection. At present, the precise endogenous ligands remain uncertain despite numerous candidates been suggested such as: that isoglobotrihexosylceramide (iGb3), β-linked glycosphingolipids (GSLs), β-glucosylceramide (β-GlcCer), gangliosides, and phospholipids. The physiological activity of the ligands identified was either shown to activate only specific iNKT cell subsets, have weak activity [175-177]. For instance, iGb3 was identified as a self-antigen when iNKT cells were absent in β-hexosaminidase B-deficient mice, which were believed to have low iGb3 levels. iGb3 was suggested to be accountable for iNKT cell development and shown to activate some human and mouse iNKT cells. Nonetheless, iGb3 synthase-deficient mice showed no iNKT cell defect. Additionally, iGb3 is essentially untraceable in mouse peripheral tissues [11], and it may not be antigenic in most humans as the gene encoding iGb3 synthase seems to not be functional.

β-GlcCer, the precursor of most GSLs, has been proposed as a possible self antigen for both humans and mice, given that it is abundant in the thymus and peripheral lymphoid tissues and levels rise in response to danger signals [180]. There have been several breakthroughs regarding other physiologically related iNKT cell agonists, but primarily from a therapeutic standpoint. For instance, it has been demonstrated that β-GlcCer can drive anti-tumour immunity and additionally enhance

the immune response to Hepatitis B, in an iNKT cell-dependent manner [178, 179]. However, it was later revealed that direct iNKT cell activation does not occur with CD1d-bound β-GlcCer [182]. This suggests that iNKT cell activation may be due to presentation of β-GlcCer derivatives such as mammalian α-linked monoglycosylceramides contaminants [183]. Originally it was believed that *α*-linked lipids were absent in mammals, but recently low levels of α-linked monoglycosylceramides (e.g., α-GalCer) have been detected which could potentially function as self iNKT cell ligands. The origin of mammalian α-GalCer is presently ambiguous, studies indicate that various bacterial communities such as *Bacteroides fragilis* from the gut microbiota, can produce this specific hexosylceramide. It has been shown that α-linked monohexosylceramides are found in bovine milk indicating that diet could also be a source of these endogenous ligands [145].

Additional to GSLs, gangliosides and phospholipids like phosphatidylinositol phosphatidylcholine, and phosphatidylethanolamine, have been proposed as potential stimulatory self-antigens for NKT cells; by activating a small proportion of iNKT cells from hybridomas in a CD1d dependent manner. However, in contrast to *α*-linked lipid antigens, the ability of phospholipids to activate iNKT cells is much weaker and has only been observed for a subset of iNKT cells. For instance, Fox et al. attempted to detect other self lipid antigens by eluting endogenous lipids from the CD1d molecule [182, 183]. Lysophosphatidylcholine (LPC) was detected and appeared to be antigenic for human cell lines [177, 182]. In a previous study LPC was isolated from the plasma of patients with multiple myeloma and shown to stimulate activation of iNKT cells. Notably, LPC is produced by phospholipase A2 (PLA2) during several inflammatory conditions, suggesting it could have an impact in the modified iNKT cell responses [186].

GD3 a natural lipid component of the cell membrane, has been observed to present antigens in a CD1d dependent manner to iNKT cells stimulating them [187]. GD3 from SK-MEL-28 a melanoma cell line, has been shown to be cross-presented by murine APCs to TH2- like NKT cells [187]. Moreover,

an increase in NKT cell frequency in the spleen was demonstrated following a GD3 injection into wild-type mice (but not CD1d-deficient) [186, 187].

Establishing self-antigens for iNKT cell has been exceedingly difficult due to the disparity in uptake of the lipid antigen, intracellular transport, loading efficiency onto CD1d, stability of the CD1d-binding cleft, clustering of CD1d (e.g. in lipid rafts) and antigen half life [190].Even when lipid antigens are at the cell surface, the affinity of each CD1d-lipid complexes for the iTCR differs; some might stay at the cell surface, whereas others quickly decay, thus changing the dynamics of the iNKT cell response and promote skewing in cytokine release [191]. Given that self-lipids are not well characterized, and no physiologically relevant self lipid has been detected with similar potency as α-GalCer, further research

into identifying potent self lipids is required especially in disease states where modified self-antigens could be presented

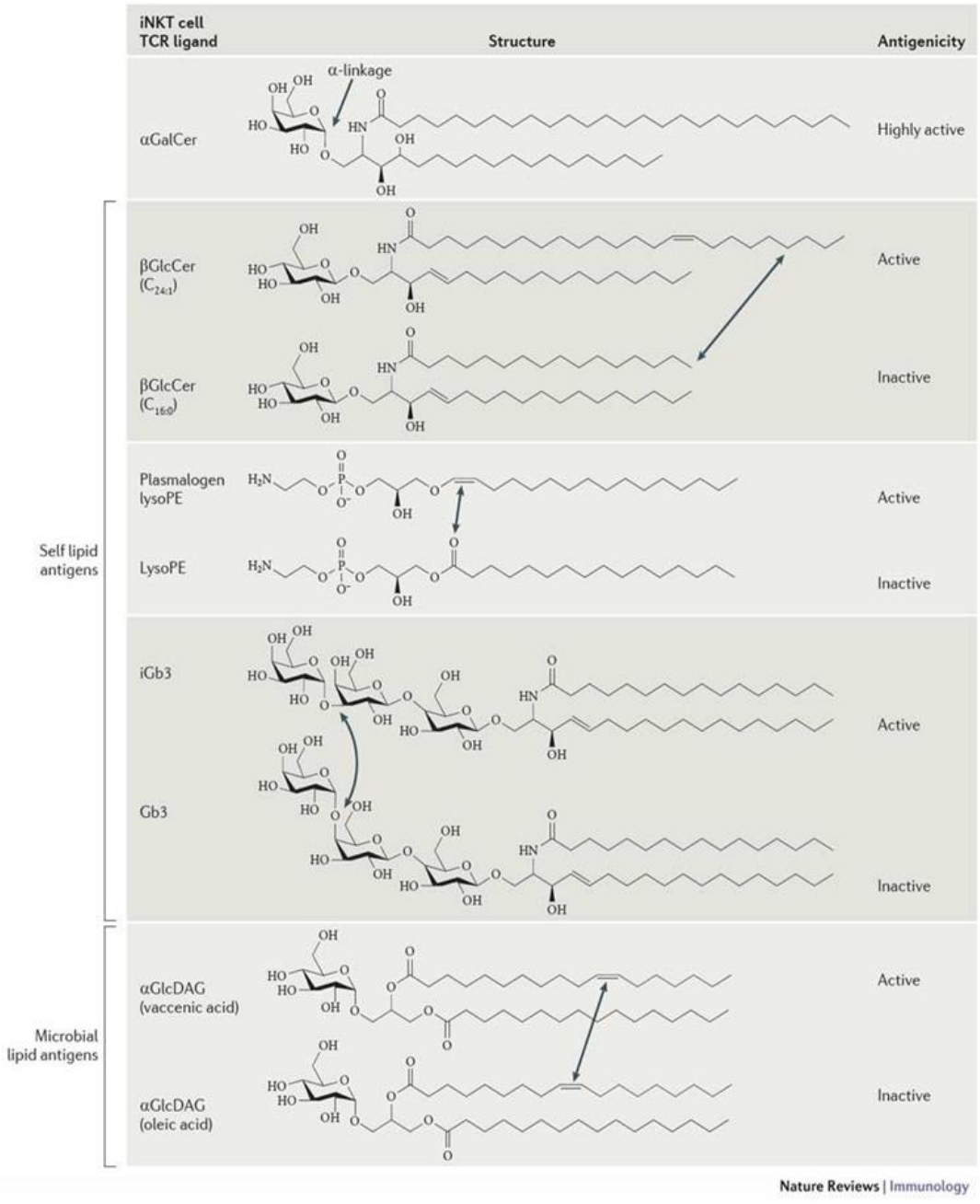


Figure 1.5 iNKT cell lipid antigens and closely related non-antigenic lipids [180].
(Taken from Brennan, P.J., M. Brigl, and M.B. Brenner, ***Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions***. Nature Reviews Immunology, 2013.)
Selected endogenous and exogenous iNKT lipid antigens: α-GalCer; β-glucosylceramide (βGlcCer), plasmalogen lysophosphatidylethanolamine (plasmalogen lysoPE), isoglobotrihexosylceramide (iGb3); and α-glucosyldiacyglycerol (αGlcDAG) from *Streptococcus pneumoniae*. Small structural alterations can change antigenicity. For each antigen, a non-antigenic lipid shown. $C_{24:1}$ and $C_{16:0}$ indicate the *N*-acyl chain structure.

Synthetic ligands

Following the discovery of potent activating properties of *α*-GalCer, linked with rapid Th1 and Th2 cytokine release, iNKT cells are poised as an attractive target to establish novel therapies to exploit the immune system. Numerous synthetic α-GalCer analogues have been produced by altering the phytosphingosine chain, galactosyl moiety or acyl chain. Some analogues can induce immune responses either by Th1 or Th2 dominance. For instance, Th2 skewed cytokine response is induced by truncated sphingosine base derivative of α-GalCer OCH and N-acyl-derivative α-GalCer20:2 [179]. While, analogues with an aromatic ring in the shingosine or the acyl tail such as α-C-GalCer and 7DW8-5 induced Th1 biased anti-cancer responses [192] as they are more effective in initiating activation and expansion of iNKT cells in comparison to α-GalCer [191]. 7DW8-5 (truncated fatty acid tail and a fluorinated benzene ring at the end) appears to have greater affinity to CD1d than α-GalCer [192] and in a vaccination study it produced 100-fold greater IFN-γ compared to α-GalCer [192]. The ability to influence and bias the immune response by activating iNKT cells with synthetic lipid antigens holds great therapeutic potential and as such the list of synthetic lipids antigens is expanding [195] .

iNKT cell distribution *in vivo*

iNKT cells comprise of a very small subset of the T-cells in the peripheral blood, this however varies between individuals ranging from undetectable levels (0-0.1%) to over 1% [180]. Mice have generally higher frequencies of iNKT cells and are mainly enriched in the liver whereas iNKT cells in humans are enriched in the omentum representing around 10% of T-cell population [178, 194].

Even though iNKT cells comprise of such a small population of the T lymphocytes, they express a semi-invariant TCR and thus are more abundant than naïve antigen specific T-cells. iNKT cells express chemokine chemokine (C-X-C) receptor (CXCR3), C-C chemokine receptor type (CCR)5 and CCR6 enabling them to migrate to sites of infection and inflammation. A low frequency of iNKT cells may

also express CCR7 retaining them in the lymph node [197]. iNKT cell homing to spleen and liver is controlled by chemokine chemokine (C-X-C) ligand (CXCL)16 release in combination with CXCR6 which is expressed on nearly all iNKT cells [198]. iNKT cells also circulate within the tissues at steady state conditions and bind with APCs expressing CD1d. This was shown in the liver by microscopy where iNKT cells travel along the hepatic sinusoids in order to bind to Kupffer cells [199].

Activation of iNKT cells

Despite their very low frequency, and limited TCR repertoire, iNKT cells upon activation rapidly proliferate and vastly influence the immune response. This is facilitated by their ability in regulating numerous immune effector functions. The presence of preformed mRNA facilitates the rapid release of copious amounts of cytokines and chemokines upon activation. Studies have illustrated that activation of iNKT cells leads to the production of a wide range of both pro- and anti- inflammatory cytokines such as: IFN-γ, TNF-α, GMCSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17 and IL-21 [198]. The capacity of iNKT cells to secrete IFN-γ in the presence of α-GalCer has become a trademark for iNKT cell activation [95, 198]. Moreover, iNKT cells display cytotoxic functions which stem from their expression of perforin, Fas ligand (FASL) and granzyme B. Studies have indicated that iNKT cells can kill APCs stimulated with α-GalCer via CD1d presentation [201].

Activation of iNKT cells by non-TCR signals

Two distinct pathways have been described to mediate iNKT cell activation. In addition to the classical activation pathway involving direct, strong TCR recognition of a CD1d presented lipid antigens, iNKT cells can be activated by a weaker signal not driven by TCR but cytokines. Even though, a weaker signal, activation of iNKT cells mediated by cytokines could be valuable for their physiological functions and the synergy of both cytokine and TCR stimuli provide robust iNKT cell activation [180]. Despite the requirement for both a TCR and a co-stimulatory signal to activate naive MHC-restricted

T cells, iNKT cell activation can be usually achieved with a TCR signal and a cytokine signal. Subsets of iNKT cells can be activated by IL-12, IL-18, IL-23 and IL-25 cytokines in their steady state as they express corresponding receptors [202]. Even though pro-inflammatory cytokines can affect conventional T cells, activation by cytokine signals normally requires previous TCR-mediated activation. It has been observed that some viral and bacterial infections drive APCs to produce and release sufficient IL-12 to activate iNKT cells i*n vivo*, even without the involvement of CD1d-restricted TCR, this is due to the high expression of functional IL-12 receptors at baseline [178, 200].

iNKT cell interactions with other leukocytes

iNKT cells are crucial players in numerous settings, such as cancer, autoimmunity, metabolic diseases, and infections. When considering iNKT cell activation, it is important to consider the microenvironment, the iNKT cell population and transactivation with other immune cells and not just cytokine production by the individual cell. Activation of iNKT cells by α-GalCer, other exogenous CD1d antigens and synthetic analogues, mediates a cross talk with several immune cells such as: DCs, B cells, and NKs; not only bridging the innate and adaptive immune system but ultimately enhancing further the immune responses [180].

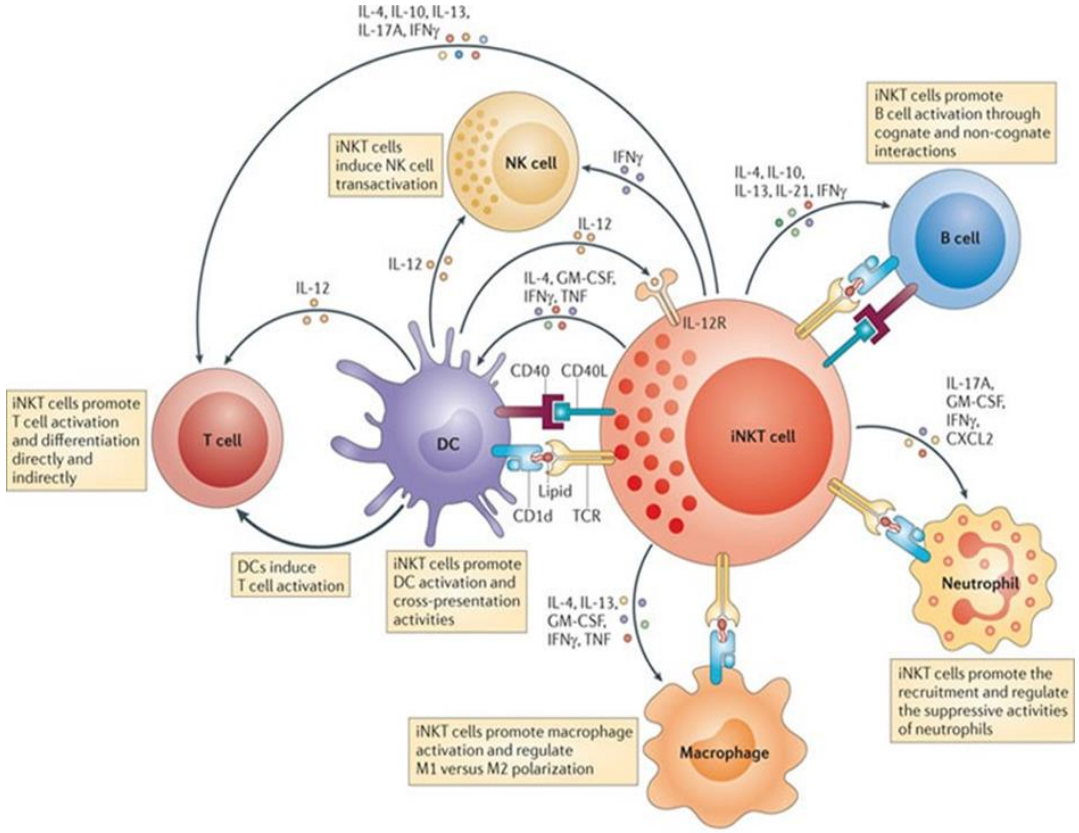


Figure 1.6. iNKT cells crosstalk with a range with of immune cells [180]. (Taken from: Brennan, P.J., M. Brigl, and M.B. Brenner, ***Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions***. Nature Reviews Immunology, 2013.)
Lipid presentation with endogenous or exogenous antigens enables iNKT crosstalk with APC cells such as neutrophils, DCs, NK cells, B-cells, macrophages in a CD1d dependent manner. In addition, co-stimulatory signals such as CD28 CD40 interact with their lignads CD80/CD86 and CD40L respectively. This leads to the activation of iNKT cells releasing a range of cytokines and in turn activation or modulation of APCs. Both APC derived cytokines and iNKT derived cytokines as consequence modulate and activate other cell MHC-restricted T cells and NK cells.

For example, CD1d is constitutively expressed on DCs and has been demonstrated *in vivo* that they are the main splenic population driving iNKT cell activation following the intravenous introduction of soluble lipid antigens [204] . Lipid antigen presentation by DCs to iNKT cells induce an iNKT response with strong IFN-γ production and additional NK cell transactivation which is qualitatively distinct from the response mediated by other APCs presenting antigens [205]. The uptake of proteins bound to lipids (for example low-density lipoprotein particles), or phagocytosed microorganisms facilitate lipid antigens to be delivered to DCs. The lipid structure can greatly influence how lipid antigens target different APCs and consequently modify the biological outcome. Importantly this study also showed that the activation between iNKT cell -DCs interaction is bidirectional. IL-12 is produced by activated DCs during infections by signals from pattern-recognition receptors (PRRs) increasing the formation of stimulatory lipid ligands for iNKT cells [206]. After iNKT cells recognise endogenous or exogenous lipid antigens presented by CD1d on the surface of an APC, CD40-CD40L interactions lead to an increase of IL-12 production by DCs [204]. IL-12 receptor is expressed on resting iNKT cells and is further upregulated in response to IL-12 derived from DCs. Similarly, ligands for co-stimulatory receptors expressed on DCs lead to iNKT activation [178, 202]. Not only does the interaction between iNKT cells and DCs lead to NK cell transactivation, it also increases the response to protein antigens by conventional CD4+ and CD8+ T cells following DC cross-presentation [205]. Therefore, via these bidirectional interactions iNKT cells and DCs synergize to further enhance both innate and adaptive immune responses. The capacity of activated iNKT cells driving T cell responses could have a significant clinical impact, for instance α-GalCer vaccine has demonstrated to also offer a protective T cell immunity to co-administered protein antigens [180].

Moreover, iNKT cells can influence B cells through their ability to produce IL-4, IL-5, IL-6, IL-13. IL-21 and express CD40L [180, 209]. α-GalCer presentation can induce iNKT-B cell crosstalk, leading to the proliferation and differentiation of B cells in the plasma and the production of high antibody levels Additionally, iNKT cell crosstalk with B cells has been shown to amplify humoral immunity, enhance

maturation of B cells, expand B cell memory pool and increase immunoglobin levels through CD40L interactions and possible IL-4 secretion. [175]. iNKT cells have been shown to provide cognate B cell help via CD40L-CD40 interaction and IFN-γ release for class switching to IgG2a, IgG2c and IgG3 [210].

iNKT cells have been associated with a range of diseases from different types of autoimmunity, allergy, infections, atherosclerosis, transplant, and cancers. There is increasing evidence to that iNKT cells play an essential role in tumour immunosurveillance in humans and in mice. Evidence goes back to 1994 where α-GalCer was injected on B16 melanoma model demonstrating an anti-tumour response [211]. The role of iNKT cells in tumour immunosurveillance was well characterized in mice using genetic knockouts and tumour murine models [209]. For example, adoptive transfer of iNKT cells inhibited the development of methylcholanthrene sarcoma tumours in iNKT cell deficient mice (Jα18−/−)[210]. However, Blankenstein's group called into question the protective role of iNKT cells in methylcholanthrene-induced sarcomas [214]. Moreover, mice lacking iNKT cells are more susceptible to developing hematopoietic cancers and sarcomas via p53 loss [212]. In addition to p53 deficiency model, TRAMP - a pancreatic model, also demonstrated that enhanced tumour growth in iNKT deficient mice ($J\alpha18^{-/-}$ or $CD1d^{-/-}$ mice) compared to wild-type mice illustrating that NKT cells control mouse spontaneous carcinoma independently of tumour-specific cytotoxic T cells [216]. Even though in iNKT cells in KPT mice, another pancreatic model did not show direct cytoxicity against tumours, they did regulate M2-type tumour associated macrophages (TAMs). iNKT cells inhibited the function of IL-10 production driven by SAA released by neutrophils in melanoma cells [217]. SAA is an acute phase protein which increases over 1000- fold from basal levels during infection peaking on the third day after the onset of the acute event and, approximately four days later, levels of SAA restored [218]. The primary role of SAA is to restore of homeostasis. SAA proteins are mainly produced by hepatocytes and adipose tissues and encoded by four distinct genes on chromosome 11 [218, 219]. In humans the dominant isotype SAA1 and SAA2 exist in an acute-phase form and SAA4 is

constitutively expressed at lower levels [220-222]. SAA3 was originally believed to exist only as pseudogene however, SAA3 mRNA was identified in two human mammary gland epithelial cells by Larson *et al.* [223] Pro-inflammatory cytokines tumour necrosis factor alpha (TNFα), IL-1β and IL-6 have been shown to upregulate SAA transcription, thus, it could be assumed that SAA could be linked with different inflammatory and autoimmune diseases such as rheumatoid athritis [224]. Additionally, SAA synthesis in extrahepatic tissues has also been reported in human cancer cell lines and carcinoma metastases [218]. In chronic inflammation, however, which is a driving force in tumour development, SAA levels increase substantially [218]. In addition to iNKT cells suppressing levels SAA by neutrophils in melanoma [217], studies have also reported increased SAA levels in both solid tumours and haematological malignancies such as in colon, breast, lung, pancreas, prostate, ovary, melanoma, Hodgkin's disease, and non Hodgkin's disease [225, 226].

iNKT cells have been shown to mediate effective antitumour immunity in three mechanisms: direct tumour lysis, by recruiting and transactivating other immune cells and inducing a Th1 cytokine cascade and targeting immunosuppressive cells in tumour microenvironment (TME) [227].

An association between the frequency and or function of iNKT cells with overall survival of patients has been observed such as in prostate cancer, neuroblastoma, medulloblastoma, lung, melanoma, breast, colon, lung and head and neck squamous cell carcinomas (HNSCC) [216]. HNSCC patients which have reduced circulating iNKT cells frequencies prior to radiation therapy demonstrate poor 3-year survival in contrast to patients with higher circulating levels of iNKT cells [217, 218] .

The direct and spontaneous role of iNKT cells in inducing an innate immune response was also demonstrated against blood cancers such as CLL, B/T lymphomas and multiple myeloma [219-223]. These studies reveal that iNKT cells have the ability to regulate or delay development of early stage or premalignant disease in a CD1d-dependent manner, as demonstrated using mouse models and iNKT cells isolated from patients [236]. Moreover, a correlation between the ability of innate

immunity to modulate blood cancers and functional capacity of iNKT cells to produce inflammatory cytokines such as IFN-γ and TNFα, as well as the induction of IL-12 production in APCs was observed [221, 225, 226]. Further to the immunostimulatory responses, activated iNKT cells acquire direct cytotoxic activity against blood cancers by releasing cytolytic molecules such as granzyme B and perforin, and through the interaction of death inducing receptors FAS and TRAIL [224, 227].

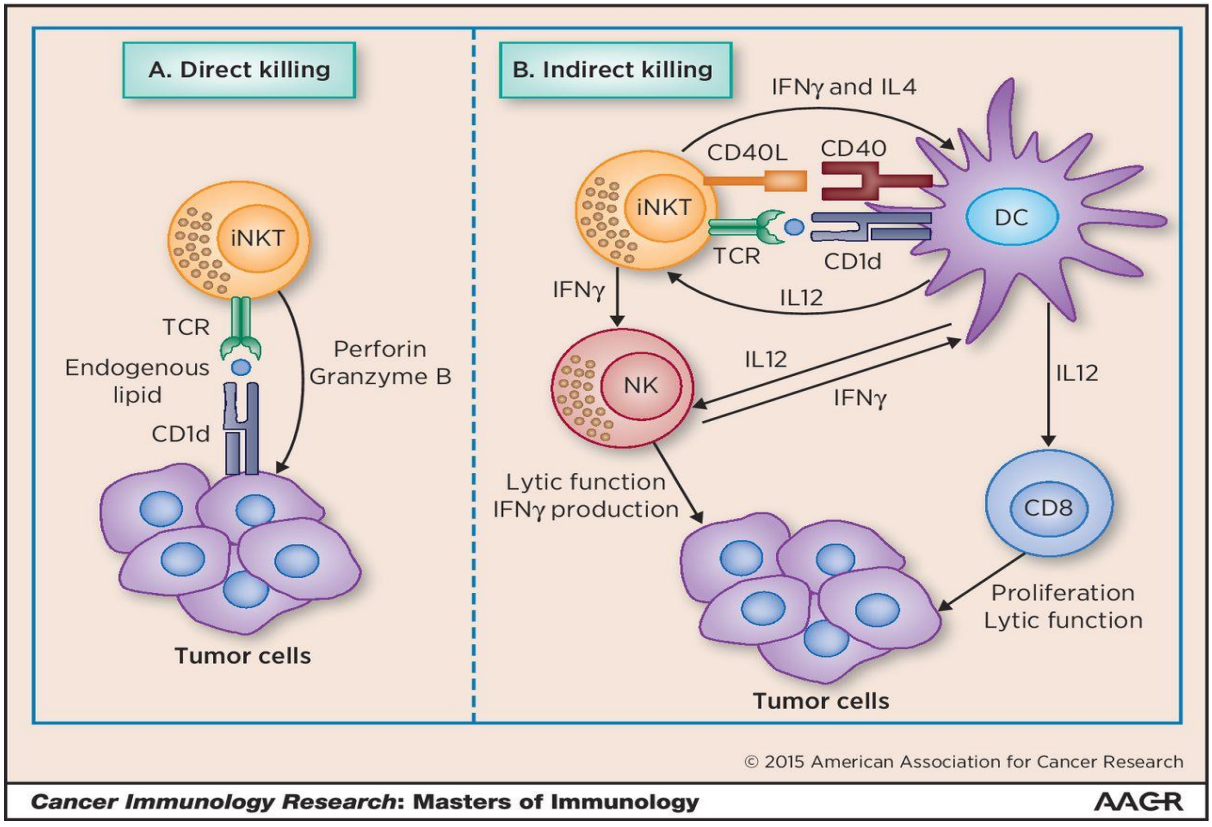


Figure 1.7: iNKT cells mediate anti-tumour responses [221]. (Taken from: Ewen-Smith, R.M., M. Salio, and V. Cerundolo, ***The regulatory role of invariant NKT cells in tumor immunity***. Cancer immunology research, 2015.
a)CD1d present lipid antigens on tumour cells to iNKT cells then iNKT cells directly kill iNKT tumour cells. B) iNKT cells kill tumours in a CD1d-independent manner by becoming activated through crosstalk with to CD1d-expressing or TLR-activated APCs. This leads to a bidirectional activation of APCs driving NK-cell and T cell activation initiating tumour-specific response, killing the tumour.

Interestingly, there is evidence to suggest that iNKT cells could play a protective role in AML. It was shown that in paediatric leukaemia patients (including AML) relapsed when failed to replenish their iNKT cells post HLA-haploidentical stem cell transplant [240]. In addition, Maldonado and group have demonstrated that low levels of iNKT in the peripheral blood of adult *de novo* AML patients correlate with poor overall survival rates [229]. These finding suggest that the absence iNKT cells could contribute to AML regrowth and emphasises the significance of iNKT cells in tumour immunosurveillance and AML immunotherapy. Taking advantage of the diverse and protective function of iNKT cells against the immune system, they can be used as novel immunotherapy. Although there is an increasing understanding of the role iNKT cells play in controlling haematological malignancies, such as multiple myeloma, less is known about their impact in AML [224, 230]. Thus, it is vital that we comprehend the role of iNKT cells in AML in order to effectively guide their effective functions and develop immunotherapeutic strategies to target AML. Therefore, it is important to understand how to effectively guide their effector functions in order to develop novel immunotherapeutic strategies.

Hypothesis and objectives.

Recent advances in the field of immunotherapy promising approach to target cancer [243]. iNKT cells are a unique population of immune cells, viewed as orchestrators of the immune system. They not only have the ability to exert cytotoxic functions, but also activate other immune cells such as DCs, NK cells and B cells be releasing a broad array of cytokines, thus amplifying the immune response [242]. There is increasing evidence demonstrating the key role of iNKT cells in mediating an anti-tumour response. More importantly AML patients with low numbers of iNKT cells are associated with poor survival rates [229]. We hypothesise that iNKT cells could have the potential to mount a strong anti-AML response which can be exploited as an effective immunotherapy, given that there is a great need for the development of clinical effective therapy. In contrast to conventional T cells, we believe that iNKT cells can adapt to the low arginine microenvironment driven by AML blasts, remain proliferative and functional while directly eradicating AML blasts.

With this notion as a starting point, this study is aims to:

1) Identify factors and mechanisms contributing to the AML microenvironment.
2) Demonstrate that iNKT cells can adapt to the low arginine AML microenvironment.
3) Determine that iNKT cells can directly interact with AML blasts and elicit cytotoxicity against leukaemia tumour cells

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Cell Culture and cellular assay reagents

2.1.1a Cells

Cells	Sources
Human peripheral blood	NHS National Blood Services or healthy donors from the University of Birmingham
AML patient blasts	Queen Elizabeth Hospital and Birmingham Childrens Hospital
	Additional cohort of AML patients ineligible for intensive chemotherapy treated with either azacitidine or azacitidine and vorinostat in a multi-centre, randomised phase II trial (RAVVA; NCT01617226)
AML Cell Lines	DMSZ

2.1.1b Tissue Culture Reagent

Reagent	Supplier
1x Phosphate Buffered Saline (PBS)	SIGMA
RPMI-1640 (with L-Glutamine and $NaHCO_3$)	SIGMA
RPMI-1640 for SILAC	Thermo Fisher Scientific
Serum free RPMI-1640 (no phenol)	SIGMA
Foetal Calf Serum (FCS)	SIGMA
Human Serum	Sigma
Dialyzed FCS	Sigma

L-Glutamine 100X	SIGMA
Penicillin/ Streptomycin (5000U/ml) (100x)	SIGMA
Sodium Pyruvate (100m) (100x)	SIGMA
HEPES (1M) (100x)	SIGMA
MEM non-essential amino acids solution (100x)	Thermo Fisher Scientific
β-mercaptoethanol (50mM) (1000x)	Thermo Fisher Scientific
Lymphoprep™	STEMCELL Technologies
Phytohemagglutinin, (PHA-M)	Thermo Fisher Scientific
Trypan Blue	Thermo Fisher Scientific
RBC lysis buffer	QIAGEN

2.1.1c Composition of Tissue Culture Media

Medium	**Composition**
Culture Medium (for AML cell lines)	RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS, Penicillin/Streptomycin, L-Glutamine, 1mM sodium pyruvate.
Complete Medium (RPMI10%)	RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS, Penicillin/Streptomycin, L-Glutamine, 1mM sodium pyruvate, 0.005μM β-mercaptoethanol, 5mM HEPEs, 1xMEM non essential amino acids.
Low arginine Medium	75% of RPMI-1640 for SILAC supplemented with 10% (v/v) dialyzed FCS, Penicillin/Streptomycin, L-Glutamine, 1mM sodium pyruvate and 25% of RPMI10%.
Citrulline Free Medium	RPMI-1640 supplemented with 10% (v/v) dialyzed FCS, Penicillin/Streptomycin, L-Glutamine, 1mM sodium pyruvate.
Medium for seahorse assay	serum free RPMI 1640 media with 5 mM glucose, 2mM glutamine, 1mM sodium pyruvate and 5mM HEPES.

2.1.1d Cytokines and cellular assay reagents

Reagent	Catalogue No.	Supplier
Human IL-2	200-02	Peprotech
Human IL4 (for DC differentiation	200-04	Peprotech
Human GMCSF (for DC differentiation	300-03	Peprotech
Recombinant Human Apo-SAA (SAA)	300-13	Peprotech
α-galactosylceramide (α-GalCer)		BioVision
Anti-human CD3 (Clone OKT)	16-0289-85	eBiosciences
Anti-human CD28 (Clone28.2)	16-0289-81	eBiosciences
CellTrace™ CFSE cell proliferation kit	C34554	ThermoFisher Scientific
BD GolgiStop™	555028	BD Biosciences
BD GolgiPlug™	554724	BD Biosciences

2.1.2 ELISA, ELISpot and LegendPlex reagents

2.1.2a Buffers for ELISAs

Buffer	Composition
5 X ELISA coating buffer	Biolegend (421701)
ELISA stop solution	Sulphuric acid (SIGMA)diluted with dH_20 1:20
Substrate solution for ELISA	1x TMB (3,3',5,5'Tetramethylbenzidine) from SIGMA (T0440)
ELISA blocking buffer	PBS containing 10% (v/v) FCS
ELISA washing buffer	0.1% PBS-T

2.1.2b ELISA

ELISA, ELISpot and LegendPlex Kits	Catalogue No.	Supplier
Human SAA ELISA	KHA0011	ThermoFisher Scientific
Human Arginine ELISA	K7733	Immundiagnostik
Human Perforin ELISA	3465-1H-6	Mabtech
Human IL-13 ELISA	DY213-05	R&D Systems
Human GM-CSF ELISA	DY215-05	R&D Systems
Human G-CSF ELISA	DY214-05	R&D Systems
Human TNF-α ELISA	DY21-05	R&D Systems
Human Granzyme ELISpot	EL2906	R&D Systems
Human IFN-γ ELISA	430104	Biolegend
Human IL-1β ELISA	437004	Biolegend
Human TSLP	434204	Biolegend
Huma Th cytokine panel	740001	Biolegend
Human cytokine panel 2	740102	Biolegend

Mouse anti-virus panel	740622	Bioloegend

2.1.3 Flow Cytometry, magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) sorting reagents.

12.1.4a Materials for MACs and FACS

Material	Description
MACS Buffer	PBS containing 0.5% BSA and 1µM EDTA
MACS MS columns	From Miltenyi Biotec
MACS LS columns	From Miltenyi Biotec
FACS Buffer	PBS containing 10% (v/v) FCS

2.1.3b Antibodies for MACS and FACS

Macs Beads	Catalogue No.	Supplier
Anti-human iNKT microbeads	130-094-842	Miltenyi Biotec
Anti-human CD14 microbeads	130-050-201	Miltenyi Biotec
Pan T cell isolation kit, human	130-096-535	Miltenyi Biotec

FACS Antibody	Conjugate	Supplier
α-GalCer/Cd1d tetramer	APC	NIH Tetramer service

Antibodies for Flow cytometry

Antibody	Clone	Isotype	Conjugate	Supplier
Primary Antibodies				
Anti-human CD33	WM53	Mouse IgG1	APC	Biolegend
Anti-human CD34	561	Mouse IgG2a	APC	Biolegend
Anti-human CD284 (TLR4)	HT A125	Mouse IgG2a	APC	Biolegend
Anti-human α-GalCer/CD1d tetramer (hCD1d PBS-57)			APC	NIH Tetramer service
Anti-huma IFN-γ	B27	Mouse IgG1	APC	Biolegend
Anti-human CD38	HB-7	Mouse IgG1	APC/Cy7	Biolegend
Anti-human Cd154	24-31	Mouse IgG1	BV-510	Biolegend
Anti-human CD40	5C3	Mouse IgG1	FITC	Biolegend
Anti-human CD282 (TLR2)	TL2.1	Mouse IgG2a	FITC	Biolegend
Anti-human CD8	SK1	Mouse IgG1	FITC	Biolegend
Anti-human TCR Vα24	REA948	Human IgG1	FITC	Miltenyi Biotec
Anti-human LAT-1		Human IgG	FITC	J-Pharma
Anti-human/mouse CD3e	APA/1	Mouse IgG1	FITC	Biolegend
Anti-human FPR3 (FPRL2)	K102B9	Mouse IgG2a	PE	Biolegend
Anti-human CD1d	51.1	Mouse IgG2b	PE	Biolegend
Anti-human CD178 (FAS-L)	NOK-1	Mouse IgG1	FITC	Biolegend

Anti-human TCR Vβ11	REA559	Human IgG1	PE	Miltenyi Biotec
Anti-human CD4	RPA-T4	Mouse IgG1	PE	BD Biosciences
Anti-human CD107a (LAMP-1)	H4A3	Mouse IgG1	PE	Biolegend
Anti-mouse CD1d	CD1.1, Ly-38	Mouse IgG2b	PE	Biolegend
Anti-human CD3	UCHT1	Mouse IgG1	PE/Cy7	Biolegend
Anti-human CD69	FN50	Mouse IgG1	PE/Cy7	Biolegend
Anti-mouse α-GalCer /CD1d tetramer (mCD1d PBS-57)			APC	NIH Tetramer service
Anti-mouse CD45.1	A20	Mouse IgG2a	FITC	Biolegend
Anti-mouse CD45.2	104	Mouse IgG2a	APC	eBioscience
Anti-mouse CD11b	M1/70	Rat IgG2b	APC	eBioscience
Recombinant Anti-ASS1		Rabbit IgG	Recombinant	Abcam
Secondary Antibody				
Donkey anti-Rabbit IgG	Poly4064	Donkey Polyclonal	PE	Biolegend

2.1.4 Immunoblotting reagents

Material	**Composition**
RIPA buffer	ThermoFisher Scientific
Protease inhibitors	Roche Applied Science
Phosphatase inhibitors	Roche Applied Science
Albumin Standard	ThermoFisher Scientific
Coomassie Plus Protein Assay Reagent	ThermoFisher Scientific
4 x 4x Laemmli sample buffer	BioRad
4-20% Criterion™ Tris-Glycine eXtended (TGX) stain-free precast gels	BioRad
Protein Ladder	Broad Range BioLabs (P77195)
10X Tris/Glycin/SDS Buffer	Biorad
Wash buffer for immunoblotting	0.1% PBST (PBS + 0.1% (v/v) Tween-20)
Antibody dilution buffer for immunoblotting	0.1% PBST + 5% BSA (w/v)
Blocking buffer for immunoblotting	0.1% PBST + 5% BSA (w/v)
ECL substrate	BioRad
CL-XPosure Film	ThermoFisher Scientific

Antibodies for Immunoblotting

Antibody	Isotype	Catalogue No	Supplier
Primary Antibodies			
Anti-human SAA	Rabbit	SC-59679	Santa Cruz Biotech
Anti-human ASS-1	Rabbit	HPA-020934	Atlas Antibodies
Anti-human OTC	Rabbit	HPA-000243	Atlas Antibodies
Anti-human LAT-1	Rabbit	53475	Cell Signalling Technologies
Anti-human Cleaved Caspase 3	Rabbit	96625	Cell Signalling Technologies
Anti-human Caspase 3	Rabbit	96645	Cell Signalling Technologies
Anti-human Cleaved Caspase 9	Rabbit	9507	Cell Signalling Technologies
Anti-human Caspase 9	Rabbit	9502	Cell Signalling Technologies
Anti-human PARP	Rabbit	9542	Cell Signalling Technologies
Anti-human Arginase1	Rabbit	93668	Cell Signalling Technologies
Anti-human Arginase2	Rabbit	55003	Cell Signalling Technologies
Anti-human β-actin	Rabbit	49705	Cell Signalling Technologies
Secondary Antibody			
Anti-Rabbit IgG (HRP linked)		7074	Cell Signalling Technologies

2.1.5 Antibody for functional assays

2.1.5a Stimulation and Neutralisation Assays

Antibody	Clone	Isotype	Company
Anti-human TLR2	TL2.1	Mouse IgG2a	Biolegend
Anti-human TLR4	HTA125	Mouse IgG2a	Biolegend
Anti-human FPR3	K102B9	Mouse IgG2a	Biolegend
Anti-human CD40	82111	Mouse IgG2B	R &D Systems
Anti-human CD1d	Cd1d42	Mouse IgG1	BD Pharmingen
JPH-203			J-Pharma

2.1.6 Mice

Strain	Description
C57BL/6(B6)	Mice are of the H-2^b haplotype
B6.SJL- PtprcaPep3 b/BoyJ (B6Ly5.1)	These congenic mice express the lymphocyte surface antigen CD45.1 (also referred to as Ly5.1) Ptprca (protein-tyrosine phosphatase, receptor type c, a allele). To distinguish lymphocytes from these mice and C75BL/6 which are CD45.2 positive

2. Methods

2.2.1 Cell Culture

2.2.1a Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood from healthy donors and AML patients (~40 ml) was collected and diluted with 110ml of RPMI. 30 ml of diluted blood was then carefully layered over 15ml of Lymphoprep solution in a 50ml tube. This was spun at 1000g for 30 minutes with no centrifuge brakes. The opaque ring between Lymphoprep and plasma was gently collected. Cells were then washed with 5ml of RPMI and spun for at 400g for 15 minutes. The cell pellet was resuspended in 50ml of fresh RPMI and spun a second time at 350g for 10 minutes to remove any remaining platelets. Cells were resuspended in RPM10% and used for experiments. All blood samples were processed in assays within 12 hours of blood sampling.

2.2.1b Red blood Lysis

For erythrocyte removal from patient whole blood or mouse cells, RBL lysis solution was added at a 1:10 ratio for 10 minutes. Lysis was stopped by adding RPMI10% and cells are spun at 400g for 10 minutes. Single-cell suspension is used for flow cytometry.

2.2.1c Isolation of patient AML blasts

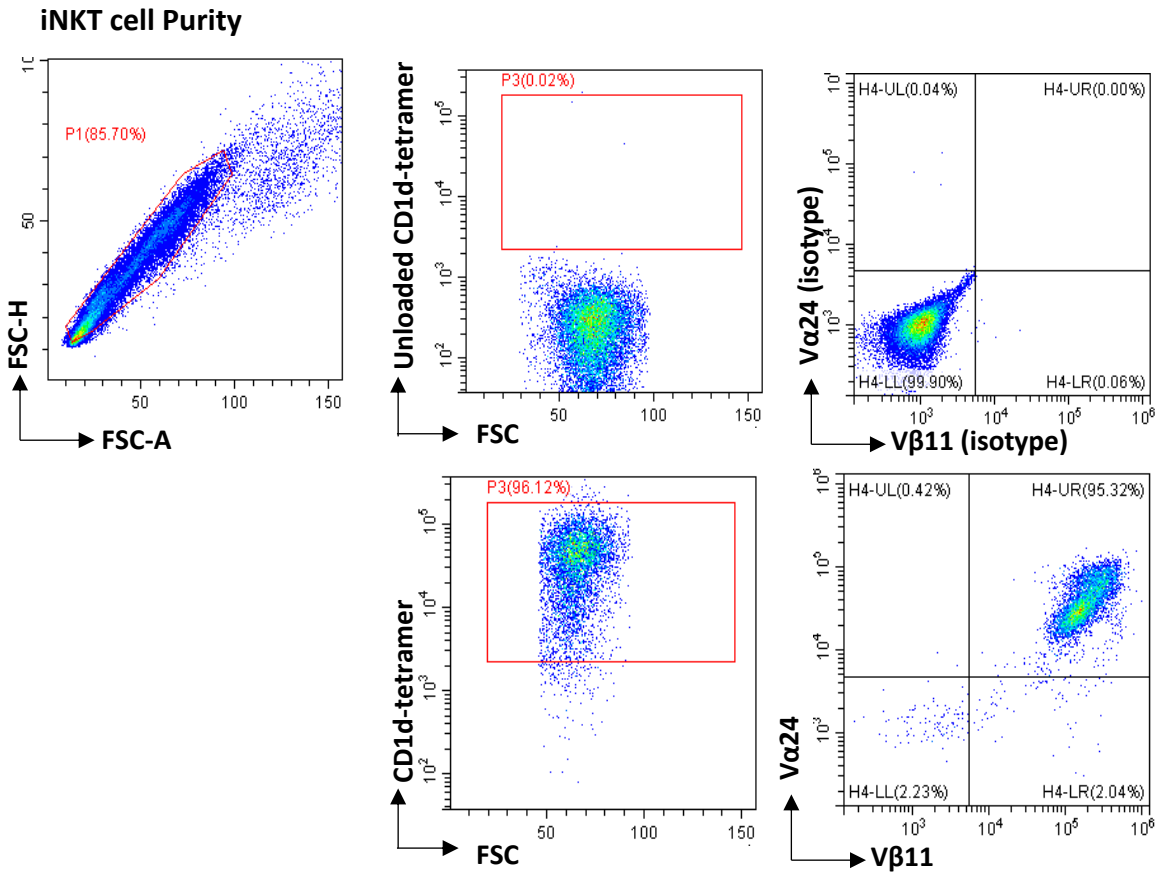
AML blasts were isolated from whole blood donated by AML patients. Total PBMCs were generated by Lymphoprep separation (section 2.2.1a) and enriched by positive selection using an anti-human CD33 or anti-human CD34 magnetic beads and MACS separation (see section 2.2.1h), following the manufacturer's protocol (Miltenyi Biotec).

2.2.1d Expansion and maintenance of human iNKT cells

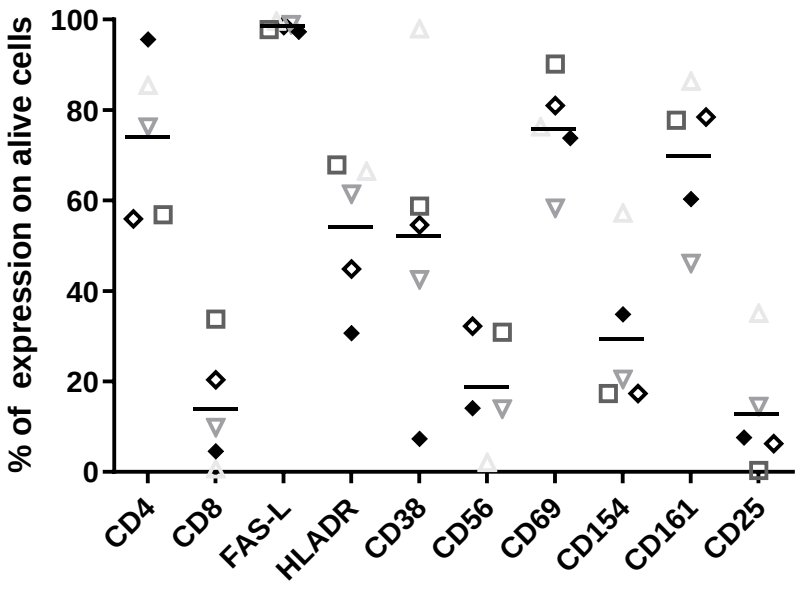
Healthy blood donors were used to generate iNKT cells. Total PBMCs were isolated by Lymphoprep separation (section 2.2.1a). 2 X 10^6 of PBMCs were cultured in 3ml of RPMI10% stimulated with

100ng/ml of α-GalCer. From day 5 to 14, fresh medium supplemented with IL-2 (1000U) was supplemented every three days. After three weeks of culture, iNKT cells were stained with α-GalCer-loaded CD1d tetramer and sorted by fluorescence-activated cell sorting (FACS)(see section 2.21i).

Purified iNKT cells were re-stimulated every 4 weeks by co-culturing them with irradiated PBMCs, supplemented with PHA (1µg/ml) and IL-2. Cells were supplemented with fresh medium with IL-2 every 3 days. iNKT cells used in experiments were 90- 95% positive for α-GalCer/CD1d tetramer, VαJ24 and Vβ11 antibodies.



Phenotype of all iNKT cells after expansion used in this study:



2.2.1e Purification of monocytes

Monocytes were enriched from PBMCs generated by healthy peripheral blood (section 2.2.1a) by positive selection with anti-human CD14 magnetic beads by MACS separation (section 2.21h).

2.2.1f Generation of human monocyte-derived dentritic cells

CD14 positive monocytes were cultured in RPMI10% supplemented with 50ng/ml recombinant GMCSF and 100ng/ml recombinant IL4 at density $2x10^6$ cell/ml in 6 well plates in 2 ml per well and differentiated for 5-7 days at 37°, 5% CO_2.

2.2.1g Isolation of human naïve T Cells

Naïve T cells were isolated from PBMCs by positive selection using Pan T cell isolation Kit by MACS (see section 2.2.1h).

2.2.1h MACS

Cell suspension was centrifuged at 400g for 5 minutes and supernatant is completely aspirated. Cell pellet is resuspended in 500ml of cold MACS buffer. For magnetic labelling, the required magnetic microbeads were added and mixed well followed by 15 minute incubation at 4^{o}C. Cells are then washed by adding 2ml of MACS buffer and spun at 400g for 5 minutes. For magnetic separation LS columns was placed in magnetic field and was calibrated by rinsing it with 5ml of MACS buffer. Cell suspension was then applied onto the column and the unlabelled flow through was collected in a 15ml tube. Column was washed three times with 3ml of MACS buffer and flow through was collected. Column was removed from magnetic field and placed in a fresh tube were 5ml of RPMI10% was added and magnetic labelled cells are instantly flushed through using the plunger. Column-retained for positive selection or the flow-through for negative selection was harvested and washed twice before using the cell suspension for experiments.

2.2.1i FACS

To generate iNKT cell lines, expanded iNKT cells were harvested and stained with α-GalCer/CD1d tetramer (see section 2.2.3a). Following the final wash, cells were resuspended in FACS buffer. Cell clumps were removed after passing them through a 70µm strainer and sorted using the MoFlo Astrios Flow Cytometer Cell Sorter with the help of BioHub at University of Birmingham.

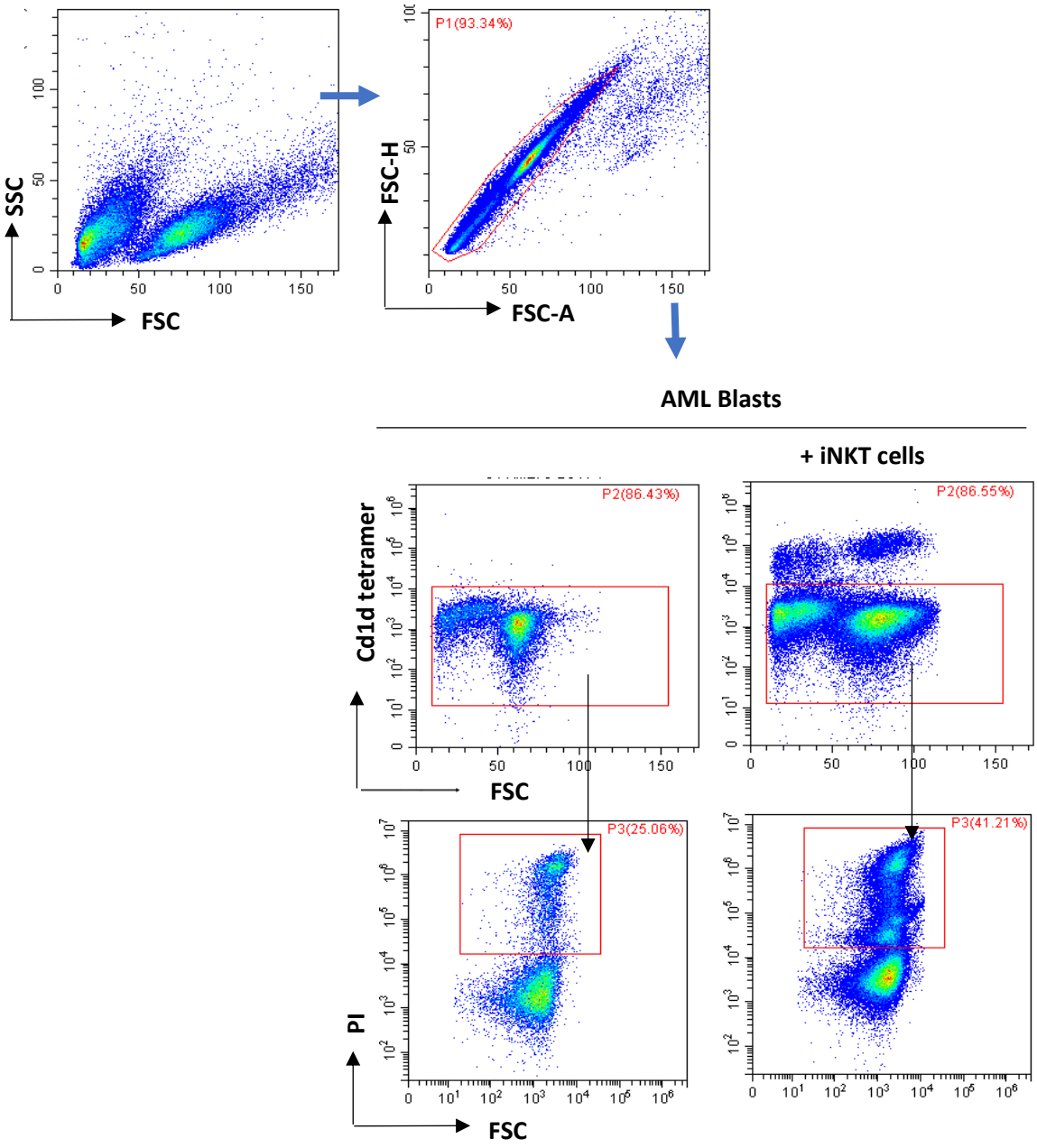
2.2.2 Cellular Assays

2.2.2a AML Blasts -iNKT co-culture assays

0.2X10^{6} AML blasts were either cultured alone or co-cultured with iNKT cells (effector: target ratio:0.25: 1 or at stated ratio) in final volume of 200µl of RPMI10% per well in a 96 well plate flat bottom for 72 hours. For antigen mediated iNKT cell activation, potent lipid antigen α-GalCer was used. α-GalCer was sonicated for 15 minutes to break liposomes formed while stored and AML blasts were pulsed with 100ng/ml of α-GalCer for 4 hours. AML blasts were washed and co-cultured with

iNKT cells as above. Supernatants were harvested and spun at 400g for 5 minutes to remove any contaminant cells and stored at -20°C to determine cytokine release. The cells were washed and resuspended in FACS buffer. AML blast viability or iNKT cells frequency was determined by flow cytometry were relevant. Gating strategy used for flow cytometry analysis is shown below. (Fig 2.1).

Gating Strategy



2.2.2b Neutralisation assays

AML Blasts at density 0.2 $X10^6$ in 200µl in a 96 well flat bottom plate were pre-incubated with blocking antibodies (anti-TLR2 (10µg/ml), anti-TLR4 (10µg/ml), anti-FPR2 (10µg/ml), anti-CD1d (5µg/ml) and anti-CD40 (6µg/ml)) for 1 hour before co-incubating with iNKT cells and α-GalCer for 72hours. The neutralising antibodies were kept in culture during stimulation. Cells were washed and resuspended in FACS buffer and viability of AML blasts was determined by flow cytometry. JPH-203 (0.125mM) was added to CD3(3µg/ml) CD28 (2µg/ml) stimulated iNKT cells for 96 hours. Supernatants were harvested for cytokine analysis. Cells were washed and resuspended in FACS buffer, viability and proliferation was determined by flow cytometry.

2.2.2c Mixed leukocyte Reaction

T cells were isolated from healthy donors by MACS as described above (see section 2.21h). $2x10^5$ T cells, enriched by positive selection, were cultured with allogenic irradiated (5000 rad) dendritic cells ($0.5x10^5$), in 200µl RPMI 5% human serum in 96 well flat bottom plates. Cells were incubated at 37°C, 5% CO2 for 4 days and then 1mCi/ well ^{3}H-thymidine (Perkin Elmer Life Sciences) was added for 12-16 hours. ^{3}H-thymidine incorporation was measured using a Wallac Microbeta Jet 1450 reader (Perkin Elmer). AML inhibition of T cell proliferation was carried out by co-culturing AML blasts from patients with T cells and irradiated DCs. To test the ability of iNKT cells to overcome AML-induced suppression of T cell proliferation. $0.25x10^5$ iNKT cells were first co-cultured with AML blasts ($1x10^5$) for 8hours, in the presence of α-GalCer (100ng/ml). After 8 hours the cells were washed and T cells ($2x10^5$) and DC ($0.5x10^5$) were added to cultures. Data are expressed as a percentage of T cell proliferation driven by allogenic irradiated DCs in the presence of AML cells, as compared to allogenic T cell proliferation in the absence of AML cells (100%).

2.2.2d Cell tracking

iNKT cells were harvested and washed with PBS twice to remove serum and resuspended in 5 $X10^6$ /ml of PBS. Cells for tracking were stained with 5µM of CellTrace CFSE for 20 minutes in the dark at 37°C. Dye was quenched by adding 4 volumes of RPM10% and incubating for 5 minutes. Cells were washed twice with RPMI10% and then cultured at 37°C, 5% CO2.

2.2.3 Analysis of Cellular and Cytokine Content

2.2.3a Flow Cytometry

Cells (maximum $1X10^6$) were harvested and transferred to a round bottom 96-well plate, washed with 200µl of FACS buffer and spun at 400g for 5 minutes. The appropriate conjugated fluorescent antibody was added at a predetermined optimum concentration for 20 minutes at 4°C. Stained cells were washed with FACS buffer and resuspended in 200µl of FACS buffed for analysis. For CD1d/α-GalCer tetramer staining, cells were firstly incubated with of CD1d/α-GalCer tetramer for 20 minutes at 37 °C. Cells were then washed in FACS buffer and stained with monoclonal antibodies, as outlined above. Viability was measured using propidum iodide (at 5µg/ml) added before analysis to stain. Unstained, isotype antibodies and single conjugated fluorescent antibodies were used as control. Samples were read on ACCURI, CYAN, and CYTOFLEX flow cytometers and analysed using FlowJo, BD Accuri and CytoFlex software.

2.2.3b Intra Cellular Staining (ICS)

To measure intracellular cytokine, protein transport inhibitors BD GolgiStop™ and/or BD GolgiPlug™ were added to the cell culture for 6 hours according to the manufacturer's protocol. Cells were harvested and surface antigen staining was carried out as above. Cells were fixed and permeabilised by resuspending cells with the 200µl of Fixation/ Permeabilization solution for 20 minutes at 4°C. Cells were washed twice with 150µl of BD Perm/Wash™ buffer. Cells were resuspended in 50 µL of BD Perm/Wash™ buffer and stained with pre-determined optimal concentration of a fluorochrome-

conjugated antibody and for purified the relevant secondary antibody staining followed. Cells were washed with 150µl of BD Perm/Wash™ buffer and resuspended in 200 µl of FACS buffer for analysis. ACCURI, CYAN, and CYTOFLEX were used to read samples and FlowJo, BD Accuri and CytoFlex software.

2.2.3c ELISA

Cytokine detection assays

For detection of multiple cytokines in plasma or culture supernants LegendPlex multiplex flow cytometry was used human Th cytokine panels and murine Anti-virus response panel). The concentration of TNFα, IL-1β, human and mouse IFN-γ, GMCSF, SAA andIL-13 and was measured by ELISA according to the manufacturer's instructions. In brief ELISA plates were incubated with 25µl of diluted capture antibody from the ELISA kit overnight at 4^0C. The capture antibody was aspirated and the wells were washed three times with ELISA wash buffer before adding 150µl of blocking buffer (FACS). The plate was covered and incubated at 37^0C for 3 hours. Solution was aspirated and 25µl of standards and supernatants were added and incubated overnight at 4^oC. Wells were washed three times. 25µl of diluted detection antibody was added and incubated at room temperature for one hour. The liquid was aspirated and wells were washed three times. 25µl of HRP conjugate was added to the wells and incubated in the dark at room temperature for 30 minutes. Plates were washed 5 times before adding 50µl of TMB solution. The plate was developed by incubating it in the dark up to 15 minutes. ELISA stop solution was added to terminate the enzyme substrate reaction after attaining the desired colour intensity. The plate was then evaluated by reading the absorbance at 450nm using a microplate reader.

2.2.3.d Arginine ELISA

The concentration of arginine in human and murine plasma or culture supernatants was quantified using a competitive enzyme immunoassay following manufacturers' instructions. In brief, the assay

uses a competitive enzyme immunoassay in which L-arginine is derivatized from samples and competes with an L-arginine-tracer for binding of polyclonal antibodies. The concentration of the tracer-bound antibody is inversely proportional to the L-arginine concentration in the samples. Absorbance was measured at a wavelength of 450nm using a spectrophotometer.

2.2.3.e Granzyme B ELISPOT

Granzyme B release was measured by ELISPOT according to manufacturer's instructions. In brief, 96 well PDVF plates coated with a Granzyme B polyclonal antibody. 2X 10^5 patient AML blasts and AML cell lines were plated in the presence of 0.25x10^5 iNKT cells. After 24 hours the plates were washed, and biotinylated detection antibody added. Following washing, avidin-HRP solution was added, and incubated for 45minutes. Following a further wash, substrate solution was added, and left to develop until spots, at which point the reaction was terminated by washing, and the plate was left to dry. Images of spots were taken using an ELISPOT reader (AID).

2.2.3f Immunoblotting

Preparation of cell lysates

Cells (1 x10^6) were harvested from experiment and spun at 600g for 5 minutes and pellet was washed with PBS. Cells were lysed by adding 1x RIPA buffer with 1x protease inhibitors and 1x phosSTOP™ phosphatase inhibitors and incubated on ice for 30 minutes followed by a 10 minute spin at 600g at 4^{o}C. Protein concentration was calculated by mixing 5µl of cell lysate with 250ml of Bradford reagent. Absolute protein concentrations were obtained by using a BioRad microplate reader to measure the absorbance at 595nm. Relative standard curve from known concentrations of BSA. Samples were normalised to the same protein concentration by diluting with cell lysis buffer.

Immunoblotting

4x laemmli sample buffer was added to 20µg/ml of cell lysate and heated for 10 minutes at 95°C. A protein ladder (3µl) enabling the identification of the molecular weight of target proteins and denatured samples were loaded on Criterion™ Tris-Glycine eXtended (TGX) stain-free precast gels precast gels (4-20%). Gels were run in 1x tris-glycine running buffer and proteins migrated through the gel at 150V until loading dye reached the bottom of the gel.

Proteins were transferred from gel onto 0.22µm PVDF membrane using ready to use Trans-Blot Turbo Midi transfer packs along with the Trans-Blot Turbo Transfer System from Bio Rad at 25V for 7 minutes.

Membranes were incubated with gentle agitation in blocking buffer consisting of 5% BSA diluted in 0.1%PBS-Tween for one hour at room temperature. Following blocking membranes, primary antibody diluted to appropriate concentration was incubated overnight at 4°C with gentle agitation. The next day wash buffer was used to wash the membrane three times for 10 minutes on rocking platform. The relevant HRP-conjugated secondary antibody was added to membranes and incubated for one hour with gentle agitation at room temperature. Membrane was washed three times with wash buffer for 10 minutes each.

The membrane was developed by adding 6µl of enhanced chemiluminescence (ECL) reagent mixture and exposed on x-ray film or imaged on a Chemidoc MP system (BioRad) and images analysed using the ImageLab software v4.1.

2.2.3g Transition Electron Microscopy

After harvesting, cells ($2X10^6$) were pelleted at 400g for 5 minutes and fixed by gently layering primary fixative 2.5% glutaraldehyde followed by 1% osmium tetroxide. Samples were dehydrated by ethanol and embedded in propylene oxide/resin mixture at 60°C for 16 hours before sectioning at 80 nm in thickness and placed on 300 mesh copper slot grids for investigation via transmission electron microscopy. Fixation, dehydration, embedding and sectioning was completed by Theresa Morris at the Centre of electron microscopy at University of Birmingham.

2.2.3h Arginase Activity Assay

The presence of arginase activity within AML patient blasts and AML cell lines (THP1, U937 and Nomo) was determined by measuring the conversion of arginine into urea. AML blasts were cultured in the presence or absence of SAA (10µg/ml) for 48hours in RPMI10% at 37^0C, 5% CO_2, cells were spun at 400g for 5 minutes. Pellets were resuspended with 50µl of lysis buffer (0.1% Triton X-100, protein inhibitors) was added to the cell pellets for 30 minutes at 37°C. Cells were centrifuged for 5 minutes at 400g. To activate the arginase enzyme, buffer consisting 50µl Tris-Hcl (25mM) and 10µl MnCl2 (10mM) were added to 50µl reaction mixture and incubated for 10 minutes at 56^0C. 100 µl L-arginine (0.5M) was added to each mixture and samples were incubated for 1 hour at 37°C. The hydrolysis of arginine by arginase was stopped by adding 800µl of blocking buffer (H2SO4-H3PO4-H2O, 1:3:7). The amount of urea produced was determined adding 40µl of 9% a-isonitrosopropriophenone and compared to a standard curve. Absorbance was measured at a wavelength of 540nm using a spectrophotometer.

2.2.3i ASS-1 activity assay

The activity of ASS-1 within iNKT cells was determined by measuring the catabolism of citrulline. $5X10^6$ iNKT cells were cultured at with $5X10^6$ AML cells (THP1, U937 and NOMO) with and without α-GalCer (100ng/ml) for 72hours. iNKT cells were positively selected with anti-human iNKT beads

according to manufactures protocol and lysed with 20 µl of lysis buffer (0.1% Triton X-100, 5 µg pepstatin, 5 µg aprotinin and 5 µg antipaina) on ice for 20 minutes. To 20 µl of each sample lysate supernatant, 10µl of L-citrulline (4mM, pH 7.5), 10µl of L-aspartic acid (4mM, pH 7.5), 10µl of $MgCl_2$ (6mM), 10µl of ATP (4mM, pH 7.5), 40µl of Tris-HCl (20mM) was added and incubated for 90 minutes at 37^{o}C to allow the enzyme reaction to occur. The hydrolysis of citrulline was stopped with 80 µl of an acid solution mixture of phosphoric acid and sulphuric acid (3:1) and 20µl of 3% 2,3butanedione monoxime, followed by vortexing and incubation at 95^{o}C for 30 minutes. The amount of citrulline at the end of the assay was determined by comparison to a standard curve with absorbance measured at 490 nm.

2.2.3j Seahorse assay

AML cell lines THP1 and KG-1a were pulsed with α-GalCer (100ng/ml) for 4 hours before being washed and co-cultured with iNKT cells at 0.25:1 ratio for 24 hours. iNKT cells were positively selected with anti-human iNKT cell microbeads according to manufacturer's instructions and immobilised onto CellTak-coated (Corning) Seahorse XFe96 plates (Agilent) at a density of 0.25x106. Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured on a XFe96 Extracellular Flux Analyzer (Agilent) in media prepared for sea horse assays at the Mitochondrial profiling centre, University of Birmingham with the assistance of Dr Jonathan Barlow. Measurements were obtained under basal conditions and upon Oligomycin (2µM), BAM 15 (3µM), Rotenone and Antimycin A (both 2µM) staggered injections during the period of 80 minutes.

2.2.4 Preparation of samples from *in vivo* experiments

2.2.4a AML mouse model-MLL-AF9

Generation of immortalised MLL-AF9 murine AML cell line has been previously described. REFS.. MLL-AF9 cells were thawed, resuspended and 0.5 X10^6 MLL-AF9 cells were transplanted into sublethally irradiated (4.5Gy) B6.SJL-Ptpr^{a} Pepcb/BoyJ (CD45.1) mice recipients. These mice either received two

doses of a-GalCer (2μg/mouse) or vehicle (PBS) on day 5 and 10. On day 17, blood was obtained T cells and iNKT cell was measured measured in the bone marrow, thymus, and spleen in addition to AML donor cells (CD45.2+). *In vivo* experiments were carried out in collaboration with Dr Karen Keeshan and group, at The Univesity of Glasgow who have years of experience on establishing this AML murine model. After mice were sacrificed mice organs were transferred to the University of Birmingham where we harvested the required cells and analysed for the required expression markers.

2.2.4b Establishing low arginine conditions in mice with recombinant arginase

A human recombinant arginase (BCT-100) was used to systemically deplete arginine, C57BL/6 mice were intravenously injected with 5 mg/kg BCT-100 from day 0 to day 5 every 24 hours and untreated mice were used as control. On day 3, α-GalCer (2μg/ml) or vehicle was intravenously injected in both groups. On day 5 mice were culled and organs were harvested. *In vivo* experiments were carried out by AXISBIO (Belfast), after mice were sacrificed mice organs were transferred to the University of Birmingham where we harvested the required cells and analysed for the required expression markers.

2.2.4c Harvesting Mouse Organs

Hind legs were dissected from mice and washed with PBS. Bone marrow was then flushed through a syringe needle with RPMI. Debris was removed by passing the cell suspension through a 70µM cell strainer and washed with RPMI. Thymus, spleen and liver were harvested from mice and washed with PBS. The organs were mechanically homogenised by the head of a 5ml syringe and washed with RPMI. Cells were spun for five minutes and red blood cells were lysed by adding 2ml of RBC lysis buffer for 10 minutes. Cells were spun for 5 minutes at 400g, the pellet was resuspended in RPMI10%, passed through a 70µM strainer and processed for flow cytometry (see section 2.2.3a).

2.2.5 Ethics

Consent was taken from all healthy and AML patients donating blood samples and ethical approval was done under the granted study: REC: 10/H0501/39.

2.26 Statistical Analysis

All experiments were statistically analysed using GraphPad Prism. Statistical significance was determined via two tailed paired or unpaired Student's t-test and Mann Whitney test. The cut off for p value was $p < .05$.

Chapter 3

Results

AML patients express raised levels of SAA.

Our group has previously established that arginine catabolism via ARG2 drives both viability of AML blast and suppression of peptide antigen-specific and unspecific T-cell responses in patients [74]. Following on from these findings, levels of arginine in plasma of AML patients at diagnosis were measured and compared to aged matched healthy donors by ELISA. Significantly lower arginine (p=0.003) levels were detected in plasma of AML patients (on average 86μmol/l) compared to healthy controls (on average 130 mol/l) (Fig. 3.1).

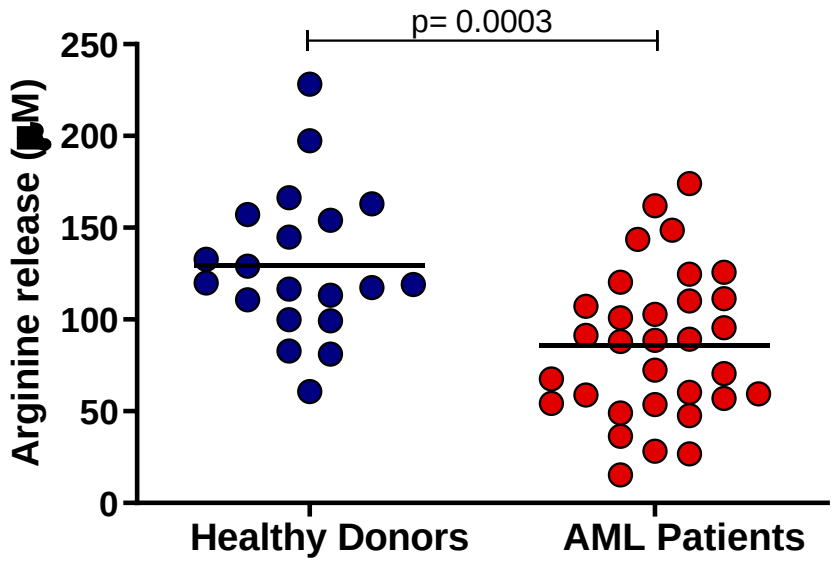


Figure 3.1: Arginine levels are impaired in plasma of AML patients.
Arginine levels in the plasma of healthy donors (n=20) and AML patients (n=32) was measured by ELISA. Arginine levels are decreased in plasma AML patients compared to healthy donors. Horizontal line across samples represents the grand mean. Statistics performed by unpaired t-test. Statistical significance considered when: $p<0.05$.

Even though the immunomodulatory activity of ARG2 has since been described in several pathological settings, the factors which regulate enzyme expression are still poorly understood especially in AML. Inflammatory cytokines can drive malignant transformation or expansion,

consequently, exploring and establishing factors controlling ARG2 expression in AML blasts is necessary. Plasma of AML patients was evaluated using ELISAs and a bead-based immunoassay for different cytokines.

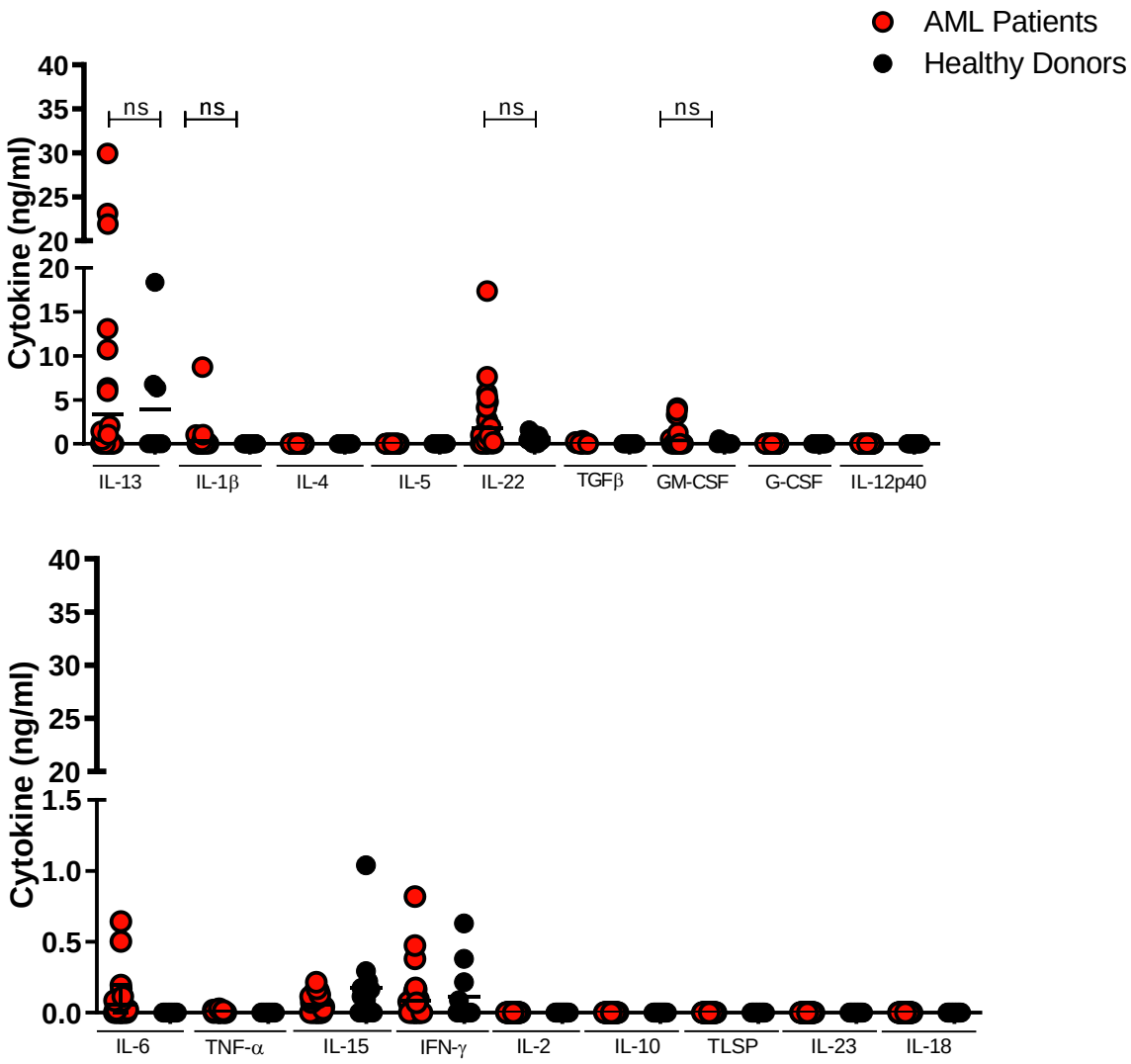


Figure 3.2: Cytokine profile in plasma of AML patients and healthy donors.
Plasma from AML patients (n=35) at diagnosis and healthy donors (n=8) was analysed for different cytokines using a multiplex assay with fluorescence–encoded beads and ELISAs. Plasma analysis revealed no difference in cytokine release between healthy donors and AML patients. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

No statistical significant differences were identified in the concentrations of Th1/ Th2 cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-12p40, IL-15, IL-18, IL-22, IL-23, IFN-γ, IL-1β, G-CSF, GM-CSF, TGF-β, TNF-α and TLSP) compared to healthy controls (Fig. 3.2). An increase in acute-phase proteins is a common characteristic of inflammation in the tumour microenvironment [232-234]. Given that the acute phase protein SAA is raised in the plasma of melanoma patients, and can modulate the phenotype of immunosuppressive myeloid cells, levels if SAA in plasma of AML patients were measured by ELISA [217]. SAA levels were significantly higher (p=0.0001) in AML patients (on average 76.1μg/ml) compared to healthy donor controls (on average 16.8μg/ml) (Fig. 3.3a). These results were revalidated in a second cohort of AML patients involved in a clinical trial, before and after the first and second cycle of Azactidine (AZA)/Vorinostat treatment. Each cycle of treatment consist of 22 days where AZA is administered on day 1 and Vorninostat day 8, blood from patients was collected at the end of each cycle (day 22) before starting treatment with the next cycle. However, despite treatment, no increase in overall response or survival rate was observed [235].

Administrating AZA/Vorinostat did not alter SAA levels. Prior to treatment on average 81.6 μg/ml of SAA was measured in AML patient plasma, while treatment plasma levels of SAA following the first and second cycle of AZA/Vorinostat were on average 71.6 μg/ml and 71.5 μg/ml respectively (Fig 3.3b).

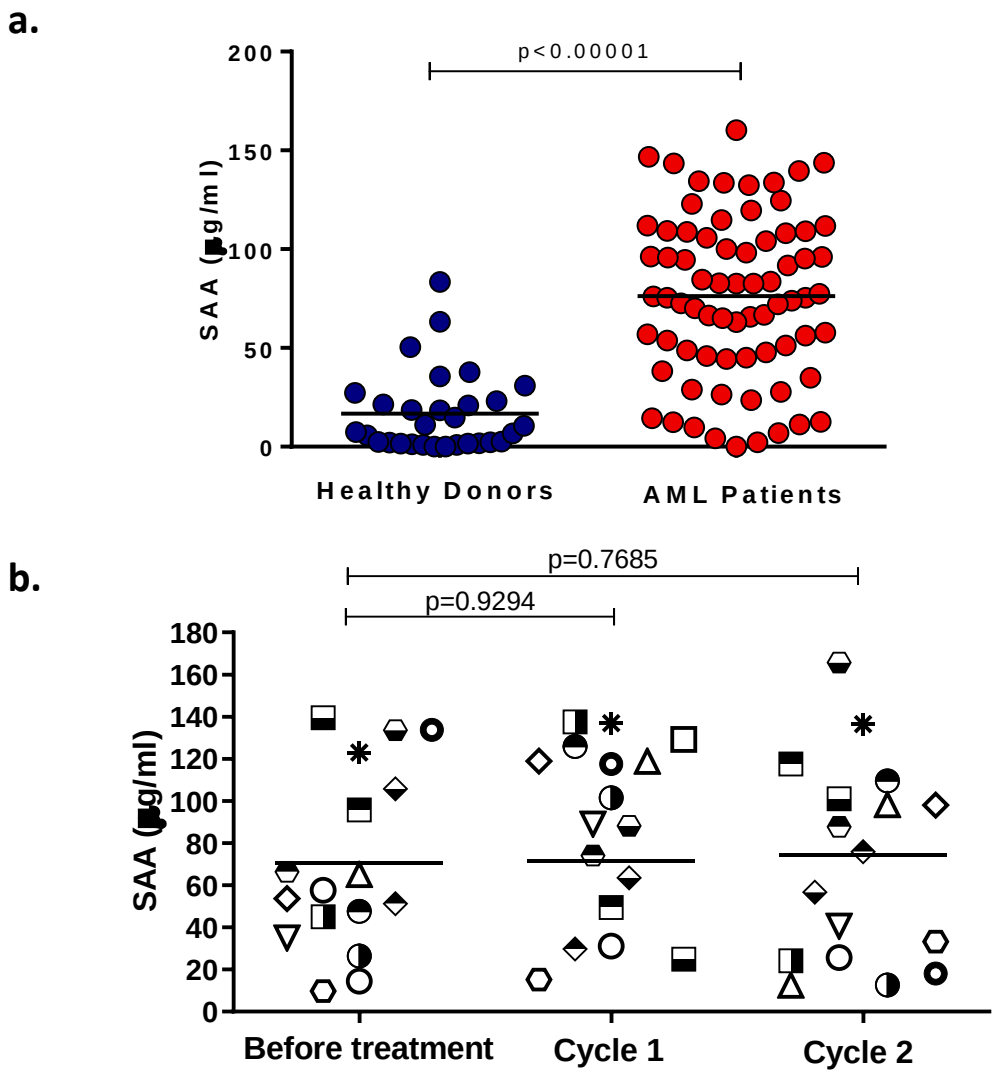


Figure 3.3: AML patients express high levels of SAA.
Levels of SAA were measured by ELISA from a) plasma of comparing patient AML blasts (n=72) with healthy donors (n=27) and b) AML patients in clinical trial treated with cycles of Azactidine/Vorinostat, before treatment (n=24) after cycle 1 (n=24) and after cycle 2 (n=24). Higher levels of SAA were measured in plasma AML patients compared to healthy donors which were not altered after Azactidine/Vorinostat treatment. Horizontal line across samples represents the grand mean. Statistics performed by unpaired t-test. Statistical significance considered when: $p<0.05$.

To understand if AML blasts are responsible for the SAA increase in patient plasma, patient AML blasts enriched by MACS were cultured in RPMI10% for 72 hours. Statistically higher (p=0.0071) levels of SAA were observed in culture supernatants of patient AML blasts when compared to RPM10% alone (Fig. 3.4).

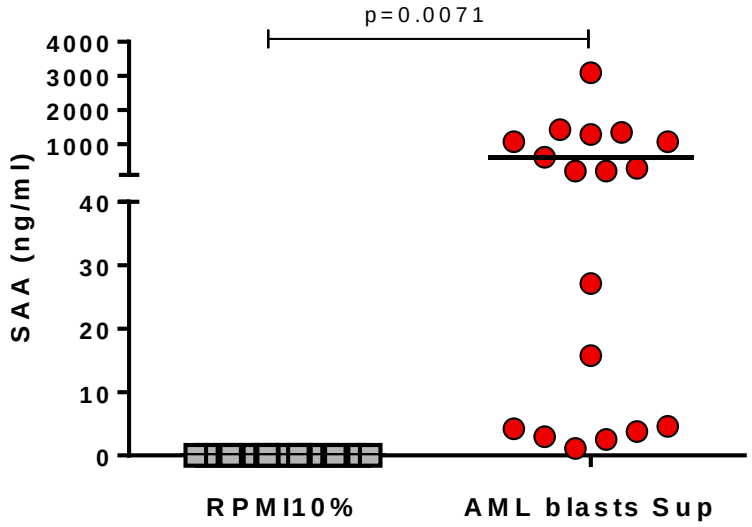


Figure 3.4: AML patient blasts release SAA.
AML blasts from patients (n=18) were cultured in RPMI10% for 72 hours. Levels of SAA detected in supernatant were measured by ELISA. Horizontal line across samples represents the grand mean. Statistics performed by unpaired t-test. Statistical significance considered when: p<0.05.

Given the high levels of SAA in the AML microenvironment and the elevated production of SAA by AML blasts, the impact of SAA on patient derived AML blasts was investigated. Recombinant human SAA (10µg/ml) was added to patient AML blasts for 72 hours. The presence of SAA significantly (p=0.0029) enhanced AML patient blasts viability *ex vivo* from 40% on average of untreated AML blast to 68% on average (Fig. 3.5a, b).

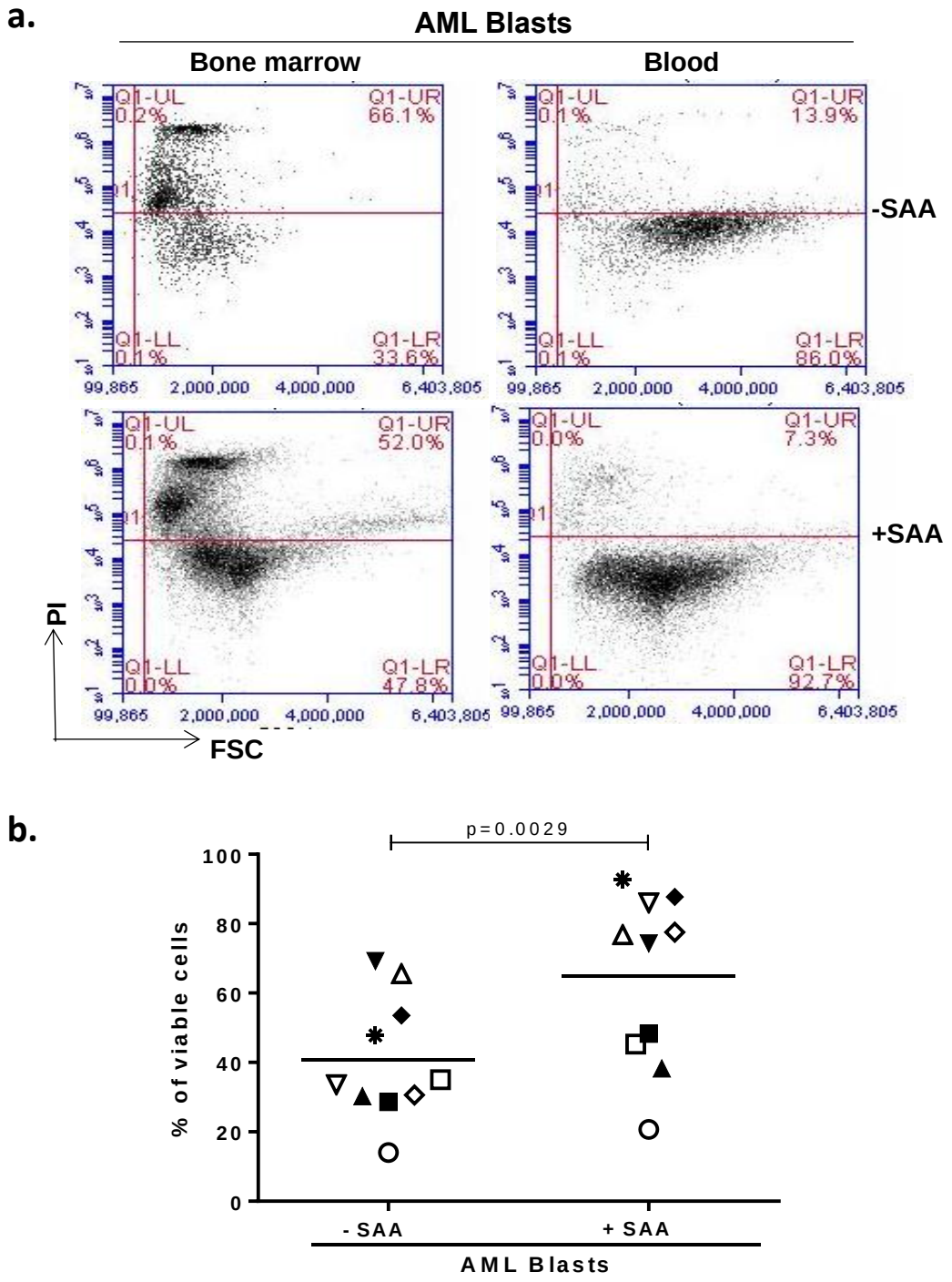


Figure 3.5: SAA enhances AML blasts viability.
a) Representative dot plot of AML patient blasts (enriched from bone marrow and blood) cultured with SAA (10μg/ml) for 72 hours. Viability was established by PI staining. b) Viability of cumulative data from AML blasts derived from patients (blood) (n=10) in the presence of SAA (10μg/ml) for 72 hours. SAA augments patient derived AML blast viability. Horizontal line across samples represents the grand mean Statistics performed by paired t-test, comparing the presence with absence of SAA in cultures. Statistical significance considered when: $p<0.05$.

SAA released from AML blasts signals via FPR2 receptor.

Toll-like receptor (TLR)2, TLR4 and formyl peptide receptor (FPR2) have been reported to mediate specific cellular responses induced by SAA. The surface expression of these SAA receptors on AML cell lines (THP1, U937, NOMO, K562, HL60 and KG-1a) and patients' blasts was determined by flow cytometry. All receptors: TLR2, TLR4, and FPR2 were expressed on surface of alive patient AML blasts, after whole blood was red blood lysed (Fig 3.6a,b) and cell lines (Fig. 3.6c).

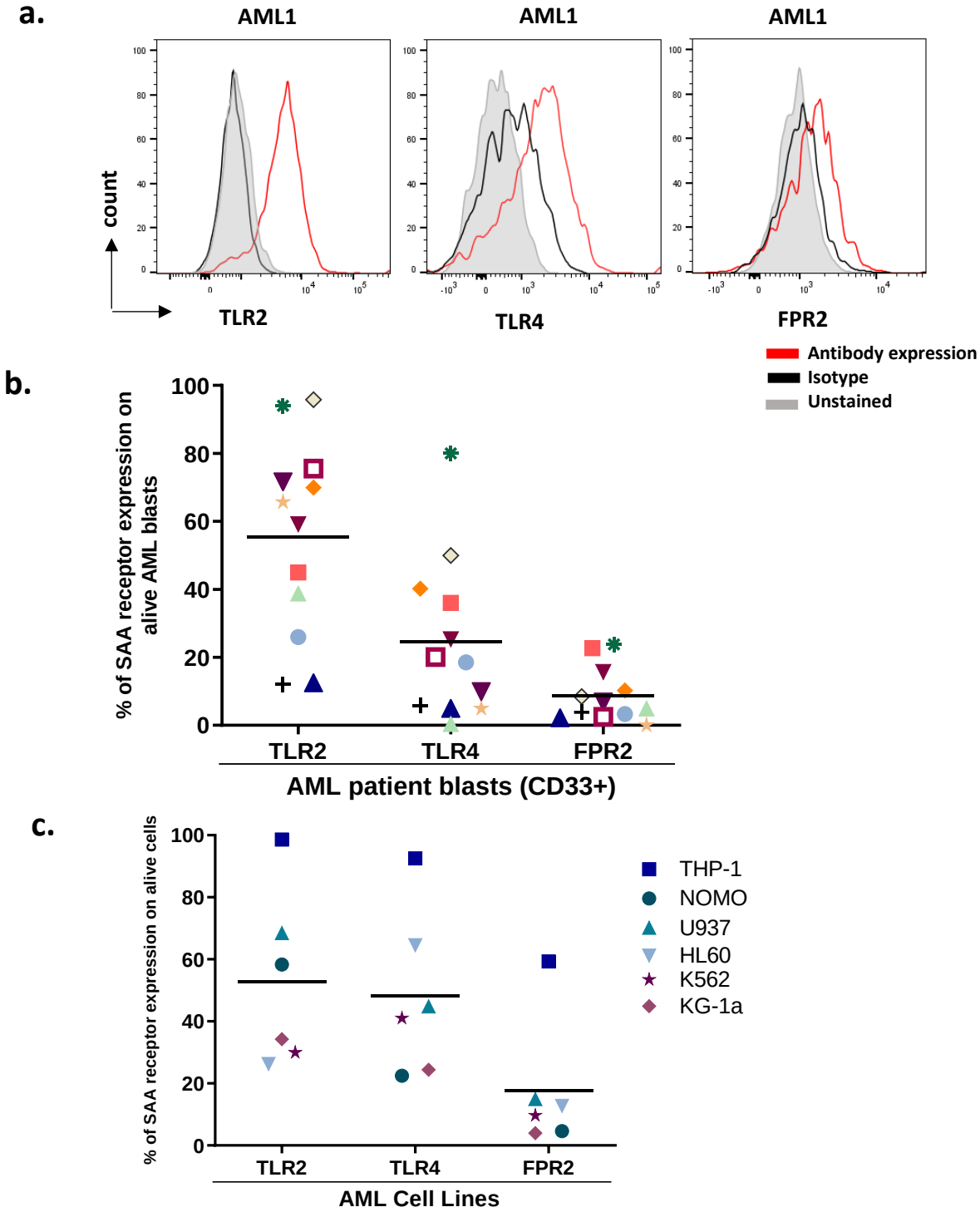


Figure 3.6: AML blasts express SAA receptors TLR2, TLR4 and FPR2.
Surface expression of SAA receptors TLR2, TLR4 and FPR2 were determined by flow cytometry in a) Example histograms in AML patients and b) cumulative data of AML patients (n=12). c) Representative (n=6) of AML cell lines. Expression shown as percentage. Horizontal line across samples represents the grand mean.

To establish which receptors were responsible for mediating SAA signals anti-TLR2, anti-TLR4, and anti-FPR2 blocking antibodies were used to neutralize the protein interaction with their own receptor. AML blasts were pre-incubated with the blocking antibodies at concentration of 10μg/ml for one hour before SAA (10μg/ml) was added to cultures for 72 hours. Viability of AML blasts was determined by propidium iodide staining in flow cytometry analysis. Whilst the addition of SAA to the *in vitro* culture of primary blasts increased their viability from 8.9% to 57% on average (p= 0.0225). The addition of anti-FPR2 antibody prior treatment with SAA abrogated the SAA mediated increase (p= 0.0266). In contrast, anti-TLR2 and anti-TLR4 antibodies had no effect on SAA mediated increase in viability (57.6% and 45.4% on average), suggesting SAA predominantly signals via FPR2. (Fig. 3.7a, b).

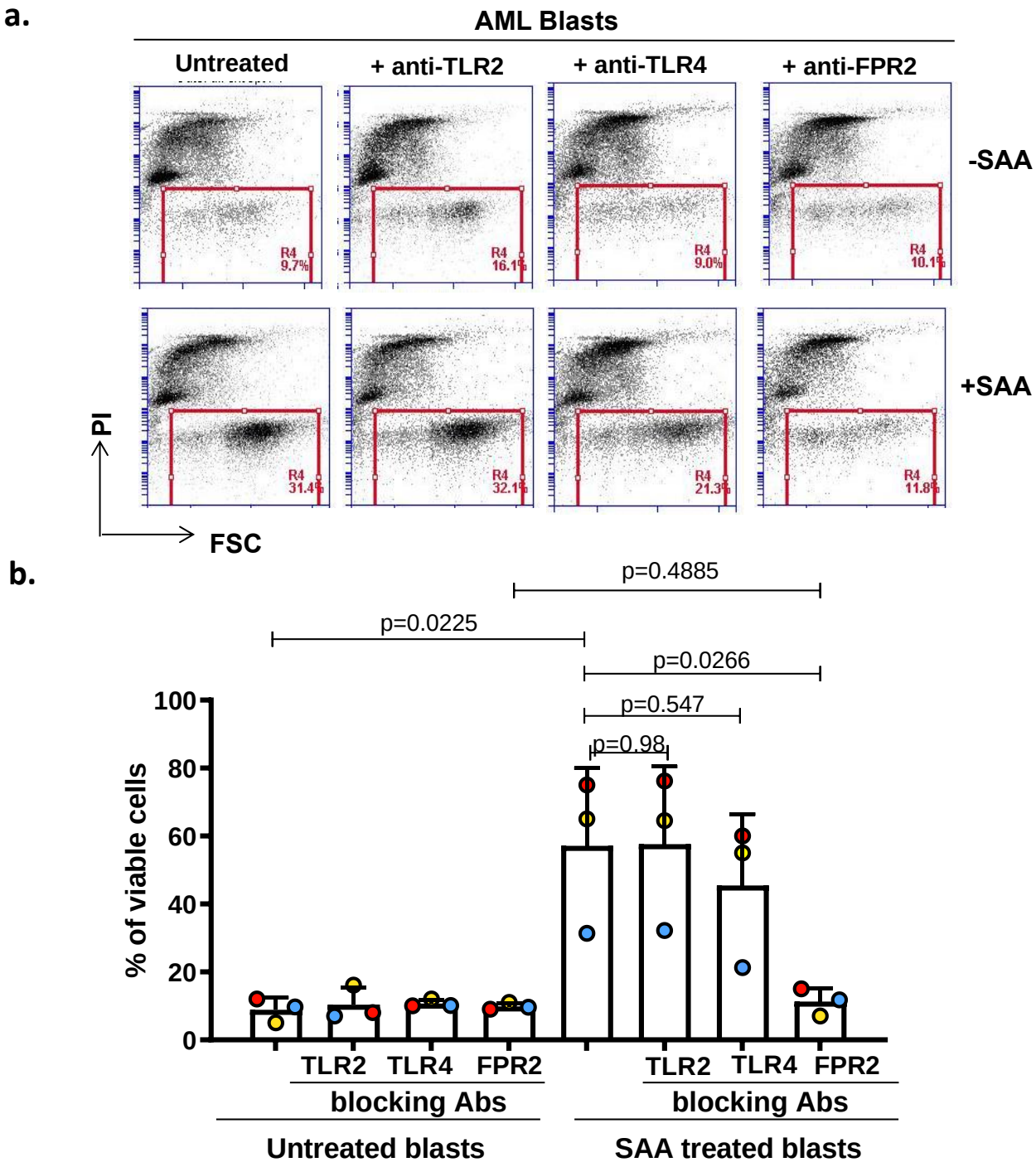


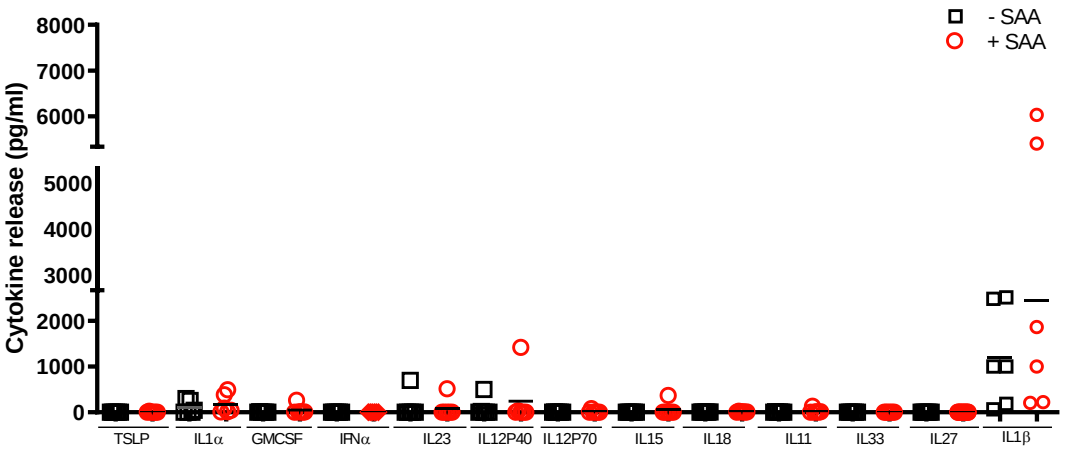
Figure 3.7: SAA from AML blasts signals through FPR2.

AML blasts were pre-incubated with anti-TLR2, anti-TLR4 and anti-FPR2 blocking antibodies (10µg/ml) for an hour before adding SAA (10µg/ml) for 72 hours. The percentage of viable cells was determined by flow cytometry. a) Example dot plot of flow gating is shown. b) Cumulative data (n=3) shown. Anti-FPR2 antibody prior treatment with SAA supressed the SAA mediated increase in viability but not with anti-TLR2 and anti-TLR4.Data represents mean± SEM. Statistics performed by paired t-test. Statistical significance considered when: $p<0.05$.

SAA drives IL-1 β release in AML blasts.

The impact of SAA mediating inflammation is evaluated by measuring release of a range of cytokines. Using a bead-based immunoassay, no statistical difference in cytokines: IL-1α, IL-11, IL-12p40, IL-12p70, IL-15, IL-18, IL-23, IL-27, IL-33, GMCSF, IFN-α and TSLP was observed in the supernatant of AML blasts treated or not treated with SAA (10μg/ml) (Fig. 3.8a). However, the immunoassay did show higher levels IL-1β in AML blasts in the presence of SAA (Fig. 3.8a). Additionally, significant raised (p=0.0056) IL-1β production was observed in supernatants of SAA treated AML blasts (n=15) compared to AML blasts alone by ELISA (Fig.3.8b).

a.



b.

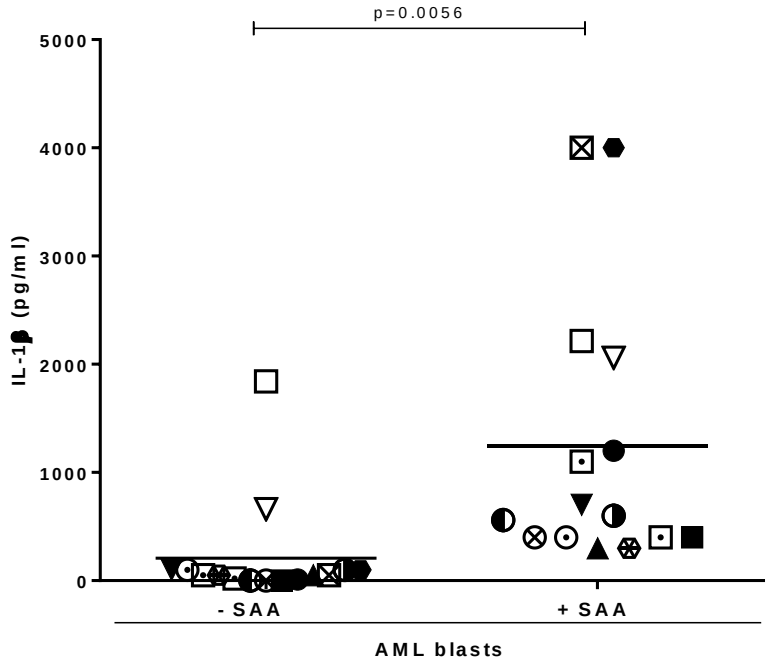


Figure 3.8: SAA upregulates IL-β in AML blasts.
a) An array of cytokines was measured on supernatant harvested from AML patient blasts (n=6) alone and in the presence of SAA (10µg/ml) for 72 hours using a multiplex assay with fluorescence–encoded beads. b) IL-1β expression was measured on supernatants harvested from AML patients (n=15) cultures in the presence and absence of SAA (10µg/ml) for 72 hours by ELISA. SAA increases IL-1β concentrations in AML blasts. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when: $p<0.05$.

SAA upregulates ARGII in AML blasts.

Taking into account that ARG2 is responsible for driving an AML immunosuppressive microenvironment, intracellular expression of ARG2 was measured on AML blasts cultured with SAA (10μg/ml) treatment for 72 hours by western blots. An increase of ARG2 expression was observed in SAA treated THP1 and NOMO cell lines when compared to the untreated samples (Fig 3.9a). Enzyme upregulation was similarly confirmed in patient derived AML blasts treated with SAA (Fig.3.9a). To understand if the increased expression of ARG2 resulted in greater enzymatic activity, a colorimetric assay of arginine hydrolysis to urea was used to calculate arginase activity in cell lysates from patient derived AML blasts as well as AML cell lines in the presence of SAA. Treatment with SAA (10μg/ml) for 72 hours led to a significant increase *(P*=0.0048) in arginase activity when compared to untreated controls (Fig.3.9b). Subsequently, extracellular levels of arginine were measured by ELISA in the supernatants harvested from patient AML blasts cultured with SAA. A statistically significant decrease (*p*=0.0093) in arginine levels was observed in SAA treated AML blasts compared to untreated AML blasts (Fig.3.9c).

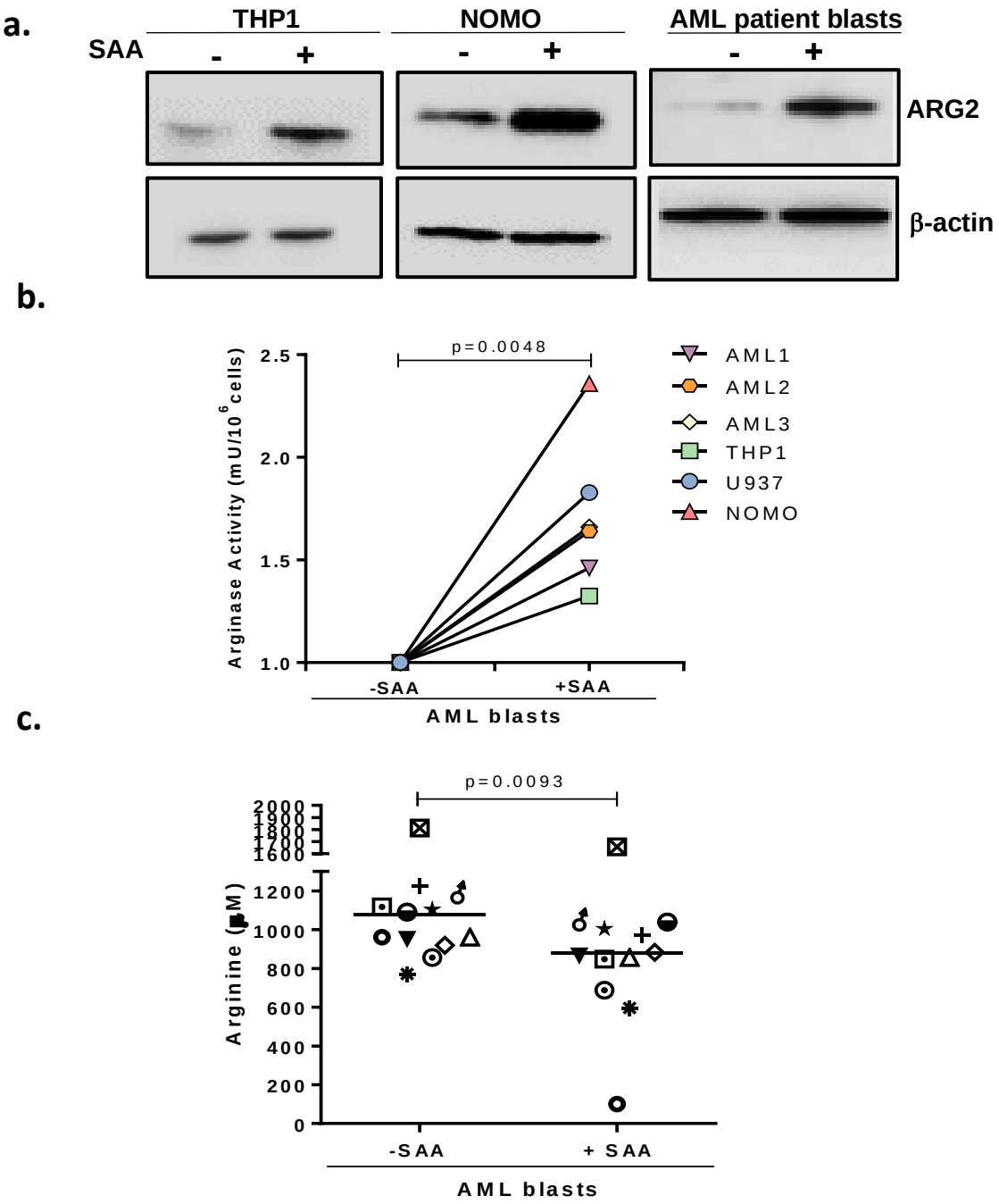


Figure 3.9: SAA leads to ARGII expression and activity in AML blasts.
AML patient blasts and AML cell lines were cultured with SAA (10μg/ml) for 48 hours. a) representative western blots (n=3) reveal upregulation of ARG2 upon in the presence of SAA of derived AML patient blasts and AML cell lines (THP1 and NOMO). β-actin was used as a loading control. b) Arginase activity is increased after SAA added to patient AML blasts (n=3) and AML cell lines (THP1, U937, NOMO). c) Reduced arginine level was measured by ELISA on patient AML blasts (n=12) in the presence of SAA, compared to untreated control. ARG2 and arginase activity on AML blasts is increased in the presence of SAA while arginine levels are reduced in plasma. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when: $p<0.05$.

AML microenvironment suppresses conventional T cells**.**

T cells play a major role in defending the body against invading pathogens by releasing cytokines following recognition of foreign antigens. The recognition and activation of T cells in response to leukemic antigens is thus the focus of several immunotherapeutic strategies aimed at eradicating leukaemia. Thus, frequency of circulating T Cells (CD3 positive cells, a marker expressed on T cell populations blood from AML patients was determined by flow cytometry. T cell expression was determined on red blood lysed blood from AML patients cell expression by gating on alive cells (CD3 positive AML blasts were excluded). On average AML patients expressed 18% of T cells compared to 54% in healthy donors (Fig. 3.10a). To further establish if AML blasts suppress T cell proliferation, patient AML blasts were co-cultured in a mixed leucocyte reaction (MLR) assay with healthy T cells and irradiated DCs for 96 hours. T cells proliferation was determined by ^{3}H-thymidine incorporation. Co-culture of patient AML blasts with allo-antigen driven T cells led to a statistically significant reduction in T cell proliferation form 100% of T cells stimulated with DC to 13.9% in the presence of AML blasts (Fig. 3.10b).

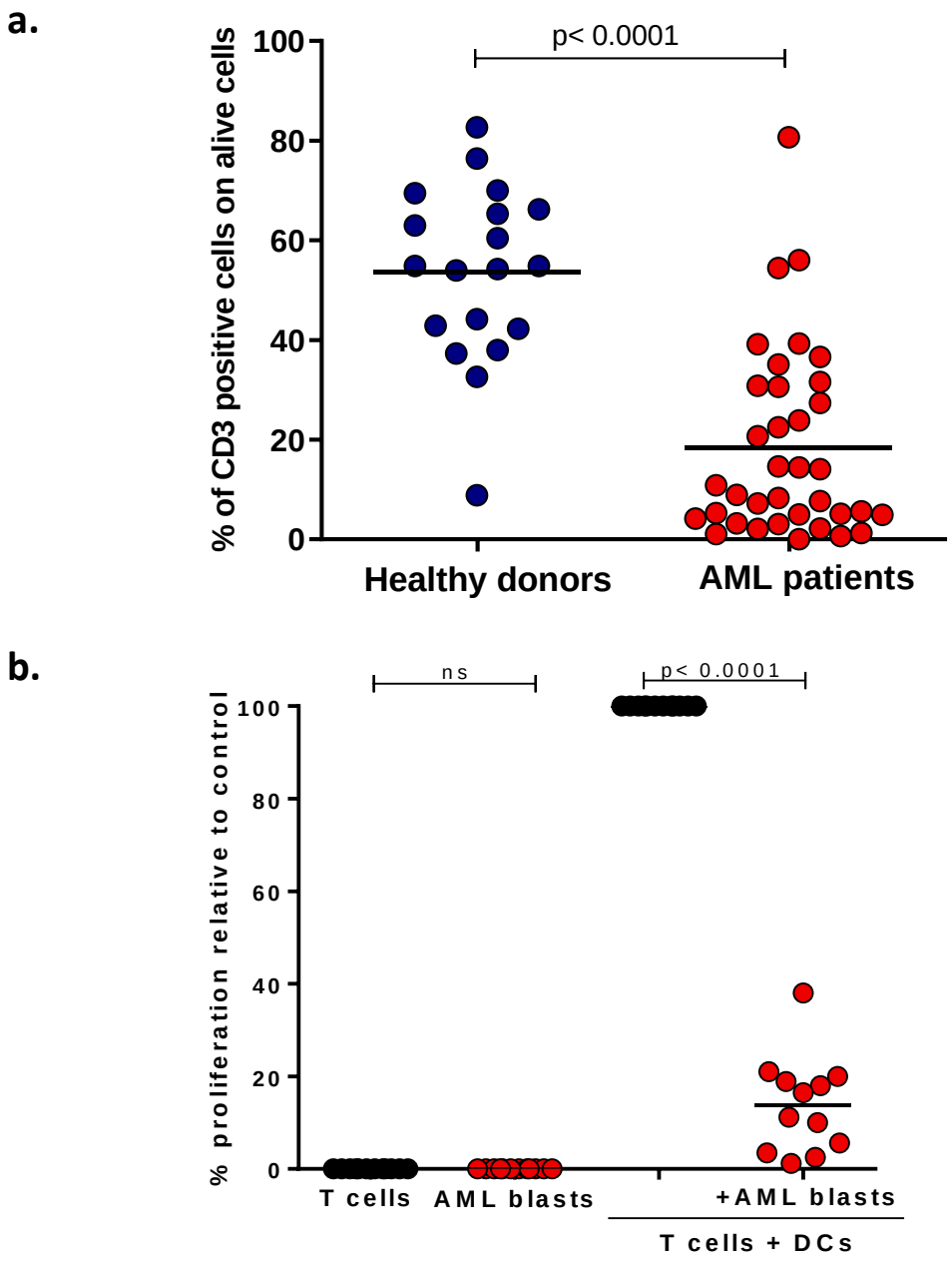


Figure 3.10: AML blasts suppress T cells.
a) Percentage of CD3 positive T cells in the blood of healthy donors (n=19) and AML (n=36) patients at diagnosis measured by flow cytometry showed lower levels in AML patients compared to healthy. b) AML inhibition of T cell proliferation was determined by 3H-thymidine incorporation. Patient AML blasts were co-cultured with healthy T cells and irradiated DCs for 96 hours before 3H-thymidine was added. T cell to DC ratio used was 1:0.25. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when: $p<0.05$.

In recent years, it is acknowledged that iNKT cells are a unique population of T cells and play a pivotal role in orchestrating strong anti-tumour responses. Upon activation, iNKT cells have the capacity to rapidly release a broad range of cytokines, secrete cytotoxic molecules and consequently stimulate numerous anti-tumour effector cells both in innate and adaptive immunity. Analysis of the frequency of circulating alive iNKT cells by flow cytometry showed no significant difference *(p*=0.7388) in iNKT cell frequencies in whole blood, following red blood lysis of healthy individuals (on average 0.24%) and AML patients (on average 0.27%) (Fig. 3.11).

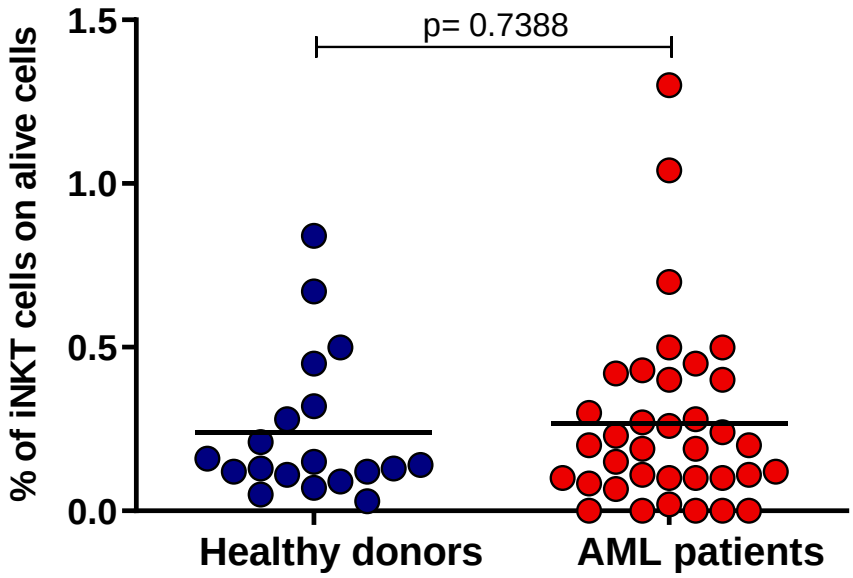


Figure 3.11: Expression of iNKT cells in AML patients compared to healthy donors.
Expression of iNKT cells in the blood of healthy donors (n=19) and AML patients at diagnosis (n=36), measured by flow cytometry with fluorescent-labelled CD1d tetramer staining on alive cells in red blood lysed whole blood. No difference in iNKT levels were observed in AML patients compared to healthy donors. Horizontal line across samples represents the grand mean. Statistics performed by unpaired t-test. Statistical significance was considered when: $p<0.05$.

Having previously demonstrated, that the low arginine microenvironment created by AML blasts hinders T cell function, the ability of iNKT cells to function in similarly low arginine conditions was explored. iNKT cells and conventional T cells were cultured in low arginine media with CD3 (3µg/ml) CD28 (2 µg/ml) stimulation for 96 hours. No significant difference in stimulated iNKT cell proliferation was observed between RPMI10% and low arginine conditions (p=0.1971), while a statistically significant decrease of T cell proliferation was observed when culturing T cells in low arginine conditions compared to RPMI10% (p<0.0001) (Fig. 3.12).

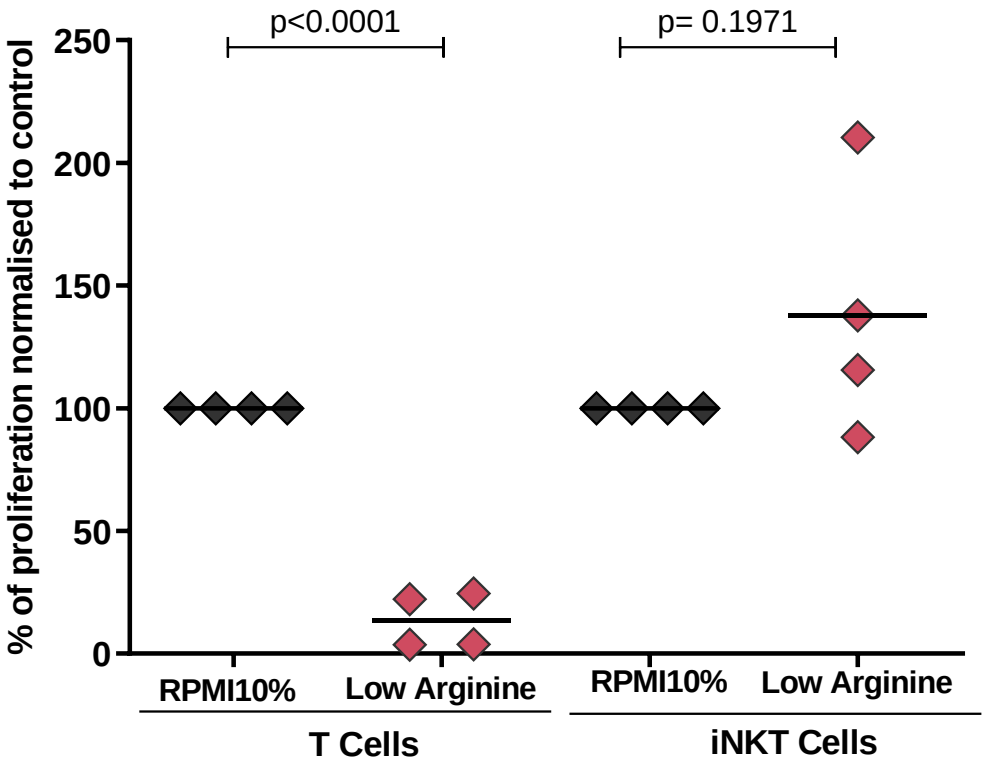


Figure 3.12: AML microenvironment does not inhibit proliferation of iNKT cells following CD3/CD28 activation in contrast to T cells.
Proliferation of stimulated iNKT cells in not affected by low arginine conditions compared to conventional T cells. Conventional T cells and iNKT cells were labelled with CFSE stimulated with CD3 /CD28 and cultured in RPMI 10% and low arginine conditions for 96 hours. Proliferation was determined by flow cytometry on alive cells. Low arginine conditions did not supress proliferation of stimulated iNKT cells in contrast to conventional T cells. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance was considered when: $p<0.05$.

To confirm the ability of iNKT cells to function in low arginine microenvironment we explored the effect of low arginine conditions have on iNKT cells *in vivo*. A human recombinant arginase (BCT-100) was used to systemically deplete arginine, C57BL/6 mice (n=8) were intravenously injected with 5 mg/kg BCT-100 from day 0 to day 5 every 24 hours and untreated mice (n=8) were used as control. On day 3, α-GalCer (2μg/ml) or vehicle was intravenously injected in both groups. α-GalCer is a bacterial-derived glycolipid presented by CD1d, with an established profile as a potent activator of iNKT cells. At day 5 blood was obtained, arginine concentrations in the serum were measured by ELISA to confirm arginine depletion (Fig. 3.13).

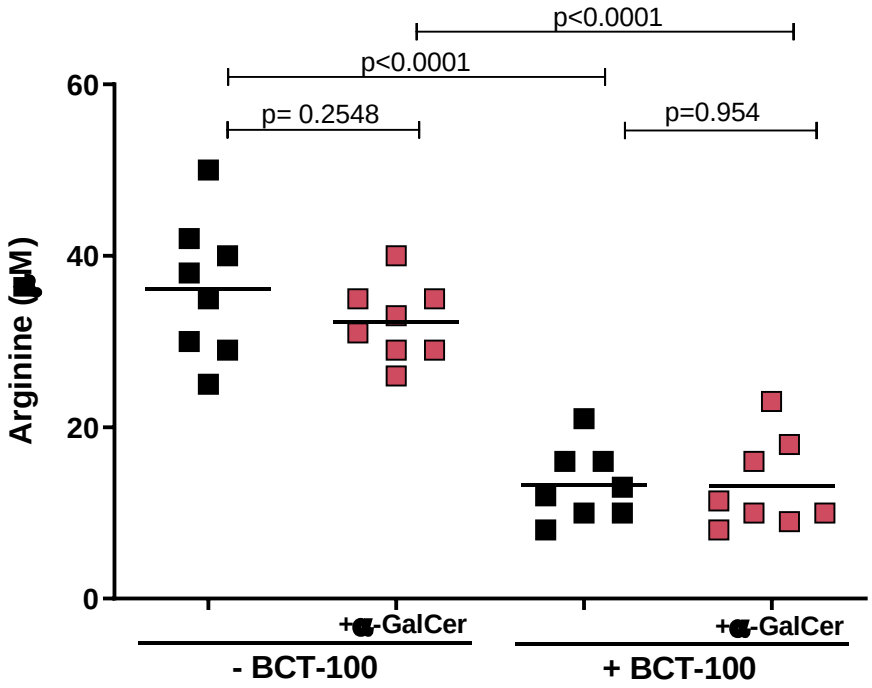
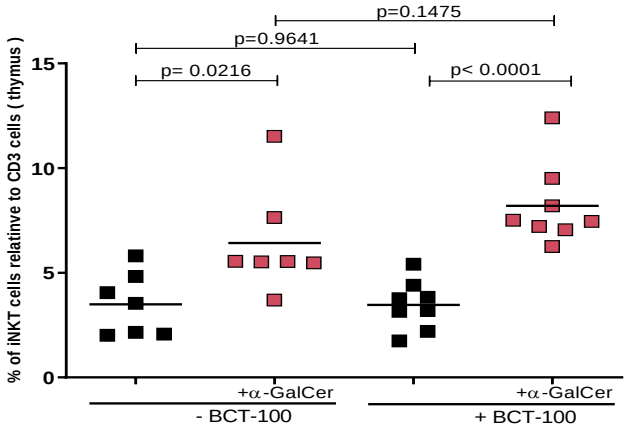


Figure 3.13: BCT-100 depletes arginine concentrations *in vivo.*
Every 24 hours, C57BL/6 mice (n=8) received recombinant arginase BCT-100 (5mg/kg) or vehicle from day 0 to day 5. On day 3, both groups of mice were intravenously administered α-GalCer (2μg/mouse) or vehicle. On day 5 blood was obtained and arginine levels in the serum were measured by ELISA. BCT-100 depletes arginine concentrations in both the presence of α-GalCer or in the absence of α-GalCer. Horizontal line across samples represents the grand mean. Statistics performed by unpaired t-test. Statistical significance was considered when: $p<0.05$.

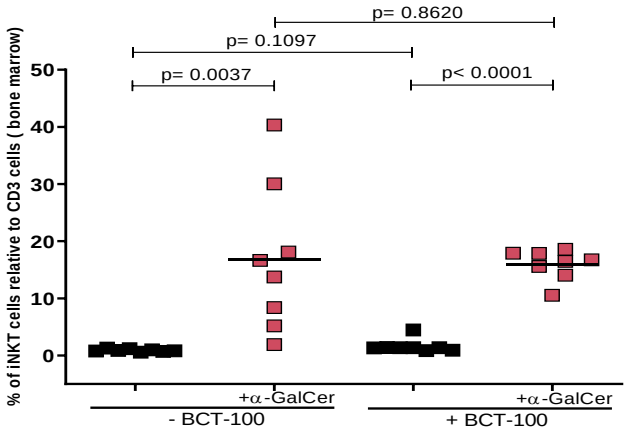
A significant decrease in arginine was observed in mice treated with BCT-100 compared to the untreated mice in the presence (p<0.001) or in the absence of α-GalCer (p<0.001).

Additionally, at day five iNKT cell expansion was measured in bone marrow (BM), spleen and thymus by flow cytometry. Increase in iNKT cells frequency in the BM, thymus and spleen was observed upon α-GalCer treatment regardless of BCT-100 administration. iNKT cell frequency in the BM of α-GalCer treated mice was 16.78% on average and in mice treated with BCT-100 and α-GalCer 15.96% on average. Respectively, iNKT cells frequencies in the thymus and spleen of α-GalCer treated mice were 6.58% and 15.31%; and in the BCT-100 treated with α-GalCer mice the levels were 8.3% and 14.9% on average respectively (Fig. 3.14).

a.



b.



c.

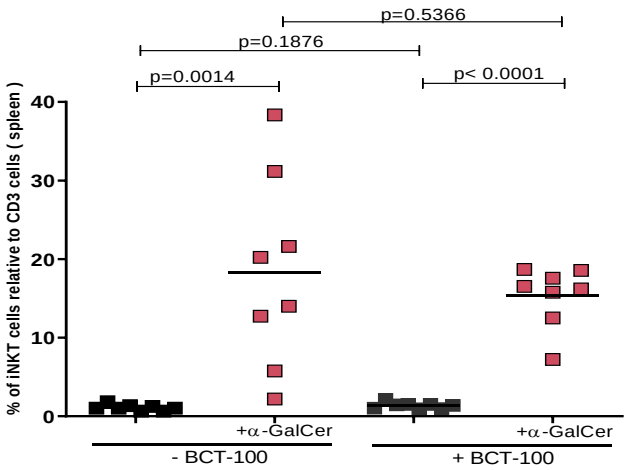


Figure 3.14: iNKT cell expansion following α-GalCer stimulation is not affected by low arginine levels *in vivo*.

Every 24 hours, C57BL/6 mice (n=8) received recombinant arginase BCT-100 (5mg/kg) or mice received vehicle from day 0 to day 5. On day 3, both groups of mice were intravenously administered α-GalCer (2µg/mouse) or vehicle. On day 5 mice were culled, and a) BM, b) thymus and c) spleen were harvested. iNKT cell expression on alive CD3 cells was determined by flow cytometry using fluorescent-labelled CD1d tetramer. α-GalCer treated mice expressed higher levels of iNKT cells. Frequency was not altered by BCT-100. Horizontal line across samples represents the grand mean. Statistics performed by unpaired t-test. Statistical significance was considered when: $p<0.05$.

Given the low arginine microenvironment does not alter iNKT cell frequencies *in vivo*, we sought to determine if iNKT cell can effectively be activated in the low arginine conditions. The titres of a range cytokines were measured in mouse serum collected on day 5 using a bead-based immunoassay. Upon α-GalCer treatment, a statistically significant increase in IFN-γ release (p=0.001) was detected and the concentration was not affected by arginine depletion by BCT-100 (Fig. 3.15). No statistical difference in cytokines: IFN-α, IL-6, IL-12p70, GM-CSF, IL-1β, IL10 and IFN-β was observed between mice receiving vehicle or α-GalCer (Fig. 3.15). With exception of IL-1β no statistical difference was observed between complete (-BCT-100) or low arginine conditions (+ BCT-100) (Fig. 3.15)

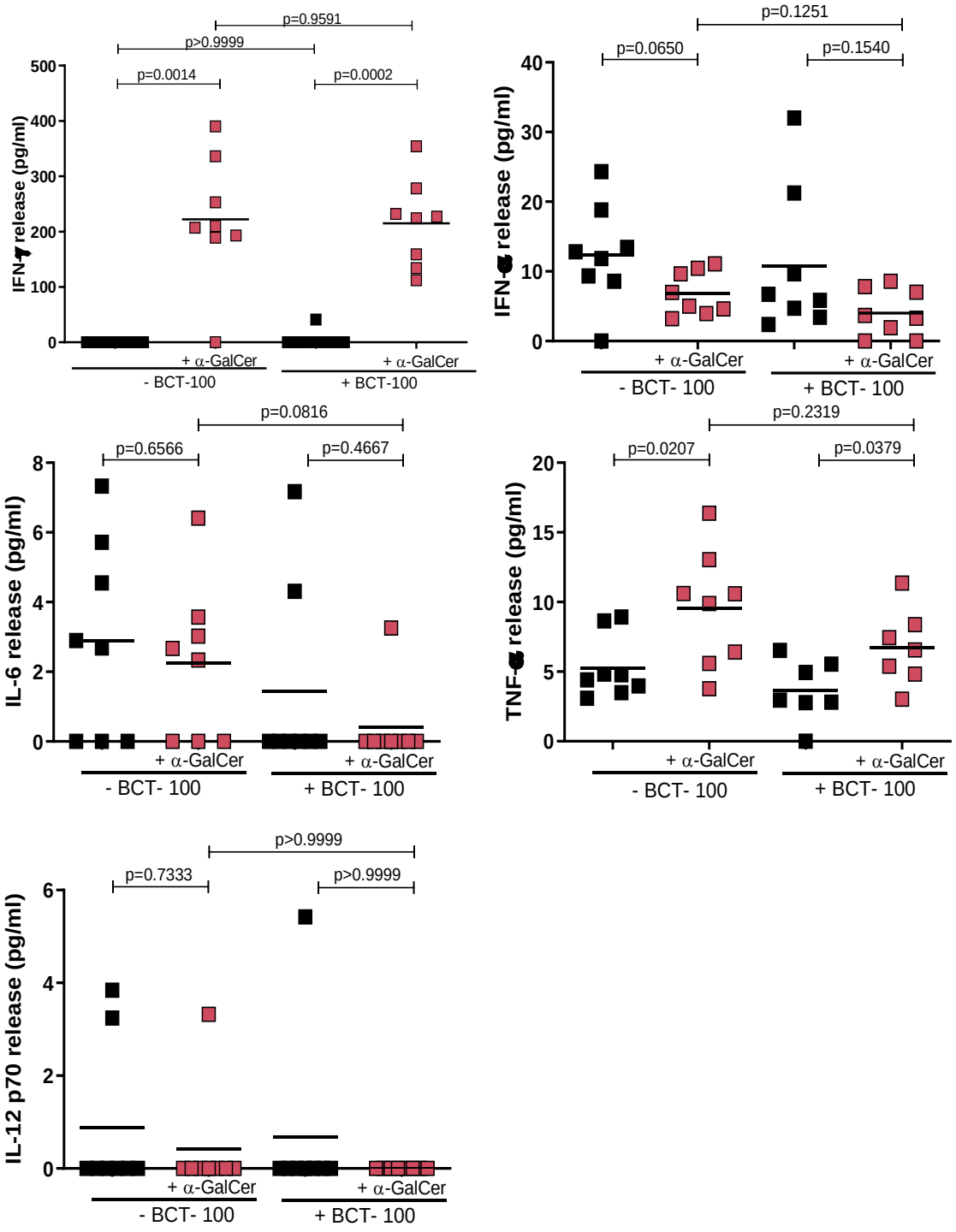


Figure 3.15 continued.

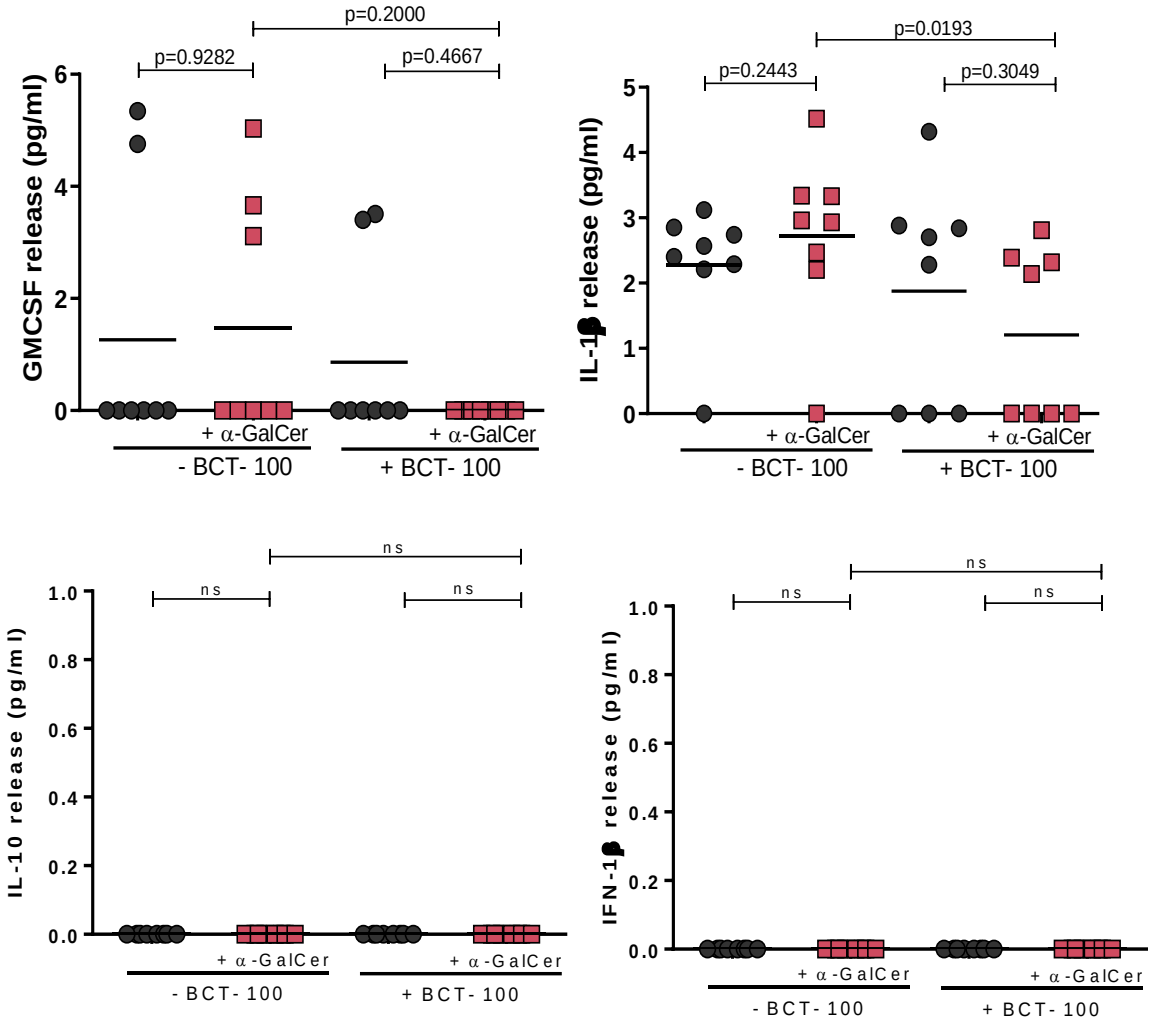


Figure 3.15: Cytokines release following α-GalCer stimulation is not altered by low arginine levels in vivo.

Every 24 hours, C57BL/6 mice (n=8) received recombinant arginase BCT-100 (5mg/Kg) or vehicle from day 0 to day 5. On day 3, both groups of mice were intravenously administered α-GalCer (2µg/mouse) or vehicle. On day 5 blood was obtained. A range of cytokines released in the serum were measured using a multi-plex assay using fluorescence-encoded beads. Increase in concentration of IFN-γ was observed in α-GalCer treated mice both the presence and absence of BCT-100. Horizontal line across samples represents the grand mean. Statistics performed by unpaired Mann-Whitney test. Statistical significance was considered when: $p<0.05$.

Discussion

Cancers pose an exceptional demand on nutrients required to sustain their growth. Such extra demands exceed their own intrinsic capacity to synthesize nutrients such as amino acids making them auxotrophic for those nutrients [236, 237]. Mussai *et al*. have previously established that AML blasts are auxotrophic for arginine owing to elevated expression and release of the arginase isoform ARG2. AML blasts thus create an arginine depleted immunosuppressive environment [75]. Data presented in this chapter showing reduced arginine levels in the serum of AML patients compared to healthy individuals is consistent with previous findings by Mussai *et al*., confirming arginine levels are depleted in AML patients, since significantly lower arginine levels in the serum compared to healthy controls were found, suggesting they express high levels of ARG2 [75] .

The interaction between immunity and cancer cell metabolism is pivotal to cancer mediated immune evasion, thus, it is critical to understand factors controlling ARG2 expression in AML blasts. One such factor known to regulate the expression of arginase is SAA. For the first time, data presented here shows that patient derived AML blasts directly release SAA. This finding is corroborated by the fact that plasma from a second cohort of AML patients participating in a clinical trial where high levels of the inflammatory molecule SAA before and after treatment who showed no objective response following [247] two rounds of induction therapy expressed. In Mussai *et al* we also show decreased plasma arginine concentrations in these AML patients, compared to healthy controls, and similarly to SAA levels are not significantly altered after cycles of AZA and vorinostat treatment [250]. Additionally, compared to healthy donors we observe significantly elevated ARG2 levels in same cohort of AML patients which is not changed following cycles of treatment [250]. Patient AML blasts were shown to express several SAA receptors; amongst which were the well characterised TLR2 and TLR4, but also the G-protein coupled receptor FPR2. FPR2 proved to be the main receptor of SAA, which drives an increase in cell viability and may partly explain the findings of elevated IL-1β in some AML patients at diagnosis [240].

Additionally, our findings reveal that SAA drives upregulation of intracellular ARG2 levels in AML blasts. Similar to protein levels, a significant increase in arginase activity was observed following SAA stimulation. This confirms that increase in ARG2 protein levels were consistent with enzyme activity. Correspondingly, a reduction in extracellular arginine was also evident. Reduced plasma arginine concentration was observed in AML patients irrespective of induction therapy regiment. Given that arginine depletion is particularly suppressive to T cell proliferation, it is likely that a failure of the patients own T cells to expand or illicit anti-cancer response in the arginine low environment [250], is partially responsible for the absence of any objective responses in these patients after induction therapy [250]. Taken together, this data suggests, SAA acts in an autocrine manner to induce ARG2 expression and activity in AML blast, promote blast viability and thus creating an arginine depleted in AML microenvironment.

One of the hallmarks of elevated arginase levels and arginase activity in the immune system is impaired T cell proliferation. Studies have shown that proliferation of both human and murine T cells is entirely supressed in arginine free medium following stimulation with anti-CD3- and anti-CD28-coupled beads or various types of mitogens [241-243]. AML patients expressed significantly lower T cell frequency compared to healthy donors implying that the AML niche impairs T cell proliferation.

For the first time, this study observes that in contrast to T cells, iNKT cell frequency in AML patients was no less than healthy controls. There is increasing evidence to suggest that iNKT cells mediate a anti-cancer immune response against several tumour types [215, 230], including in AML. The fact that iNKT cell frequencies are not reduced in AML patients and given that they are not affected by immunosuppressive microenvironment orchestrated by AML blasts this suggests they could be used to effectively target AML.

Additionally, stimulated iNKT cells can function within the low arginine microenvironment created by AML, both *in vitro* and *in vivo*. Following stimulation *in vitro* iNKT cells effectively proliferate in low

arginine and complete conditions equally. In contrast, MLRs indicated that AML blasts supress T cell proliferation. Similarly *in vivo* arginine depletion achieved by injecting mice with a pegylated human recombinant arginase (BCT-100) did not alter iNKT cell expansion induced by administration of the prototypic ligand α-GalCer. iNKT cell expansion which was not altered by low arginine conditions in the BM, thymus, and spleen, confirming that iNKT cells retain their ability to proliferate in low arginine conditions. Furthermore, increase in IFN-γ (a hallmark of iNKT cell activation) concentrations were observed in α-GalCer treated mice as evidence of iNKT cell activation. No changes in IFN-γ concentration were observed in low arginine conditions. Taken together, our findings *in vivo* demonstrate that iNKT cells not only retain their proliferation in low arginine conditions but also their activity. The capacity of iNKT cells to adapt in low arginine conditions, retaining their functionality is critical, highlighting their significance in anti-AML immunity.

Chapter 4

Results

AML blasts interact with iNKT cells

Having shown that iNKT cells retain their ability to proliferate in low arginine conditions (chapter 3), the tumoricidal potential of iNKT cells against AML was explored. The anti-tumour effect of iNKT cell-targeted immunotherapy relies mainly on direct tumour killing and by effectively activating or augmenting the immune response orchestrated by other immune cells. Our aim was to investigate if iNKT cells could directly interact with AML blasts and to identify possible molecular mechanisms in which iNKT cells can reverse the immunosuppressive activity of AML blasts.

To examine if AML blasts mediate iNKT cell activation different concentrations of iNKT cells were co-cultured with AML blasts in the presence or absence of α-GalCer for 72 hours. iNKT cell activity was determined by IFN-γ production, a hallmark of iNKT cell activation. Analysis of supernatants by ELISA detected high IFN-γ concentrations in iNKT cells co-cultured with AML blasts, which further increased in the presence of α-GalCer (Fig. 4.1a). IFN- γ concentrations detected were directly proportional to iNKT cells frequency in co-cultures (Fig. 4.1a). To establish IFN-γ measured in the supernatants is produced by iNKT cells not by the AML-blasts, ICS was performed. Patient AML blasts were co-cultured with iNKT cells in the presence or absence of α-GalCer for 12 hours. Protein transport inhibitor containing mononesin (GolgiStop) was added after 6 hours to the cultures to enhance intracellular cytokine staining. IFN-γ production by iNKT cells increases to 17.6% in the presence of AML blasts and is further augmented to 44.8% of in the presence of α-GalCer. Therefore, patient AML blasts (n=21) were isolated and co-incubated with iNKT cells at an effector: target (E: T) ratio 0.25:1 in the presence or absence of α-GalCer for 72 hours. IFN-γ levels in the supernatants harvested were measured by ELISA. A significant increase (p=0.001) of IFN-γ was measured in co-cultures of iNKT cells with AML blasts compared to iNKT cells alone, which was further increased when α-GalCer was added in the co-cultures (p=0.067) (Fig. 4. 1c).

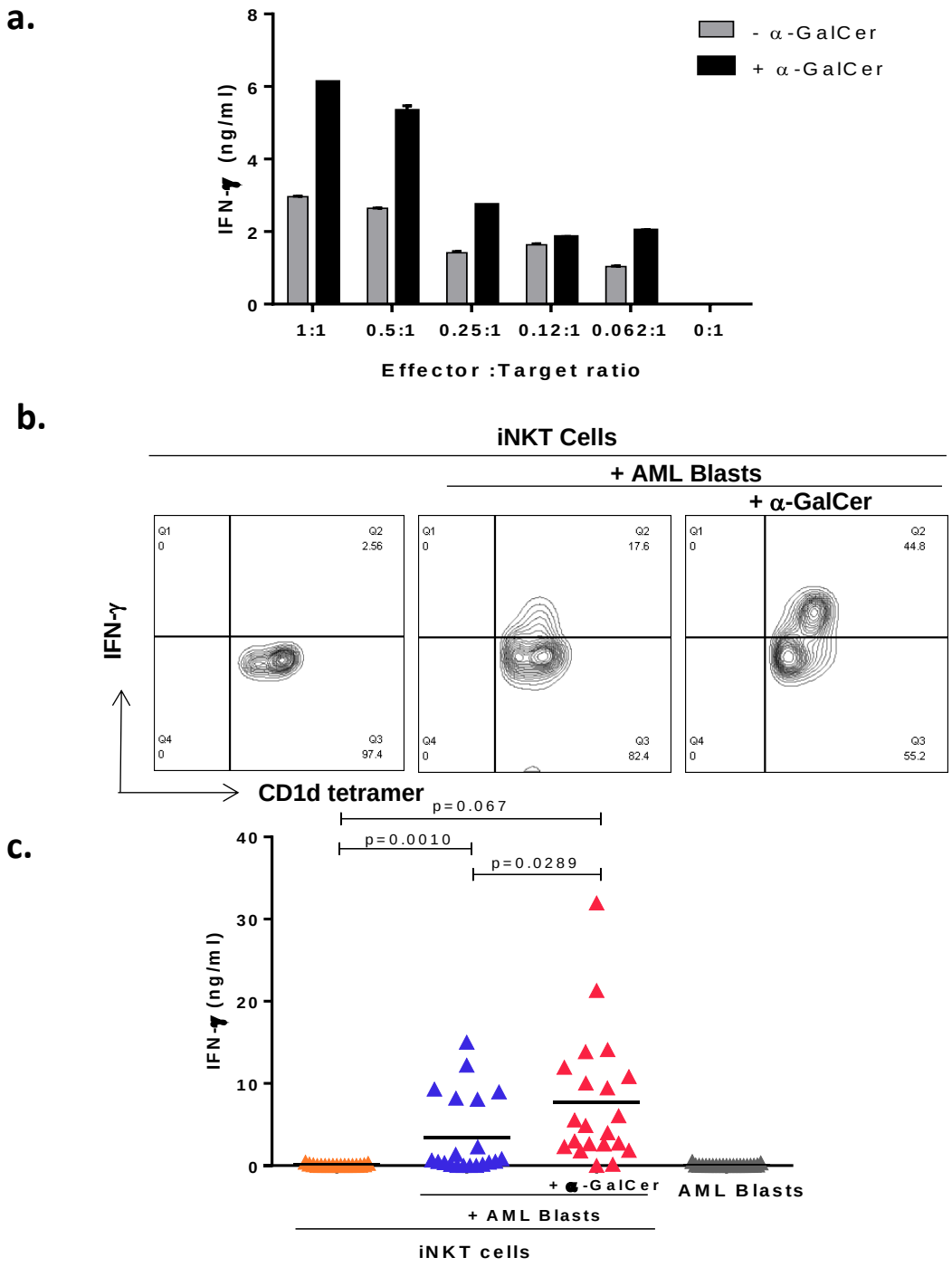


Figure 4.1: AML blasts in the presence of α-GalCer activate iNKT cells stimulated.
iNKT cell activity was determined by IFN-γ release. AML blasts were cultured alone or with α-GalCer (100ng/ml) in the presence of iNKT cells a) different ratios of iNKT cells were used (n=2) b) IFN-γ was determined by ICS after 24 hour of co-incubation. iNKT cells were identified by gating on cells positively stained with fluorescent-labelled CD1d tetramer (n=3). c) Patient AML blasts (n=21) were incubated with iNKT cells and α-GalCer for 72 hours. Supernatant was harvested and IFN-γ was measured by ELISA. E:T used was 0.25:1. IFN-γ was upregulated following co-culture of iNKT cell with the target cells. Horizontal line across samples represents the grand mean). Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

A hallmark of iNKT cells is their capacity to produce a broad range of immune modulatory and proinflammatory cytokines upon antigenic stimulation. A bead-based immunoassay was used to explore the release of Th1 and Th2 cytokines by iNKT cell following 72 hours of AML blasts co-culture in the presence or absence of α-GalCer. Supernatants of iNKT-AML co-cultures had statistically significantly higher IL-2, IL-5 and GMCSF concentrations when compared to iNKT cells cultured alone. However, in the presence of α-GalCer, a statistical significant increase in cytokines: IL-2, IL-5, IL-6, IL-10 and GMCSF was measured compared to control (iNKT cells alone), while release of IL-4, IL-9, TNF-α was not detected (Fig 4.2).

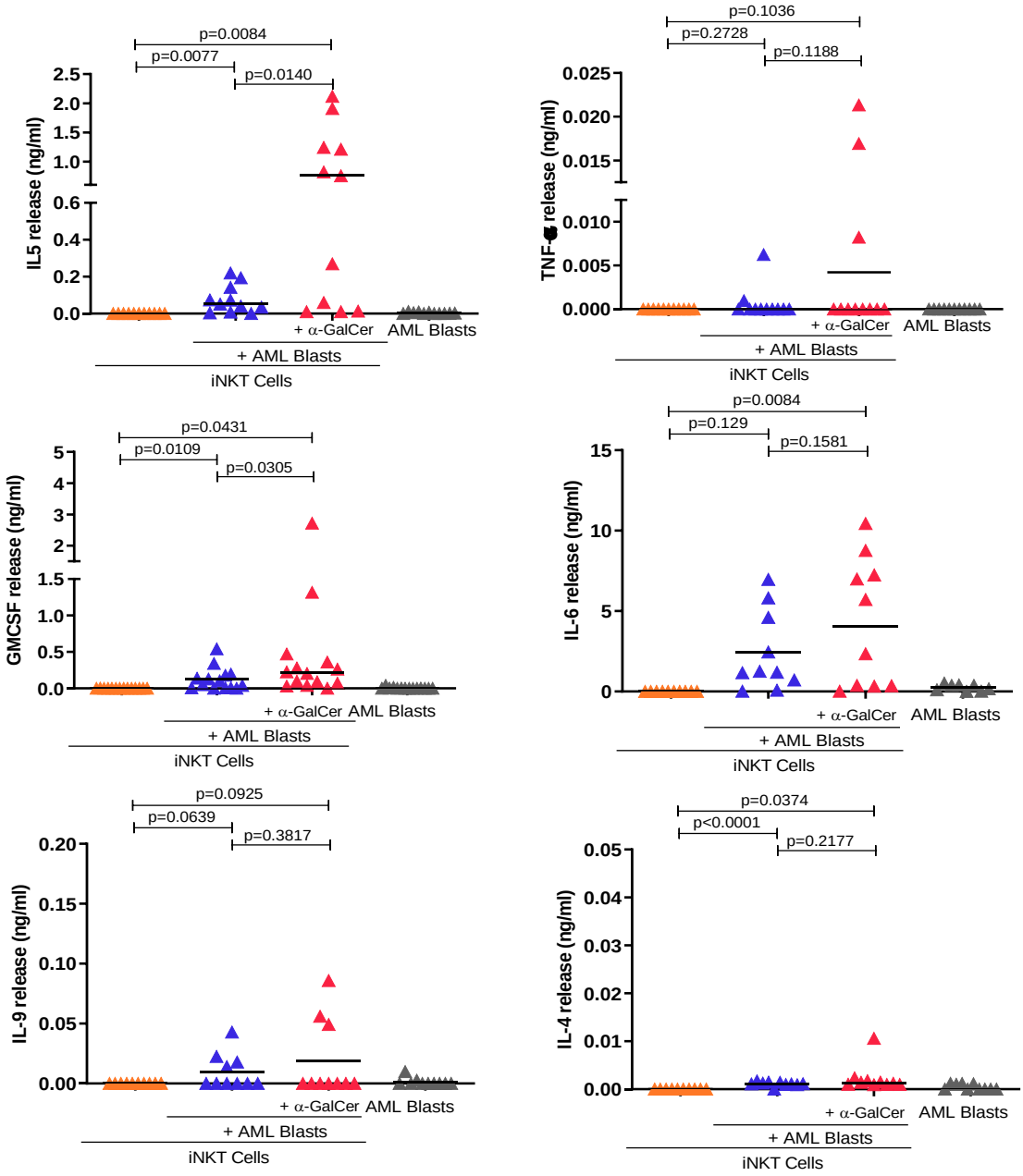


Figure 4.2: AML blasts: iNKT cell interaction drives the release of a range of cytokines.
AML blasts were cultured alone and AML blasts with α-GalCer (100ng/ml) in the presence of iNKT cells for 72 hours. Concentration of various cytokines released in culture supernatants was measured using a multiplex assay by using fluorescence–encoded beads and ELISAs. iNKT cells co-cultured with α-GalCer loaded AML blasts lead to statistical significant increase in IL-2, IL-5, IL-6, IL-10 and GMCSF observed in co-cultures with cultures. The E:T ratio used was 0.25:1. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

Co-culture of AML cells with iNKT cells can modulate iNKT cell phenotype across activation. Thus, the expression of characteristic surface receptors CD69, CD38, CD154, CD25, CD154, FASL was measured on iNKT cells by flow cytometry following co-culture with THP1 cells in the presence or absence of α-GalCer for 72 hours. THP1 cells were the used as a tool to ensure AML mediated iNKT cells activation since higher concentration of IFN-γ was measured following co-culture of THP1 and iNKT cells in presence of α-GalCer, suggesting stronger iNKT activation compared to other AML cell lines. Flow cytometry on alive iNKT cells, revealed that iNKT cells alone express CD69, CD161, CD38, FASL, CD25 and CD154 surface markers. An increase in CD69 and CD25 expression on iNKT cells was observed following co-culture with AML cells. CD38 and FASL expression on iNKT cells were further augmented after iNKT cells were co-cultured with α-GalCer pulsed THP1 cells. Even though CD161 and CD154 were expressed on iNKT cells alone, no difference in expression was observed upon co-culture with THP1 cells in the presence or absence of α-GalCer (Fig 4.3).

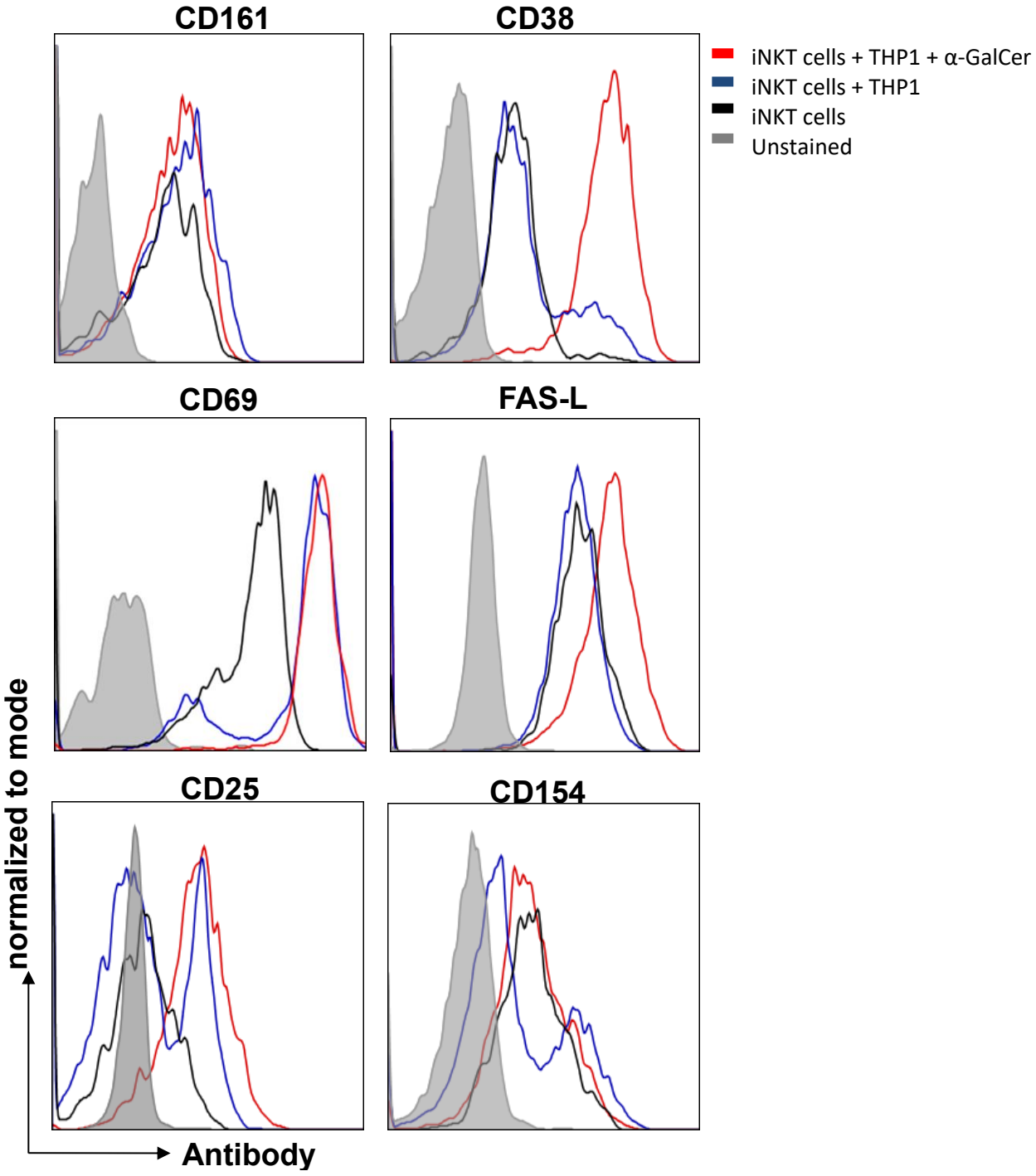


Figure 4.3: Expression of surface markers on iNKT cells after co-cultures with AML blasts. Representative histograms of flow cytometric analyses (n=3). Extracellular expression of CD161, CD38, CD69, FASL, CD25 and CD1541 on iNKT cells showed all markers are constitutively expressed. iNKT cells were cultured alone or co-cultured with THP1 (E:T 0.25:1) with or without α-GalCer (100ng/ml) stimulation for 72 hours. Upregulation in CD38, FASL, CD69 and CD25 was observed upon activation of iNKT cells. Alive iNKT cells were identified by gating on cells positively stained with fluorescent-labelled CD1d tetramer and PI.

iNKT cells pulsed with α-GalCer are metabolically active.

Immune cells use distinct metabolic pathways to support their activation state. The metabolic activity determined by glycolysis and mitochondrial respiration of iNKT cells upon stimulation, was explored using a Seahorse XF Analyzer to measure their real time oxygen consumption rate (OCR) (Fig. 4.4) and extracellular acidification rate (ECAR) (Fig. 4.5). The OCR and ECAR are associated with mitochondrial respiration and lactate production, respectively. iNKT cells were co-cultured with THP1 cells in the presence or absence of α-GalCer for 24 hours iNKT cells were positively selected by MACS and challenged with a Mito Stress Test. Basal, glycolysis, glycolytic capacity, basal respiration and maximal respiration were measured. While there was no significant difference in oxidative phosphorylation rates (Fig. 4.4), there was a significant increase (p=0.0143) in basal ECAR in iNKT cell co-culture with THP-1 in the presence of α-GalCer, compared to THP1 alone and iNKT control (Fig 4.5).

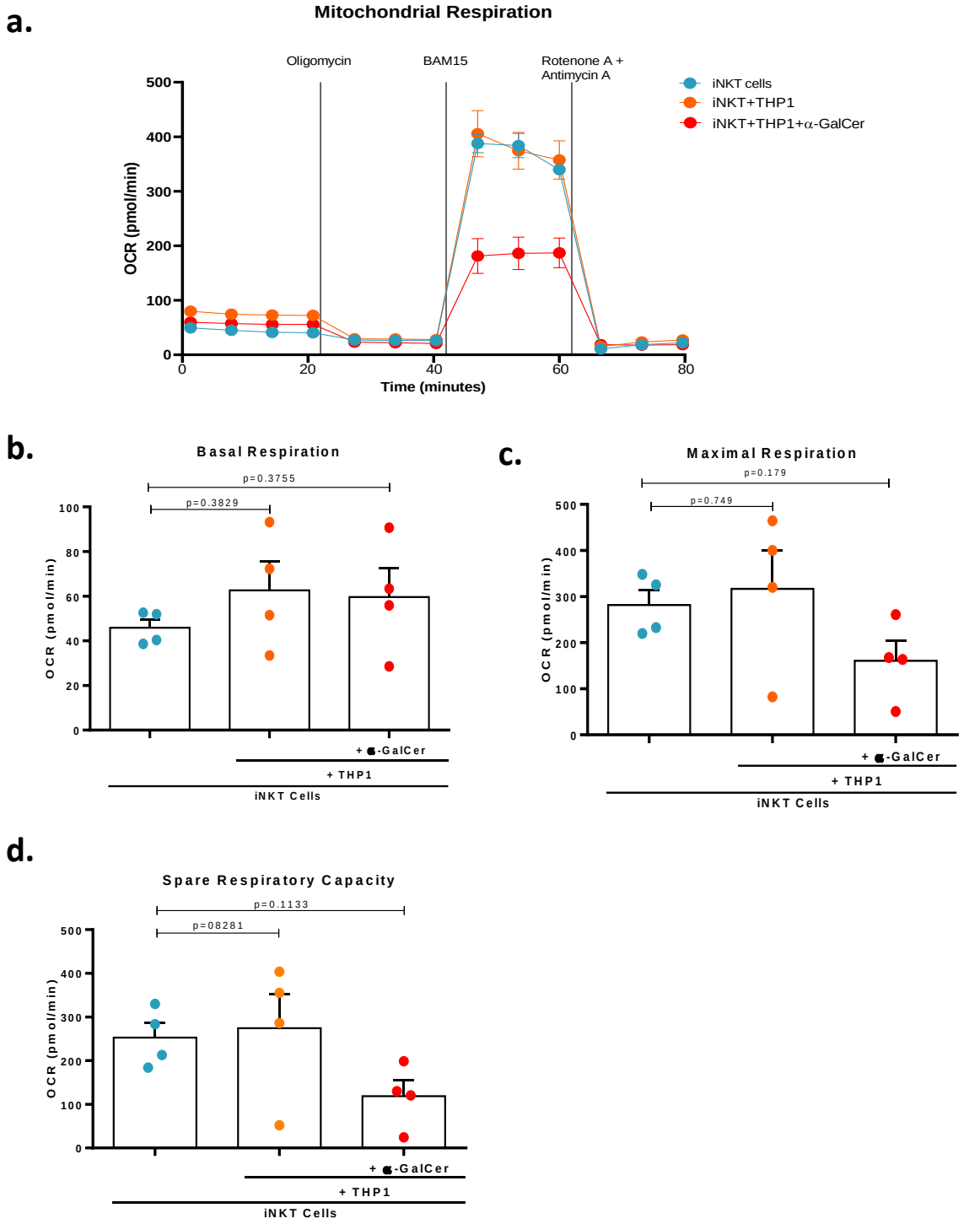


Fig 4.4: No change in OCR on iNKT cells after α-GalCer -dependent co-culture with AML THP1 cells.

iNKT cells were co-cultured with THP1 cells in the presence or absence of α-GalCer for 24 hours (n=4). a) Representative graph showing the comparison of OCR of purified iNKT cells using a seahorse assay. b) Basal respiration, c) maximal respiration and d) spare respiratory capacity shown from four independent experiments. Oxidative phosphorylation rates are not changed by iNKT cell activation. E:T ratio used was 0.25:1. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

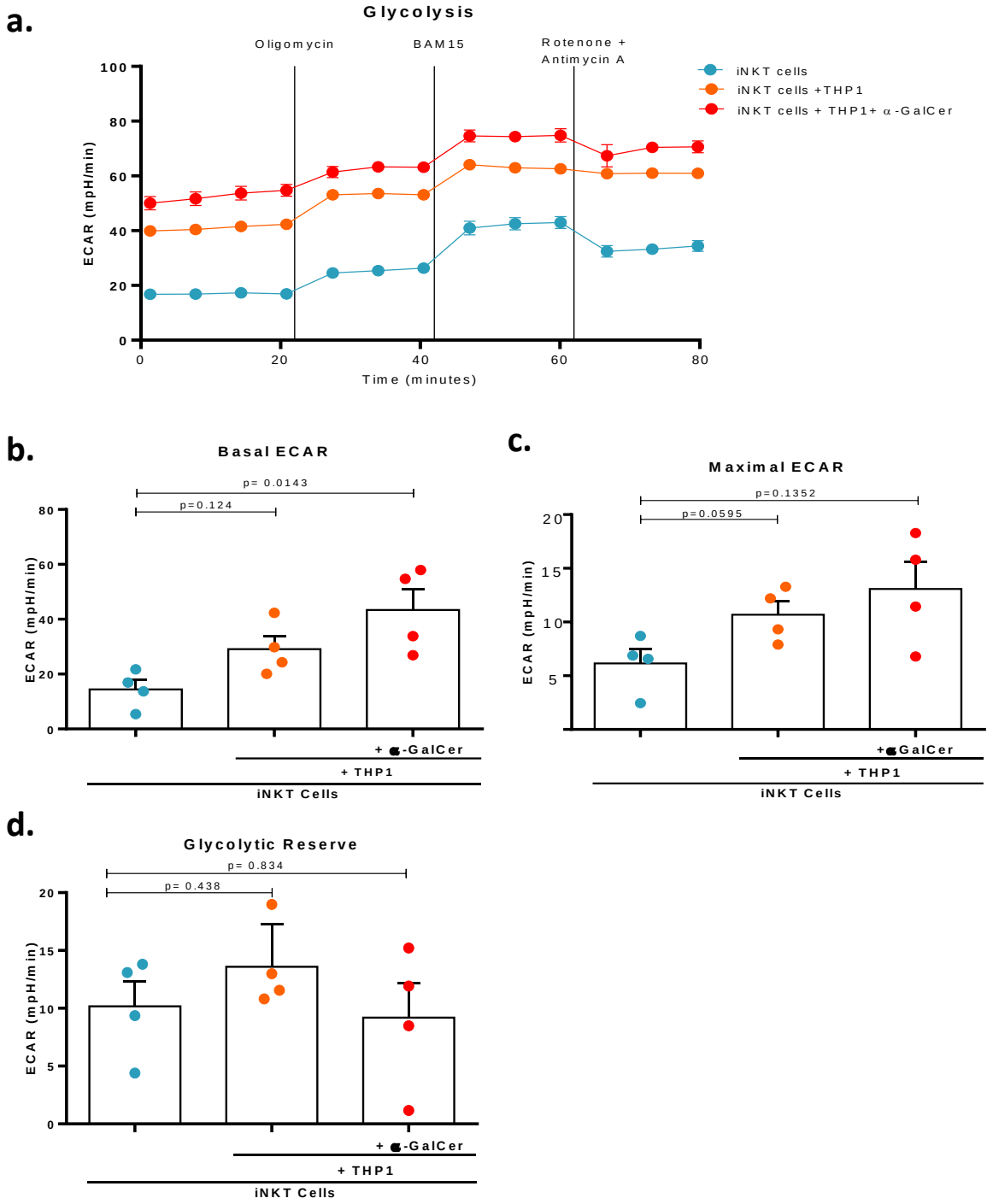


Figure 4.5: A significant increase in basal ECAR on iNKT cells after α-GalCer -dependent co-culture with AML THP1 cells.

iNKT cells were co-cultured with THP1 cells in the presence or absence of α-GalCer for 24 hours (n=4). a) Representative graph showing the comparison of ECAR of purified iNKT cells using a seahorse assay. b) Basal ECAR, c) maximal ECAR, and d) Glycolytic reserve on purified iNKT cells shown from four independent experiments. Significant increase in basal ECAR on α-GalCer –activated iNKTs compared to iNKT cells alone. E:T ratio used was 0.25:1. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

Taking into account that α-GalCer can activate iNKT cells following AML presentation, the potential of antigen presentation influencing iNKT cell proliferation was explored. iNKT cells were labelled with CFSE and co-cultured with AML cell lines THP1, U937 and NOMO and AML patient blasts in the presence or absence of α-GalCer for 72 hours. Proliferation of iNKT cells was determined by flow cytometry analysis of a CFSE dye dilution assay. AML cell lines co-cultured with iNKT cells enhanced the proliferation of iNKT cells in the presence of α-GalCer (Fig 4.6a). Moreover, proliferation of iNKT cells was also determined by flow cytometry when co-cultured with patient derived AML blasts in the presence or absence of α-GalCer (Fig 4.6b). Proliferation of iNKT cells significantly increases in the presence of α-GalCer (Fig 4.6b).

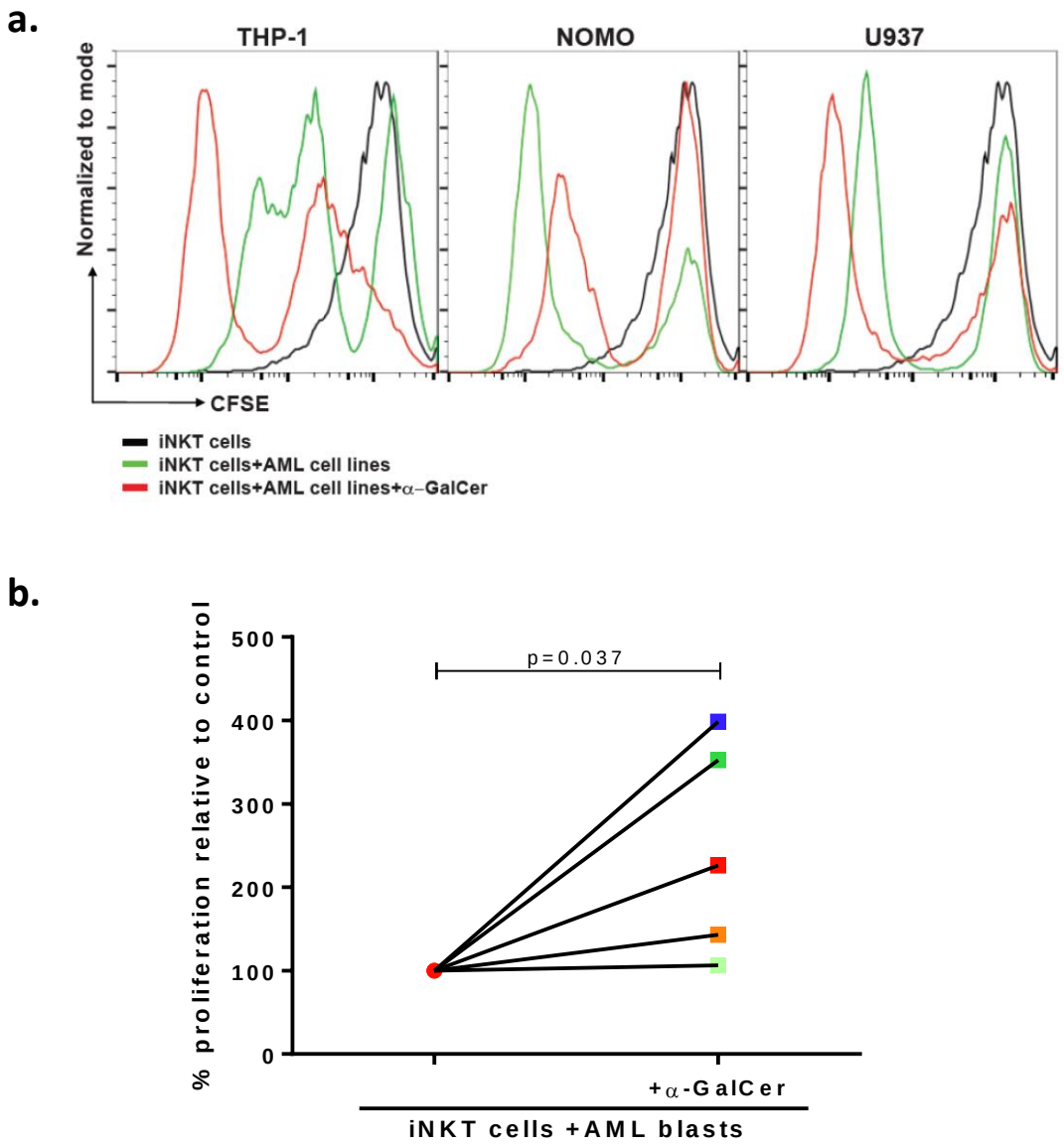


Figure 4.6: α-GalCer presentation by AML cell lines induces iNKT cell proliferation.
a) AML cell lines THP1, U937 and NOMO labelled with CFSE were co-cultured iNKT cells in the presence or absence of α-GalCer for 72 hours. Representative flow cytometry histograms showing CFSE dilution in proliferating. (n=3) b) % of proliferation of iNKT cells was also determined following co-cultures with patient derived AML blasts (n=5) in absence of α-GalCer compared with the presence of α-GalCer. α-GalCer mediated stimulation leads to an increase in iNKT cell proliferation. E:T ratio used was 1:0.25. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

iNKT cells activation following co-culture with AML blasts induces AML apoptosis.

Next the ability of iNKT cells to directly target AML blasts was investigated. Patient AML blasts were co-cultured iNKT cells at different E:T ratios in the presence or absence of α-GalCer. Viability was determined by flow cytometry after 72 hours using PI staining. Patient AML blasts alone (0:1 E:T ratio) were 92.% alive, however as the iNKT cell ratio increased viability of AML blasts decreased. 4.65% of AML blasts were alive when equal numbers of iNKT cells to α-GalCer pulsed AML blasts (1:1 E:T ratio) where used (Fig. 4.7a). Moreover, cytotoxic response of iNKT cells against patient AML blasts was determined in the presence or absence of α-GalCer using PI staining after 72 hours. Cytotoxicity against AML blasts significantly increased when co-cultured with iNKT cells since 35% on average of AML blasts were killed. However, the presence of α-GalCer enhanced further the cytotoxic response of iNKT cells, killing 58% on average patient AML blasts following co-cultures (Fig. 4.7b).

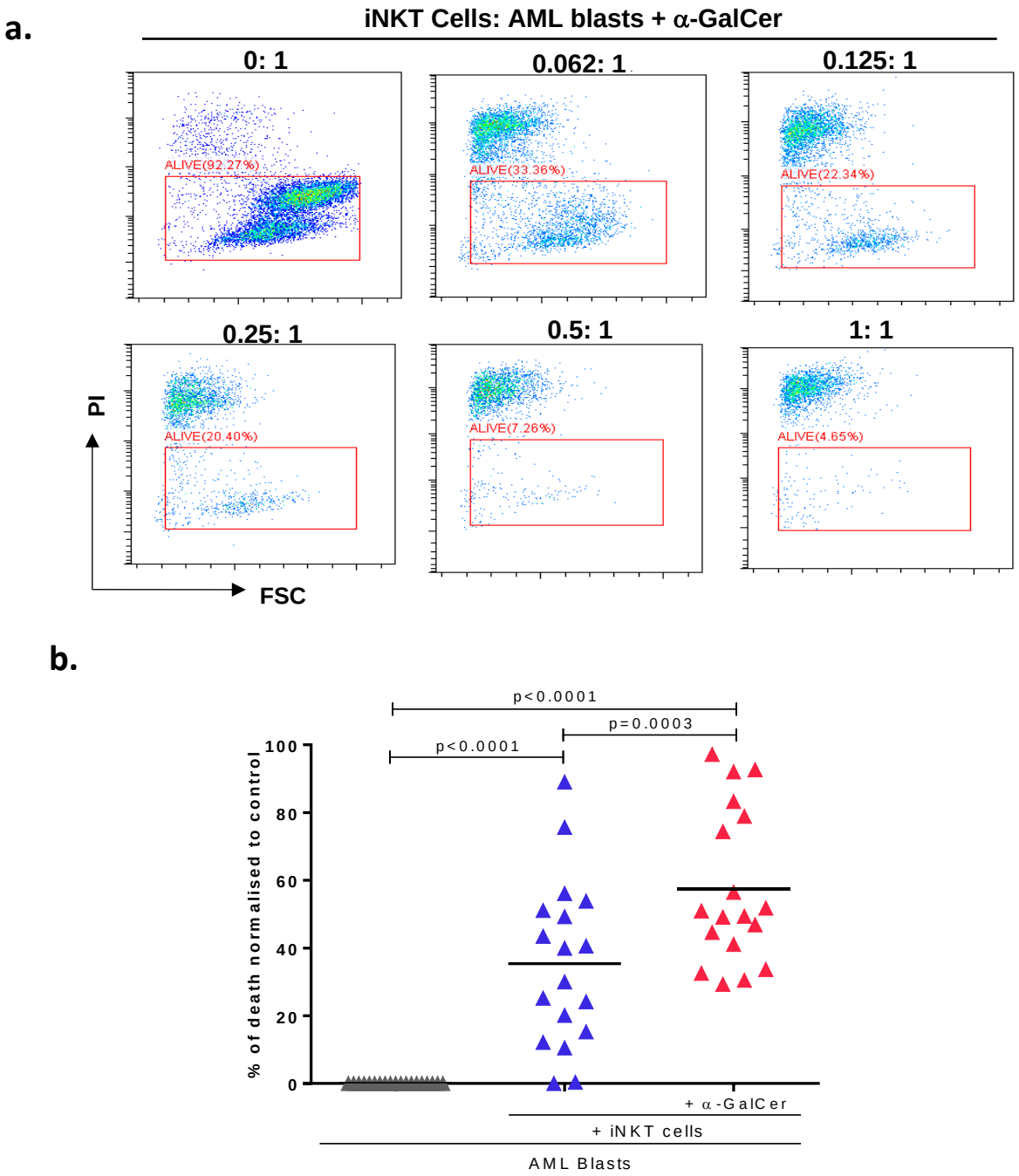


Figure 4.7: Activated iNKT cells via α-GalCer stimulation can modulate AML Cell viability.
Viability of AML blasts alone and AML blasts pulsed with αGalCer (100ng/ml) in the presence of iNKT cells for 72 hours. Flow cytometry was used to determine percentage of AML blasts viability using PI staining. a) Different ratios of iNKT cells were added to the AML Blasts (E:T ratios- 1:1, 0.5:1, 0.25:1, 0.125:1, 0.062:1 and 0:1) were cultured (n=3). b) Viability of AML patient blasts (n=18) was determined after in the presence of absence with α-GalCer (100ng/ml) co-cultured with iNKT cells (E:T ratio 0.25:1) for 72 and viability. Co-cultures of iNKT cell with AML blasts led to AML blasts lysis. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

One of the main mechanisms iNKT cells use to mediate a cytotolytic response is via cytotoxic granule release. Granzyme (particularly B) and perforin are the key components of lytic granules which are secretory lysosomes present in all cytotoxic populations. Perforin release was measured by ELISA on supernatants harvested from cultures of AML blasts alone, iNKT alone, and/or AML blasts co-cultured with iNKT cells in the presence or absence of α-GalCer after 72 hours of incubation. No significant increase of perforin release was observed after co-culturing AML blasts with iNKT cells or in the presence of α-GalCer (Fig. 4.8).

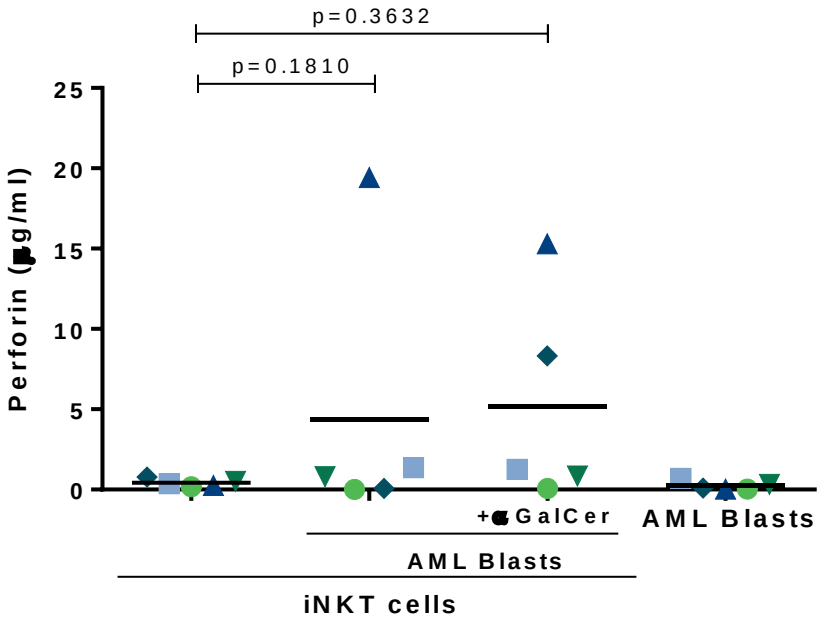


Figure 4.8: Perforin release from iNKT cells mediate a cytotoxic response on AML Blasts.
AML blasts from patients (n=5) were cultured alone or pulsed with α-GalCer (100ng/ml) and co-incubated with iNKT cells for 72 hours. Perforin concentration determined by ELISA showed no difference between activated iNKT cells compared to iNKT cells alone. The ratio of E:T used was 0.25:1. Horizontal line across samples represents the grand mean. Statistics performed by paired t test. Statistical significance considered when $p<0.05$.

Therefore, granzyme B production and release was measured by ELISpot following 24 hours of cultures of iNKT cells co-cultured with patient AML blasts and AML cell lines THP1, U937 and NOMO in the presence or absence of α-GalCer. An increase of granzyme B was observed in co-cultures of AML blasts (cell lines and patient) with iNKT cells, compared to iNKT cells alone (Fig. 4.9). However, an even greater increase in granzyme B was revealed in co-cultures with AML-iNKT cells in the presence of α-GalCer in comparison to iNKT cell alone, thus suggesting granzyme B could be the mechanism mediating iNKT cell cytoxicity (Fig.4. 9).

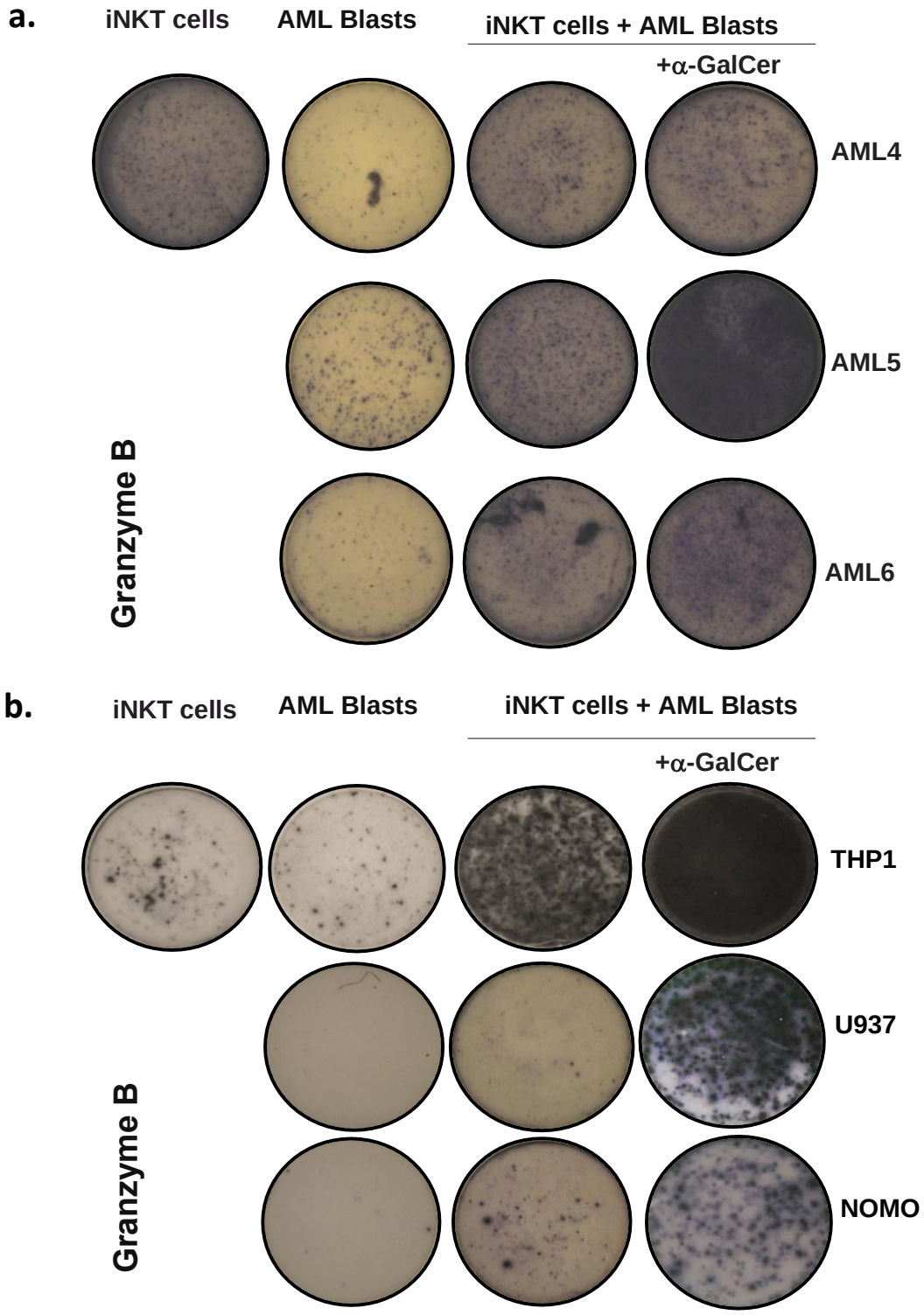


Figure 4. 9: Activated iNKT cells release Granzyme B

a) Patient derived AML blasts (n=3) and b) AML cell lines THP1, U937 and NOMO were cultured alone or pulsed with α-Galcer (100ng/ml) and co-incubated with iNKT cells for 24 hours. Increase in granzyme B was observed in iNKT cells co-cultured α-Galcer-loaded with AML cells by ELISpot. E:T ratio used was 0.25:1.

The core of lytic granules is enclosed by a lipid bilayer comprising lysosomal-associated membrane glycoproteins (LAMPs), such as LAMP1 (CD107α). Flow cytometry was used to measure levels of CD107α to determine if iNKT cell degranulation occurs. Patient derived AML blasts and AML cell lines (THP1, U937 and NOMO) were co-cultured with iNKT cells in the presence or absence of α-GalCer for 24 hours. CD107α was significantly elevated when iNKT cells were co-cultured with AML cells (p=0.0265) and further increased in the presence of α-GalCer (p=0.0064) (Fig.4.10b). The results of ICS for CD107α coincided with increased granzyme B release as quantified by ELISpot in both AML cell lines and AML patients (Fig 4.9 ,4.10).

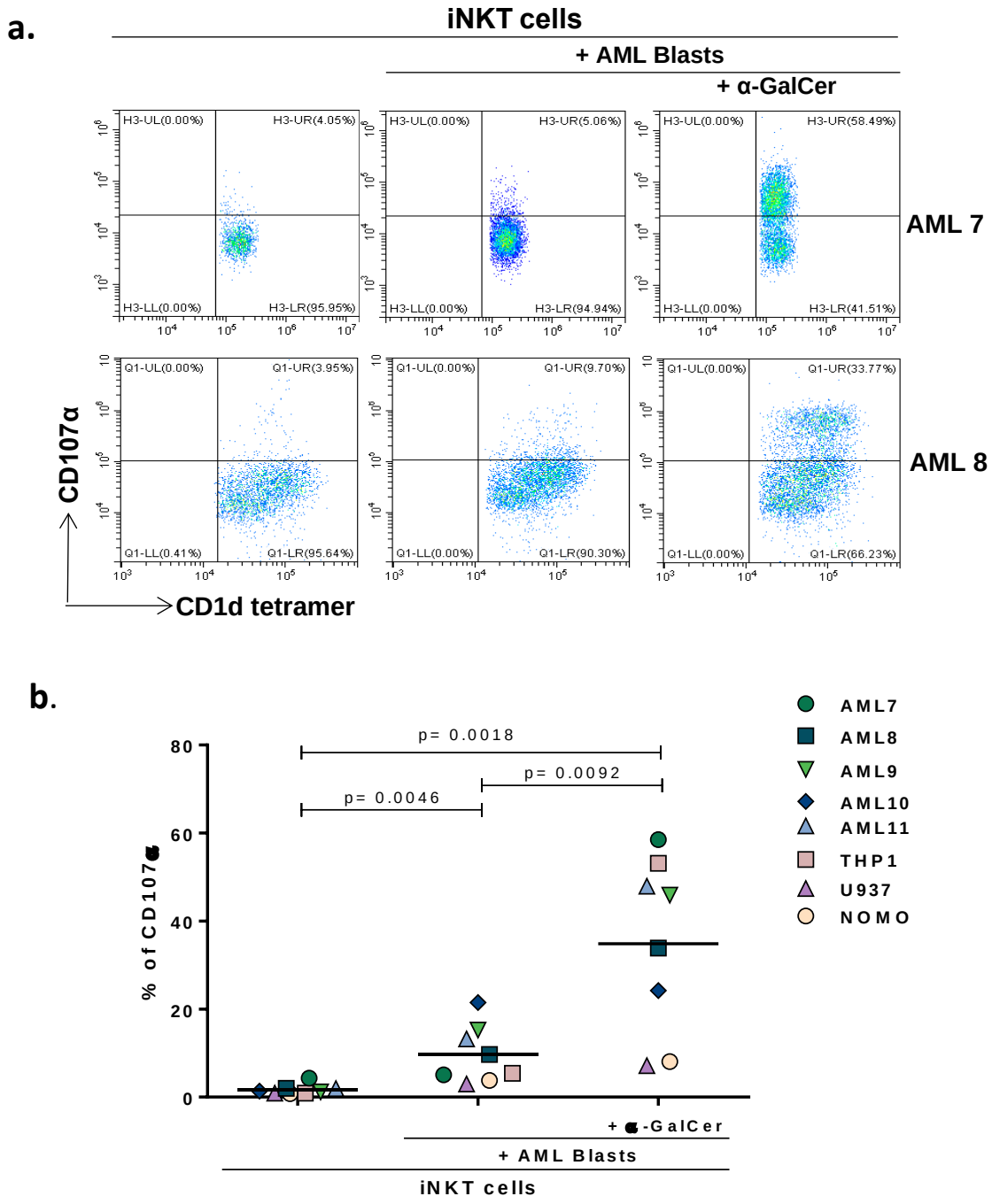


Figure 4. 10: Activated iNKT cells lyse AML blasts via CD107α release

AML blasts from patients (n=5) and cell lines (THP1, U937 and NOMO) were cultured alone or pulsed with α-GalCer (100ng/ml) and co-incubated with iNKT cells for 24 Hrs. CD107α degranulation was determined by ICS. a) Representative dot plots of CD107α release by iNKT cells upon antigen stimulation and b) pooled experiments of showing significant increase in CD107α release on activated iNKT cells following patient AML-iNKT co-cultures. E:T ratio used was 0.25:1. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

Granzyme B is the most potent pro-apoptotic granzyme. To understand if iNKT cells induce granzyme B driven apoptosis on AML blasts, AML cell lines THP1, U937 and NOMO were co-cultured with iNKT cells in the presence or absence of α-GalCer for 72 hours and apoptosis was detected using Annexin V and PI staining by flow cytometry. Increase in double positive Annexin V/ PI AML cells was observed after co-culture with activated iNKT cells pulsed with α-GalCer, indicating cells are in late apoptosis (Fig 4.11).

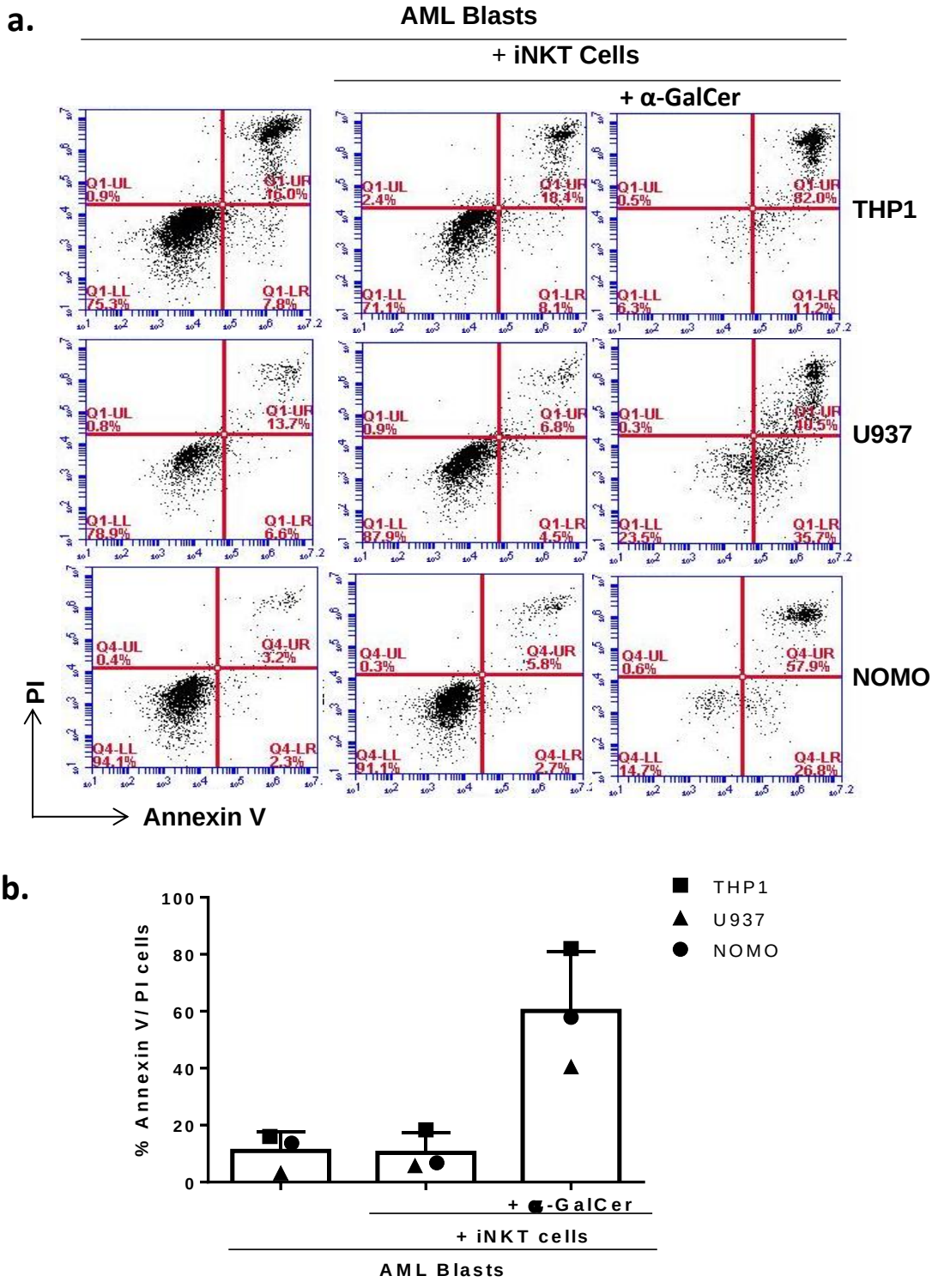


Figure 4.11: Antigen stimulated iNKT cells induce Annexin V/PI positive AML blasts.
AML cell lines (THP1, U937 and NOMO) (n=3) were co-cultured with iNKT cells in the presence or absence of α-GalCer for 72 hours. E:T ratio used was 0.25:1. a) Representative dot plots b) pooled data of Annexin V and Pi positive cells determined by flow cytometry demonstrating increase in Anexin V/PI positive cells in AML blasts following co-cultures with iNKT cells in the presence of α-GalCer.

Granzyme B can induce apoptosis in a caspase- dependent and caspase-independent manner. Expression levels of different apoptotic marker proteins was measured in AML cell lines THP1, U937 and NOMO were co-cultured with iNKT cells in the presence or absence of α-GalCer for 48 hours. After co-culture AML blasts were positively sorted by MACS and lysed for western blot. We also observed cleavage of Caspase 3, Caspase 9, and PARP by western blot on AML cell lines following co-culture with iNKT cells in the presence of α-GalCer, signifying apoptosis (Fig 4.12).

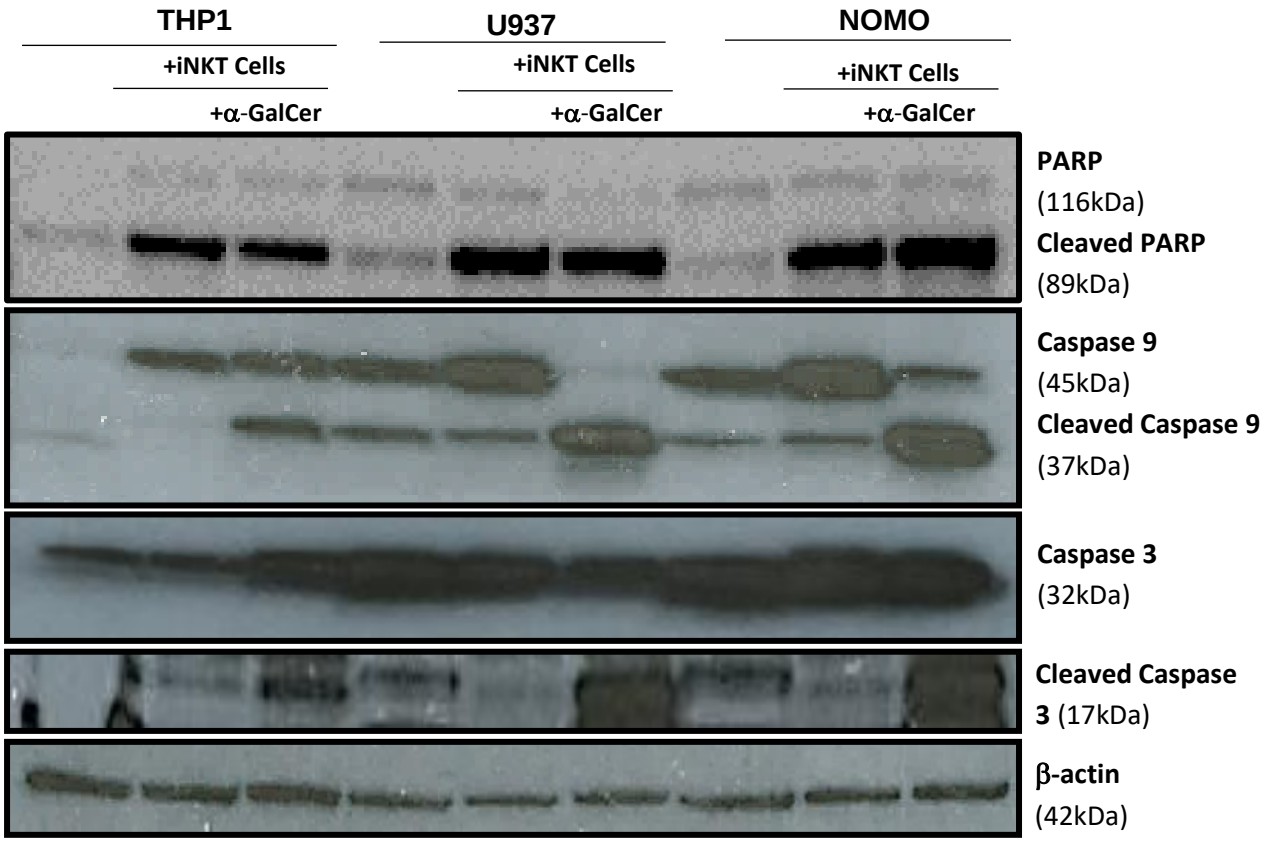


Figure 4.12: AML blasts undergo apoptosis following iNKT cell activation.
AML cell lines THP1, NOMO and U937 were pulsed with α-GalCer (100ng/ml) and co-culture with iNKT cells. Following 48 hour incubation. E:T used was 0.25:1 AML blasts were positively isolated and lysed. PARP, Caspase 9, and Caspase 3 cleavage was observed via western blot. β-actin was used as a loading control.

To consolidate findings showing that iNKT cells induce apoptosis against AML blasts we used transitional electron microscopy (TEM) given that it has been described the “gold standard” for identifying apoptotic cells. TEM was performed on AML cell lines THP1, U937 and NOMO. As previously described, AML cells were co-incubated with iNKT cells in the presence or absence of α-GalCer for 72 hours and AML blasts were positively selected using MACS sorting and fixed for TEM. Images of AML blasts which were loaded with α-GalCer co-cultured with iNKT cells showed disintegrated cell membrane, loss of chromatin and accumulation of cell debris. Such features are characteristics of apoptosis. In contrast, AML blasts cultured alone were identified as viable cells portraying normal morphology including intact cell membrane (Fig 4.13).

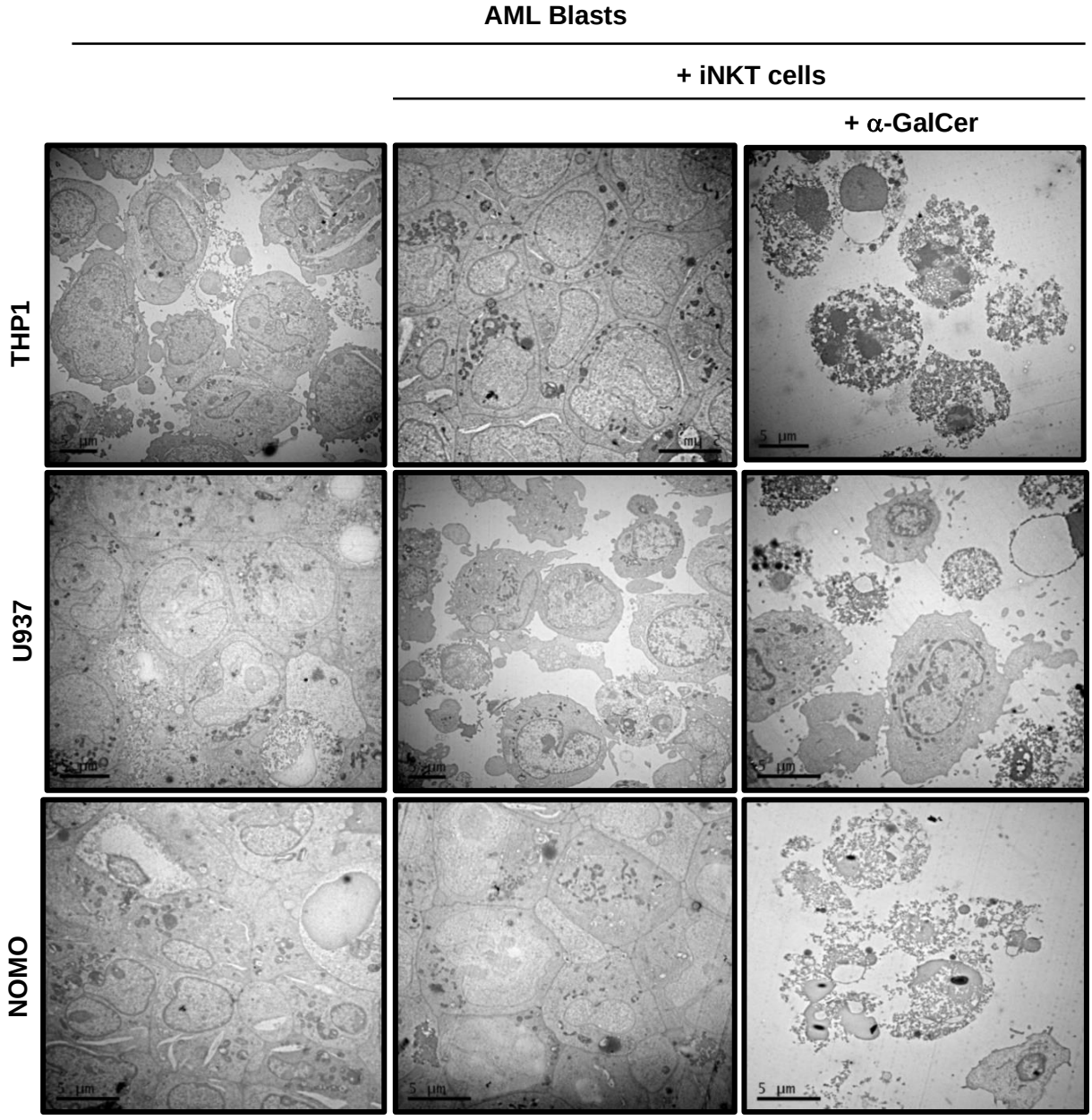


Figure 4.13: α-GalCer activated iNKT cells can kill AML cell lines.
AML cells lines (THP1, U937 and NOMO) were cultured alone or co-cultured with iNKT cells (E:T ratio used was 0.25:1) with or without α-GalCer (100ng/ml) stimulation for 72 Hrs. Isolated AML cell lines were harvested and processed for transient electron microscopy. AML blasts undergo apoptosis following co-cultures with iNKT cells in the presence of α-GalCer. Scale used:5µM

Since iNKT cells recognise lipid antigens via CD1d presentation, CD1d surface expression was measured by flow cytometry on AML cell lines and patient derived AML blasts (Fig. 4.14a). CD1d expression on CD33 positive AML blasts was observed, ranging from 0.1%-83.25% (Fig. 4.14b). Equally, AML cell lines THP1, U937 and NOMO expressed high CD1d levels 80%, 75%, and 60% respectively, while K562, HL60 and KG-1α have 2.39%, 1.05%, and 0.19% respectively low CD1d expression (Fig. 4. 14b).

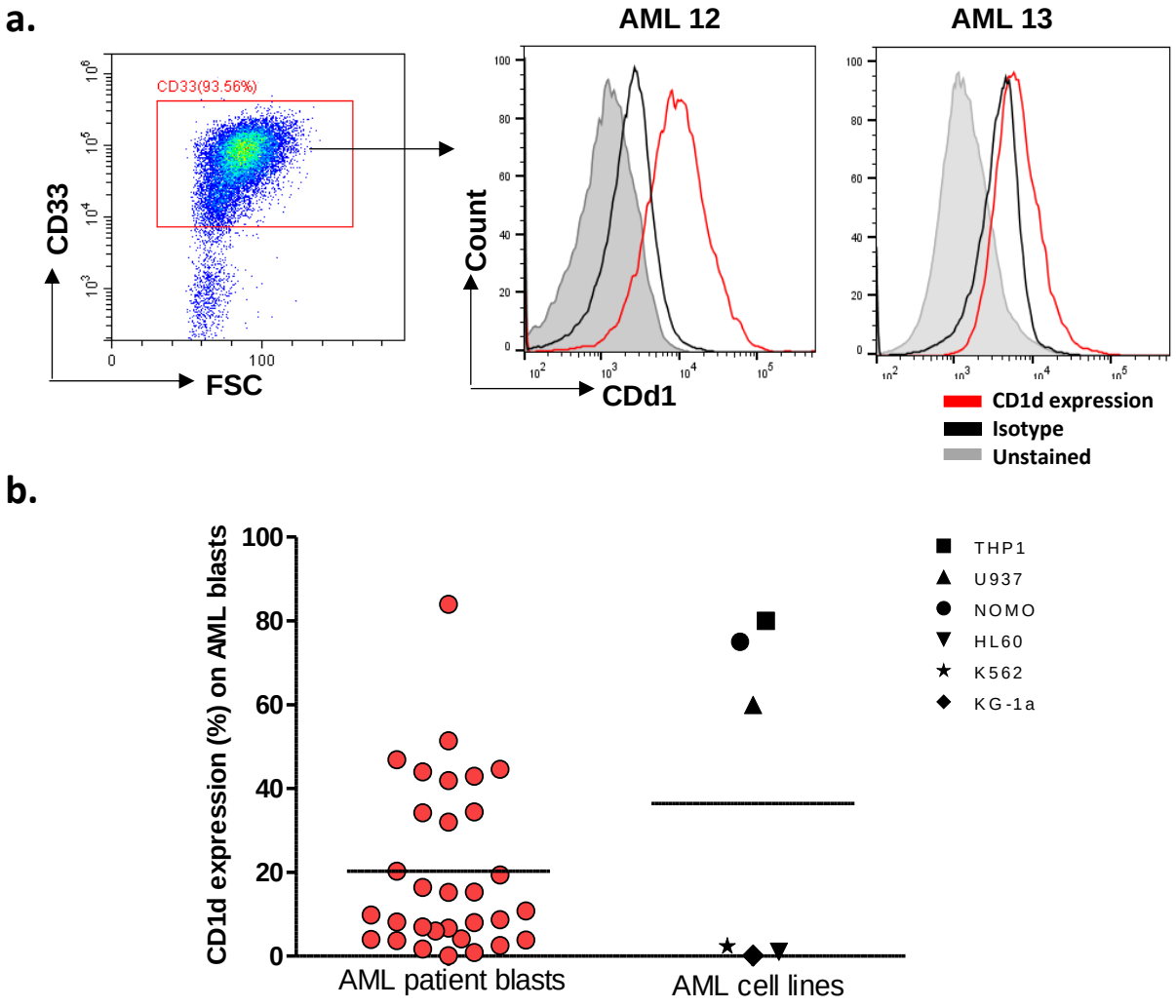


Figure 4. 14: Surface CD1d expression on CD33 positive AML Patient blasts and AML Cell Lines. a) Representative dotplot of CD33 positive blasts on AML patients lysed blood. CD1d expression was determined on CD33 positive blasts. Representative histogram overlays of CD1d expression on AML blasts (CD33 positive) derived from patients from blood. b) Surface CD1d expression was determined from pooled AML blasts derived from patients (n=31), and AML cell lines: HL60, THP1, K562, U937, NOMO, KG1-a were stained with anti-human CD1d antibody and were subject to flow cytometry. Horizontal line across samples represents the grand mean.

To understand if antigen driven stimulation on iNKT cells is driven by CD1d, IFN-γ release by iNKT cells was measured following the co-culture with CD1d negative cell lines HL60, K562 and KG1-a in the presence of absence of α-GalCer for 72 hours. No IFN-γ was detected in cell culture supernatants after iNKT co-culture with CD1d negative cell lines even in the presence of the potent stimulant α-GalCer. Conversely, IFN-γ was detected in CD1d positive cell lines (THP1, U937 and NOMO) in the same conditions. These results suggest that CD1d on AML blasts is necessary for iNKT cells activation (Fig. 4.15).

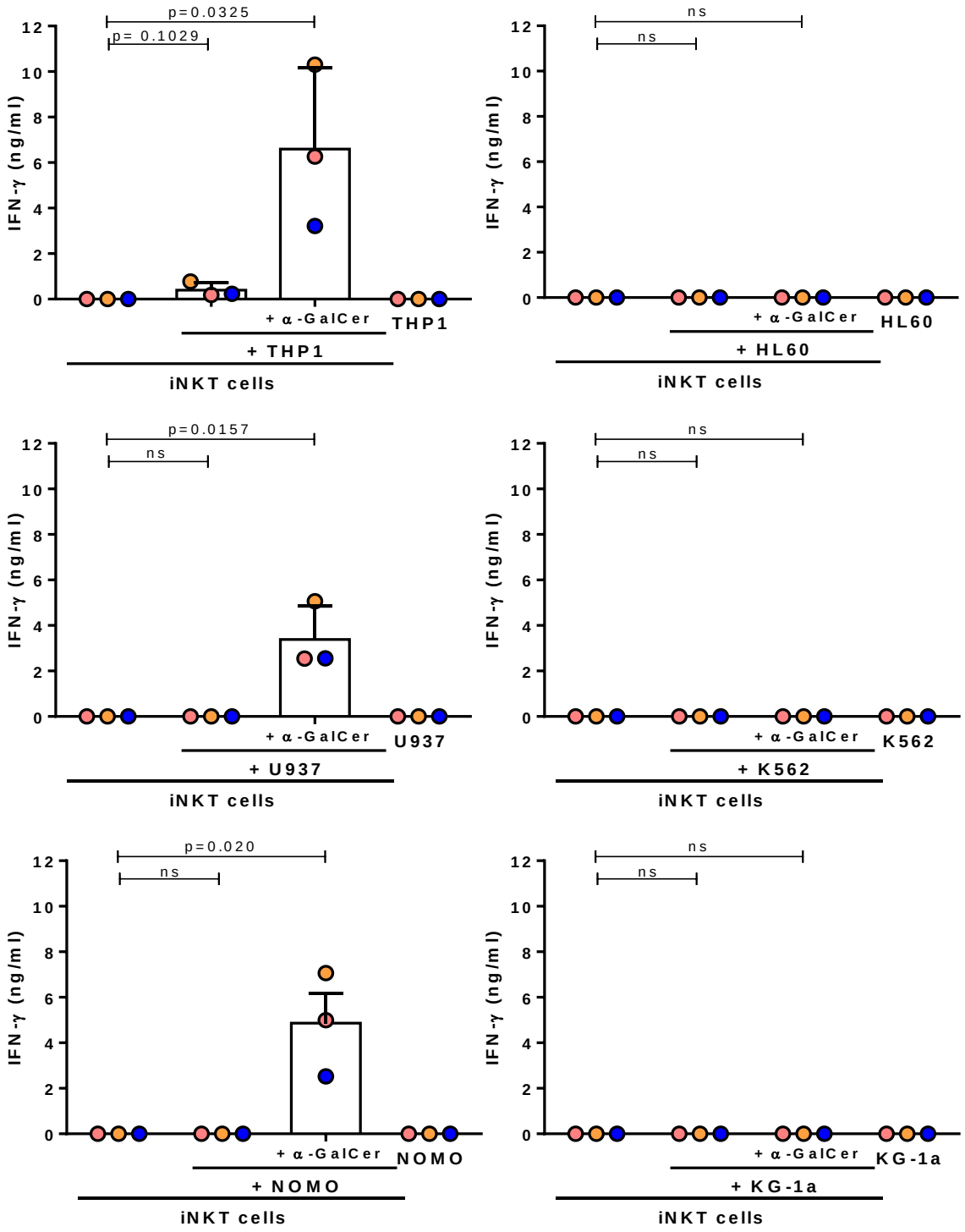


Figure 4.15: AML Cell lines HL60, K562 and KG1-a do not activate iNKT cells via α-GalCer presentation.

iNKT cell activation of supernatants was defined by IFN-γ release AML Cell lines (THP1, U937, NOMO, HL60, K562, KG1-a) were co-cultured with iNKT cells alone and pulsed with α-GalCer for 72 hours. Supernatant from cultures were harvested and ELISA was used to determine IFN-γ concentrations. No IFN-γ was detected in cultures of iNKT cells with CD1d negative AML cell lines in the presence or absence or α-GalCer. The ratio of AML Cells: iNKT cells was 1:0.25. (n=3) Data represented as mean ±SEM. Statistics performed by two-way ANOVA Statistical significance considered when $p<0.05$. ns=not significant.

To understand the changes in cellular bioenergetics of iNKT cells following co-cultures with CD1d negative AML cells, the glycolytic flux and oxidative phosphorylation of iNKT cells measured following co-incubation with KG1-a cells loaded with α-GalCer using a Seahorse XF Analyzer as previously described. No difference in the OCR and ECAR was observed between iNKT cells alone and iNKT cells co-cultured with KG1-a loaded with α-GalCer (Fig. 4.16 & 4.17).

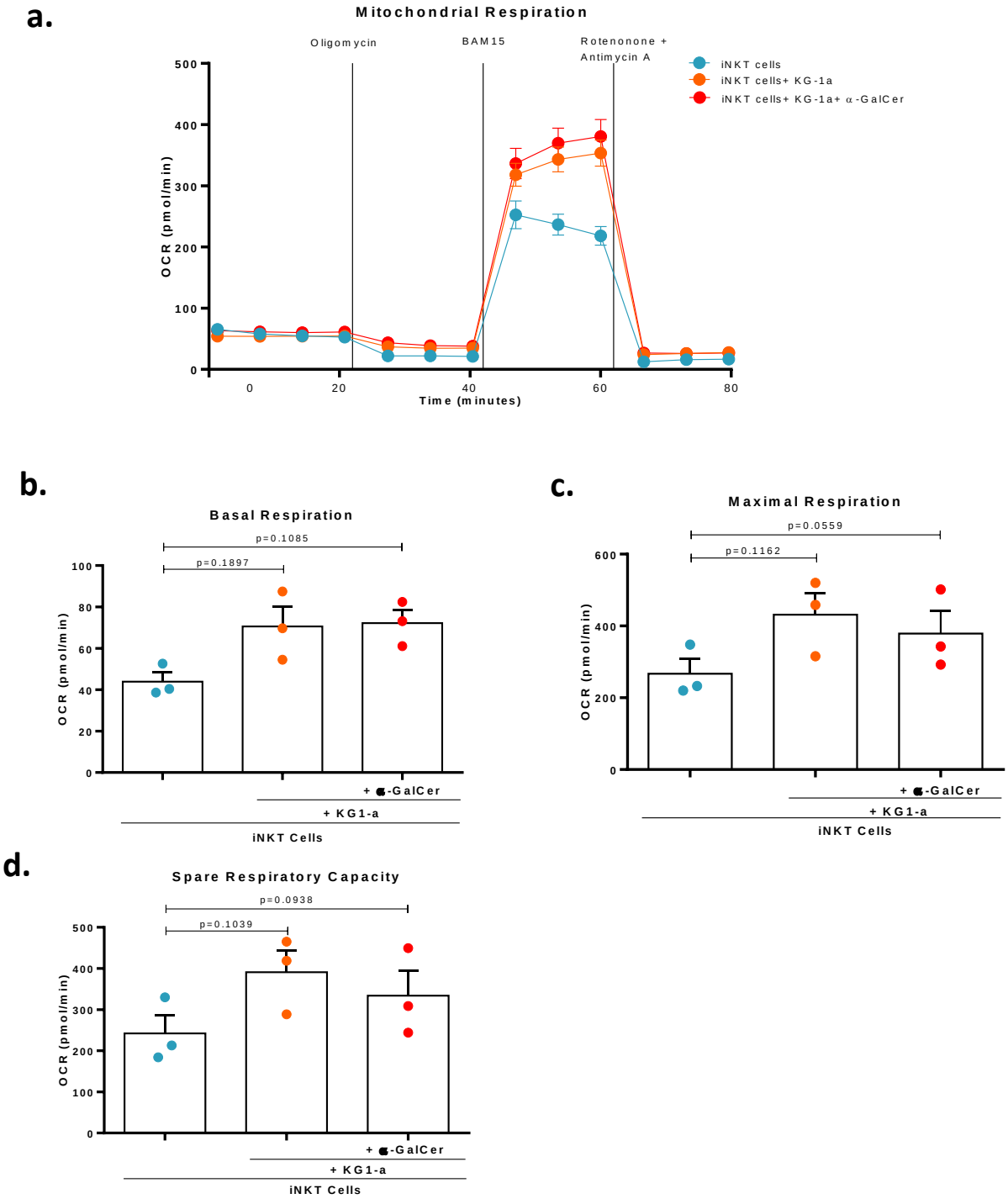


Figure 4.16: No change in OCR on iNKT cells following co-cultures with α-GalCer and KG-1a cells. iNKT cells were co-cultured with KG-1a cells in the presence or absence of α-GalCer for 24 hours (n=3). a) Representative graph showing the comparison of OCR of purified iNKT cells using a seahorse assay. b) Basal respiration, c) maximal respiration and d) spare respiratory capacity shown. Oxidative phosphorylation of iNKT cells was not altered after co-cultures with AML blasts in the presence or absence of α-GalCer. E:T ratio used was 0.25:1. Statistics performed by paired t test. Statistical significance considered when $p<0.05$.

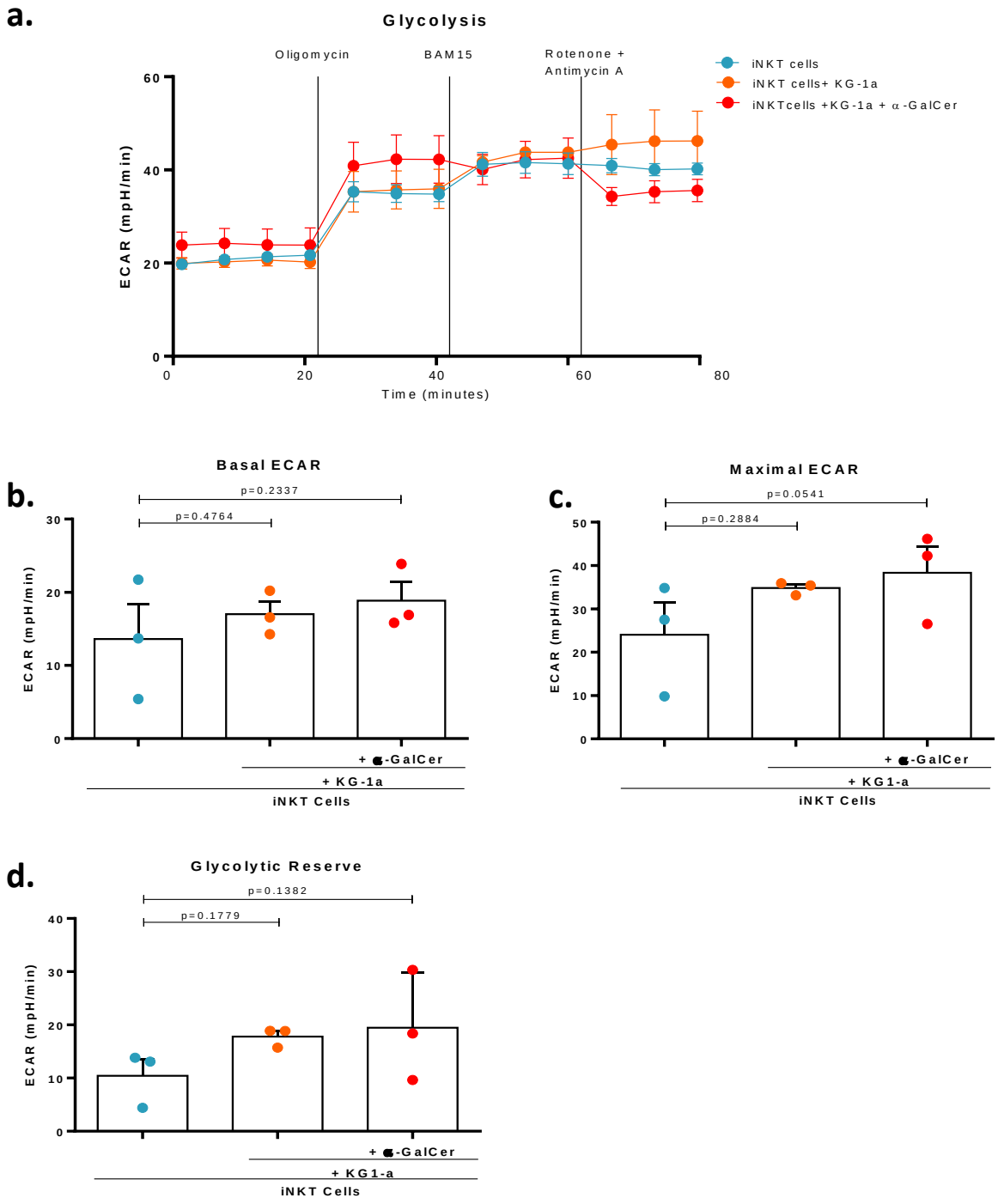


Figure 4.17: A significant increase in basal ECAR on iNKT cells following co-cultures with α-GalCer and KG-1a cells.

iNKT cells were co-cultured with KG-1a cells in the presence or absence of α-GalCer for 24 hours (n=3). a) Representative graph showing the comparison of ECAR of purified iNKT cells using a seahorse assay. b) Basal ECAR c) maximal ECAR and d) Glycolytic reserve on purified iNKT cells shown. Glycolytic flux of iNKT cells was not altered after co-culture with AML blasts in the presence or absence of α-GalCer. E:T ratio used was 0.25:1. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

Finally, we investigated the cytotoxic potential of iNKT cells on CD1d negative cells. CD1d positive cell lines THP1, U937 and NOMO as well as CD1d negative cell lines HL60, K562 and KG1-a were pulsed with α-GalCer and co-incubated with iNKT cells for 72 hours. PI staining and analysis of flow cytometry was used to identify viable cells. Unlike with CD1d positive cells, no killing of CD1d negative AML cell lines: HL60, K562 and KG1-a was observed following co-culture with iNKT cells in the presence α-GalCer for 72 hours (Fig 4.18).

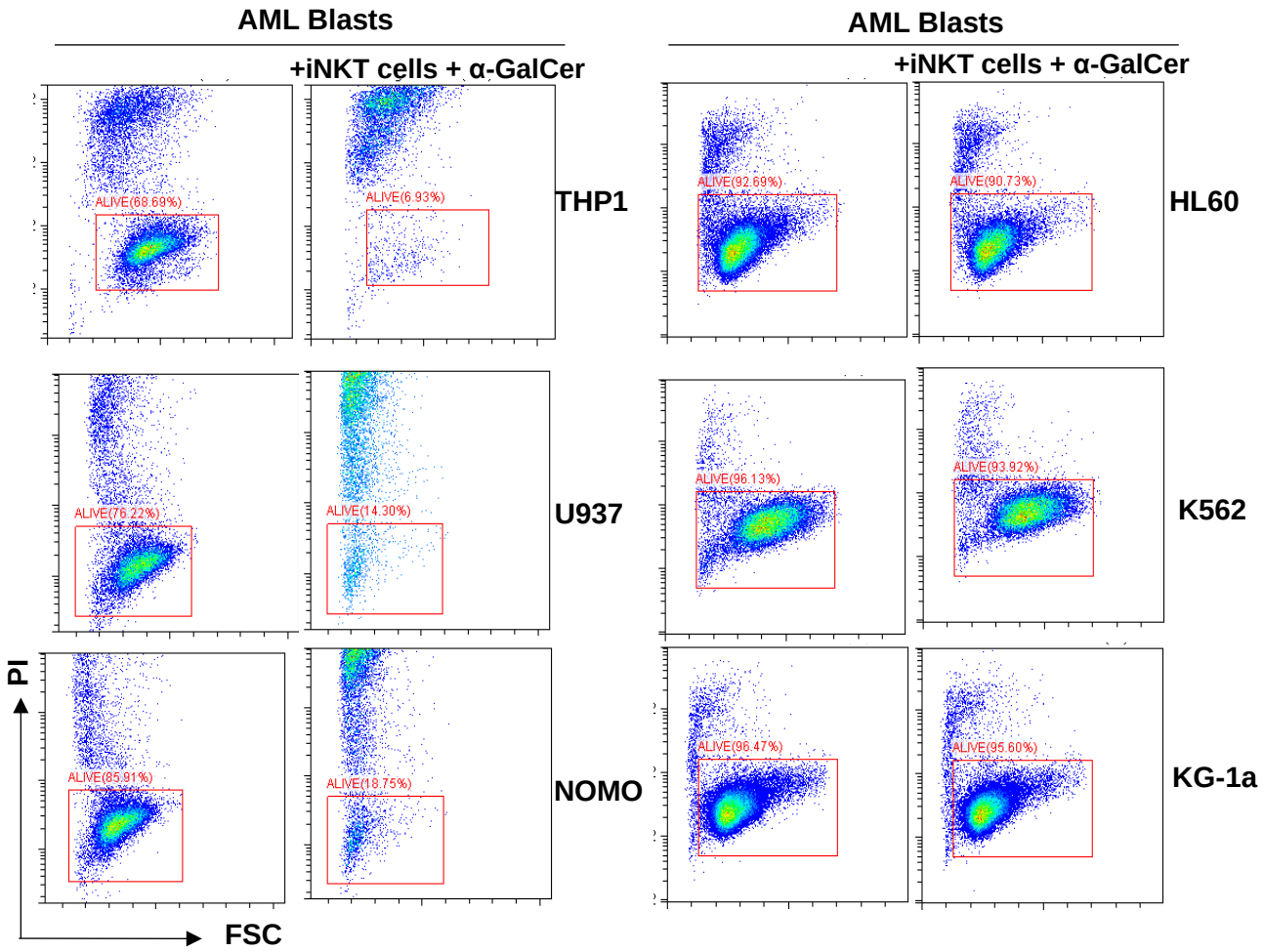


Figure 4.18: iNKT cells kill AML blasts in a CD1d dependent manner.
Viability of CD1d positive (THP1, U937, NOMO) and CD1d negative (HL60, K562, KG-1a) AML cells lines was determined by flow cytometry using PI staining following iNKT cells co-culture with α-GalCer. iNKT cells did not kill CD1d negative cells in the presence of α-GalCer. E:T ratio used was 0.25:1. Representative dot plots of 3 repeats.

To investigate the requirement for CD1d in mediating iNKT cell-AML interactions, a specific monoclonal anti-CD1d blocking antibody was used to neutralize CD1d-iTCR interactions. A dose dependent increase in AML viability was observed when increasing the concentrations of anti-CD1d mAb were added to α-GalCer pulsed patient AML blasts and iNKT cells (Fig 4.19a). A minimum of 5μg/ml mAb was sufficient to completely abrogate the iNKT cell cytotoxic effect (Fig 4.19a) Thus, 5μg/ml of anti-CD1d mAb was added to co-cultures of iNKT cells with α-GalCer loaded AML blasts derived from patients, AML blasts viability was restored (p=0.0156) (Fig. 4.19b).

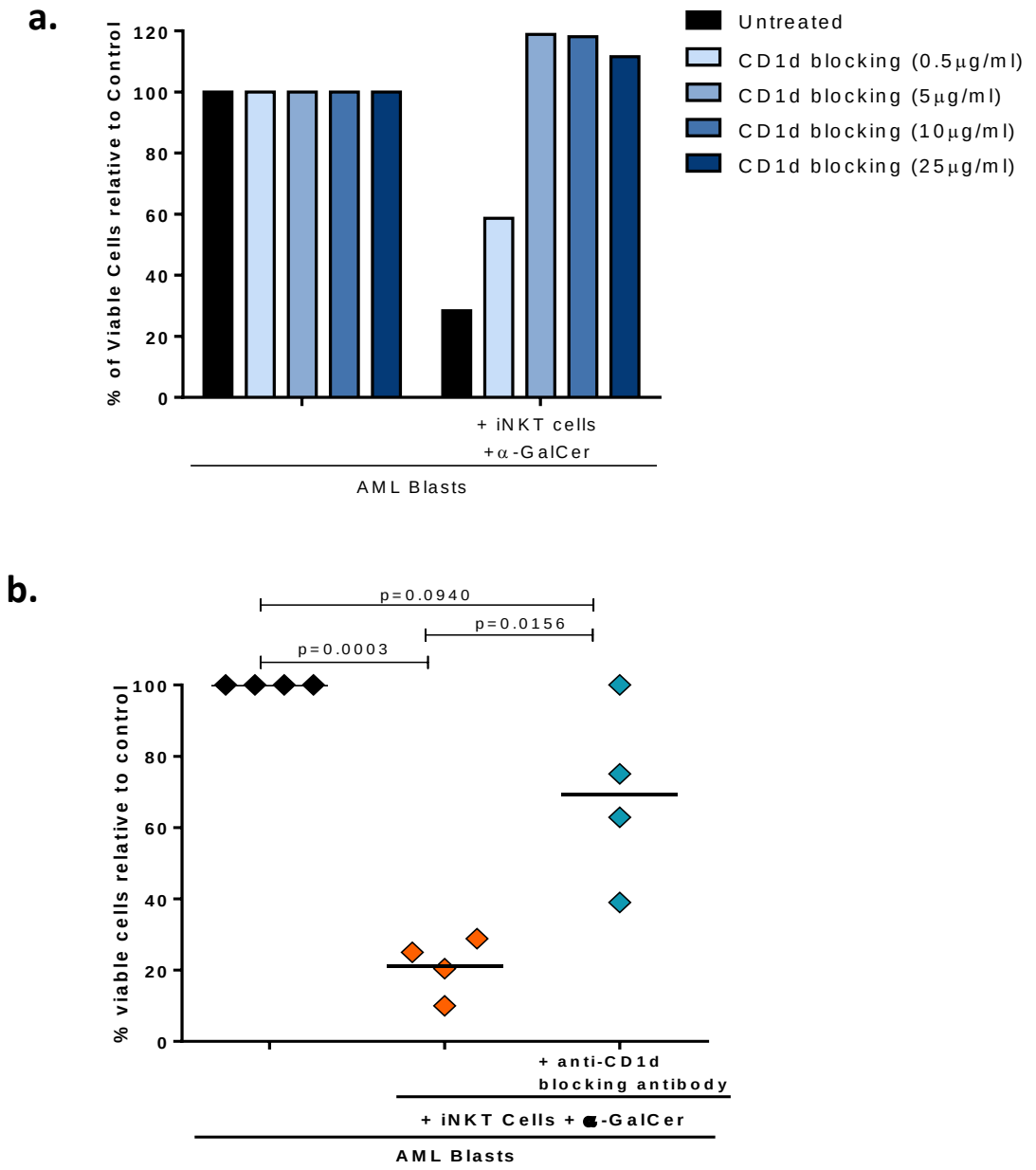


Figure 4.19: CD1d mediates iNKT cell- AML interaction.

Anti-CD1d blocking mAb was added to patient derived AML blasts (n=4) for 1 hour. a) at increasing concentrations of CD1d blocking antibody were used (0.5,5,10,25 μg/ml). b) at 5 μg/ml. Following CD1d blocking, AML blasts were pulsed with α-GalCer in the presence of iNKT cells for an additional 72 hours. Viability of blasts was determined by flow cytometry using propidium iodide. E:T ratio used was 0.25:1. Anti-CD1d blocking restored AML blast viability in the presence of α-GalCer and iNKT cells. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

Retinoic Acid does not enhance iNKT cell–mediated regulation on AML blasts.

Given that iNKT cell -AML blasts interaction is CD1d dependent, factors capable of regulating CD1d expression could enhance the efficacy of iNKT cell activation driven by AML blasts were explored. Studies have shown that *all-trans* Retinoic acid (ATRA)- an active metabolite of vitamin A can modulate CD1d expression in monocytes and THP1 cells [248]. Since AML is of myeloid lineage, ATRA (1μM) was added to low CD1d expressing AML blasts derived from patients for 72 hours, higher concentrations were toxic to AML blasts. Significant upregulation (p=0.042) of CD1d expression was observed following ATRA treatment on AML blasts from 6.7% to 15.2% on average (Fig 4.20a, b). Thus, to understand if the augmented CD1 expression can enhance the tumuoricidal capacity of iNKT cells, AML blasts were pre-treated with ATRA and co-cultured with iNKT cells in the presence α-GalCer. Flow cytometry was used to determine viability of AML blasts, following 72 hours of incubation by staining with PI. Nonetheless, ATRA treatment did not alter lysis of AML blasts by activated iNKT cells (Fig. 4.20c).

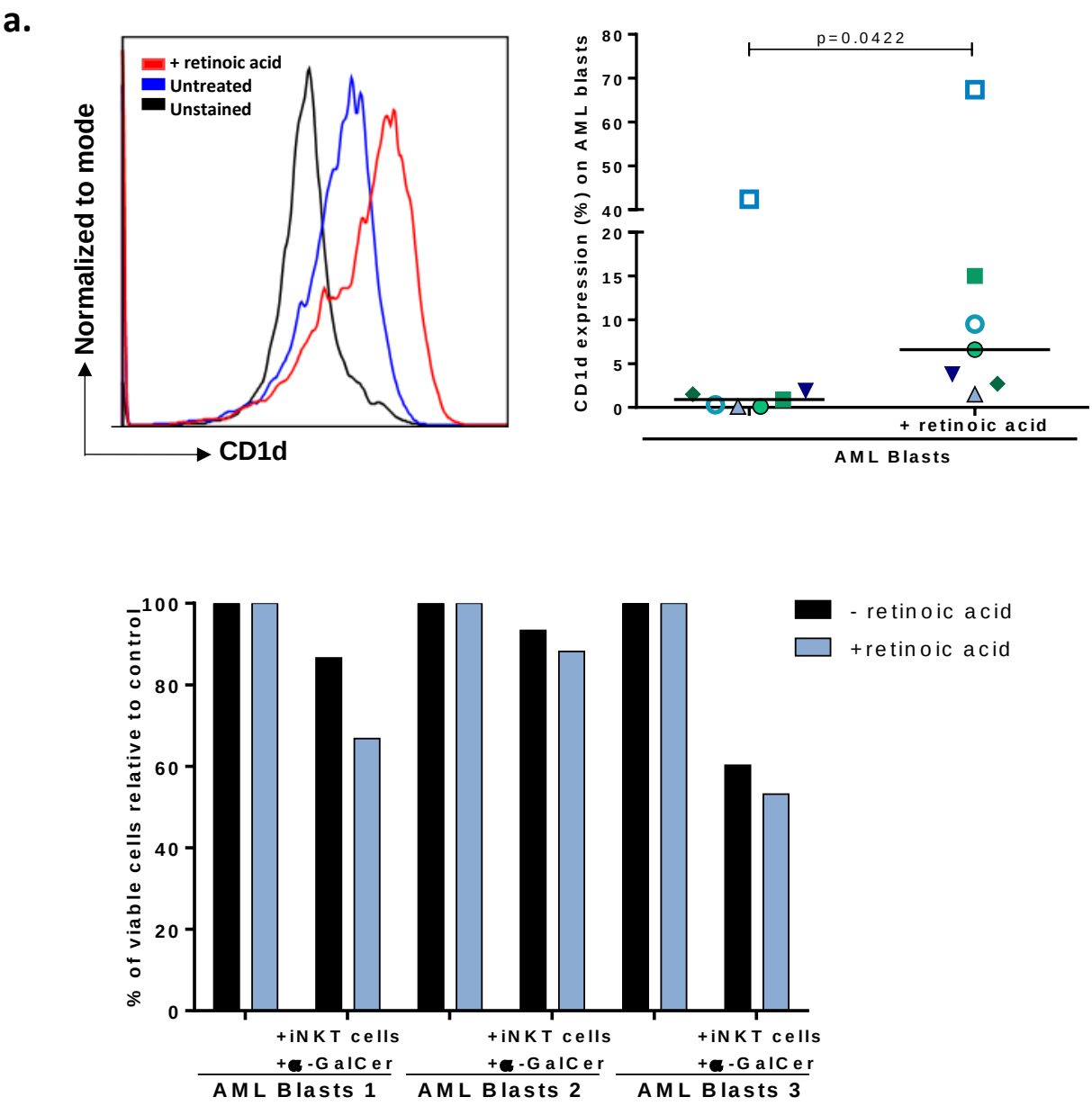


Figure 4.20: Retinoic Acid treatment does not alter iNKT cell cytotoxic response on patient AML Blasts.

a) Representative histogram and b) pooled experiments from patient AML blasts (n=7) being treated with ATRA (1μM) for 72 hours. Cells were stained with anti-human CD1d antibody were subject to flow cytometric analysis. c) Patient AML blasts (n=3) were co incubated with iNKT cells (0.25:1 E:T ratio) pulsed with α-GalCer and treated with RA (1μM) for 72 hours. Cells were harvested and stained with PI to determining cell viability by flow cytometry. ATRA treatment enhanced CD1d expression on AML blasts but did not enhance AML blast killing following co-cultures with iNKT cells in the presence of α-GalCer. Horizontal lines and bar charts across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when: $p<0.05$.

iNKT-AML interaction is CD1d/CD40 dependent.

In addition to iNKT cells recognising lipid antigens in the context of the antigen presentation molecule CD1d (signal 1), it has been demonstrated that co-stimulatory molecules can positively or negatively affect iNKT cell activation and function and skew the immune response (signal 2). Upregulation of CD40 (signal 2) following activation is necessary for production TH1 inflammatory response in anti-tumour immunity [158]. Therefore, whole blood from AML patients was lysed and CD40 surface expression was measured by flow cytometry on cell lines and patient derived AML blasts. Patient AML blasts expressed a range of CD40 on surface (from 0.2-66.91%) and in AML cell lines: THP1, NOMO, U937, K562, HL60 and KG1-a expression was 93%, 25%, 22%, 4%, 4% and 1% respectively (Fig. 4.21).

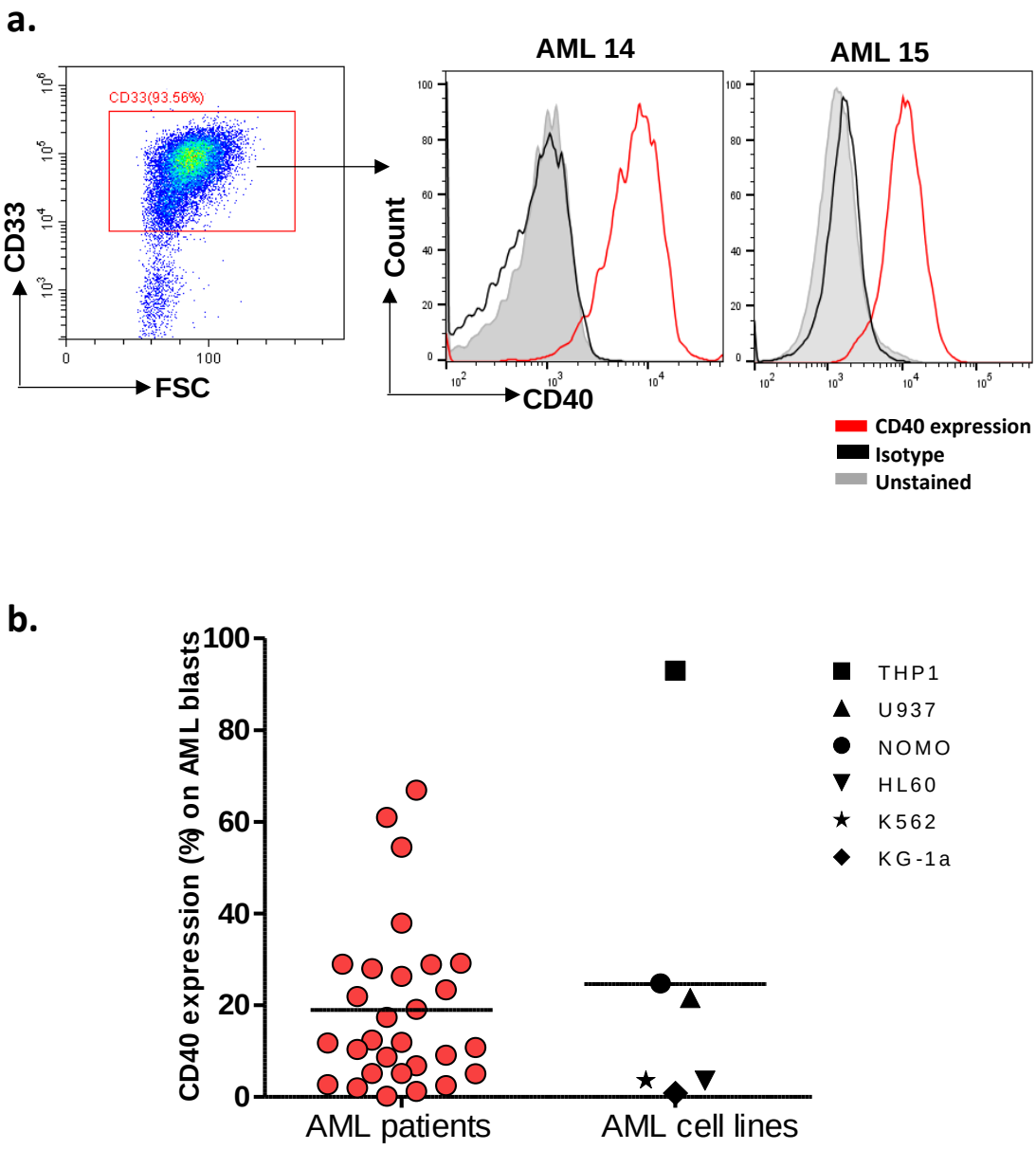


Figure 4.21: Percentage of surface CD40 expression on AML Patient blasts and AML Cell Lines.
a) Representative dot plot of CD33 positive AML blasts derived from patients' lysed blood. CD40 expression was determined on CD33 positive AML blasts. Representative histogram overlays of CD40 expression on AML patient blasts (CD33 positive) from blood. b) Surface CD40 expression was determined from AML patients (n=29), and AML cell lines: HL60, THP1, K562, U937, NOMO, KG-1a were stained with anti-human CD40 antibody and were subject to flow cytometry. Horizontal line across samples represents the grand mean.

To understand if co-stimulatory signal, CD40 is required for AML-iNKT cell mediated activation, a specific monoclonal anti-CD40 blocking antibody was used to block CD40-CD40L interactions. AML blasts were pre-incubated with anti-CD40 mAb (at increasing concentrations) for one prior to co-culture with iNKT cells in the presence of α-GalCer for 72 hours. A dose dependent increase in AML viability was observed when increasing the concentrations of anti-CD40 mAb were used to α-GalCer pulsed patient AML blasts and iNKT cells and minimum of 6μg/ml mAb was sufficient to completely restore AML blast viability (Fig. 4.22a). Therefore, 6μg/ml of anti-CD1d mAb was added to co-cultures of iNKT cells with α-GalCer loaded AML blasts derived from patients, AML blasts viability was restored (p=0.0207) (Fig. 4.22b).

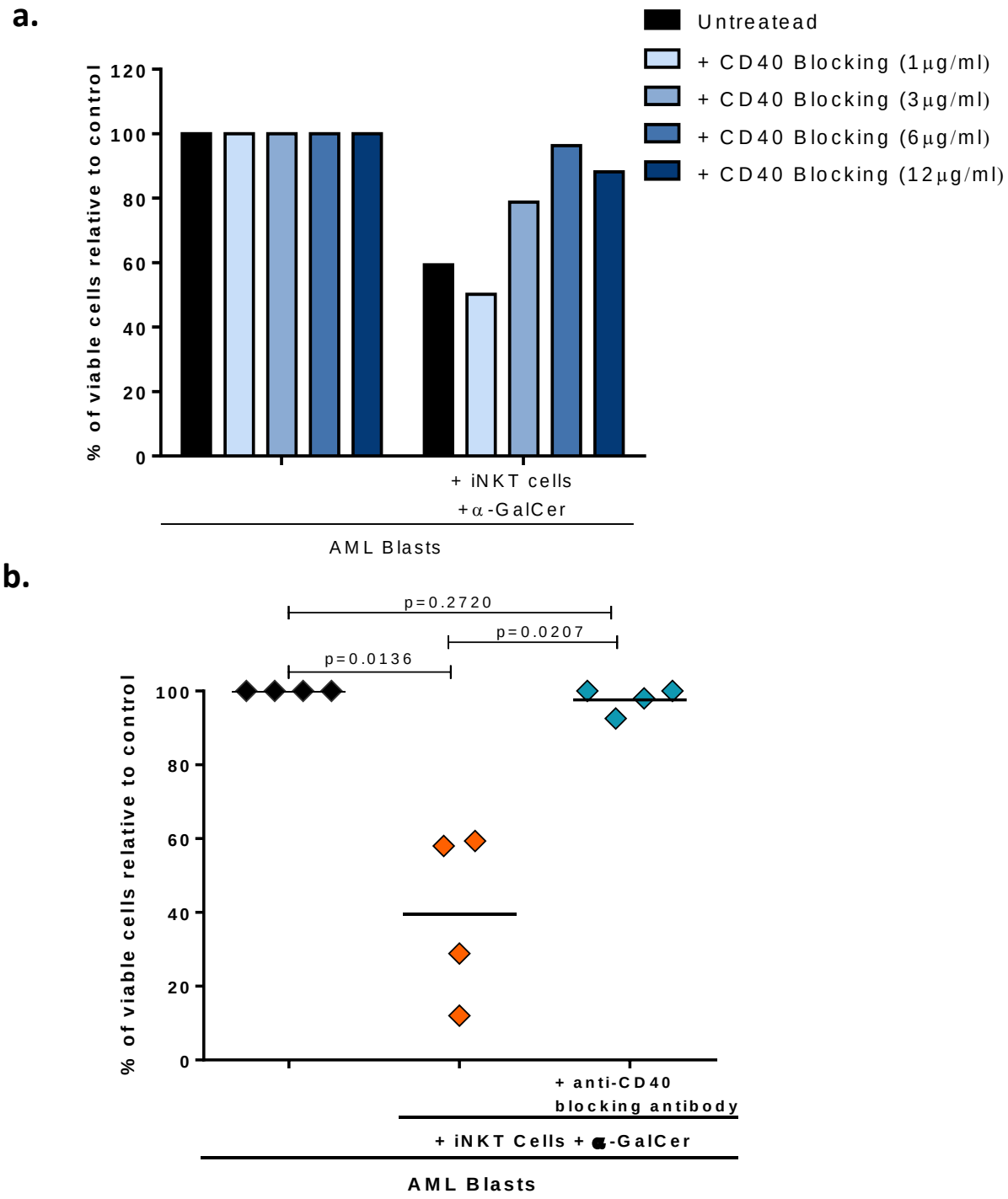


Figure 4.22: CD40 mediates iNKT- AML cell interaction.

a) Anti-CD40 blocking mAb (1,3,6,12 μg/ml) was added to patient AML blasts for 1 hour (n=4) prior to the co-culture with iNKT cells in the presence of α-GalCer. b) Prior to 72 hour co-culture of patient AML blasts with α-GalCer (100ng/ml) and iNKT cells, CD40 blocking antibody was added (6μg/ml). Viability of blasts was determined by flow cytometry using PI uptake. E:T ratio used was 0.25:1. Anti-CD40 blocking abrogated iNKT cell cytotoxic effect on AML viability in the presence of α-GalCer. Horizontal line and bar charts across samples represents the grand mean. Statistics performed by paired t-test Statistical significance considered when $p<0.05$.

Having demonstrated that α-GalCer stimulation, not only elicits a potent iNKT cell activation and proliferation in a CD1d/CD40 dependent manner against AML blasts but also a direct cytotoxic response. The influence of low arginine microenvironment driven by AML blasts, on antigen mediated-iNKT cell activation and proliferation was explored. Therefore, patient derived AML blasts were co-incubated with iNKT cells in the presence of α-GalCer for 72 hours in RPMI10% and low arginine media. Supernatants were harvested and IFN-γ levels were measured by ELISA. Upon antigen presentation iNKT cells statistically increase IFN-γ was measured (*p*=0.0024) in RPMI10% (on average 6.48 ng/ml) and when cultured in low arginine conditions (on average 7.95ng/ml) (*p*=0.0037) in a similar manner. Thus, low arginine conditions do not alter or supress iNKT cell activation (Fig. 4.23).

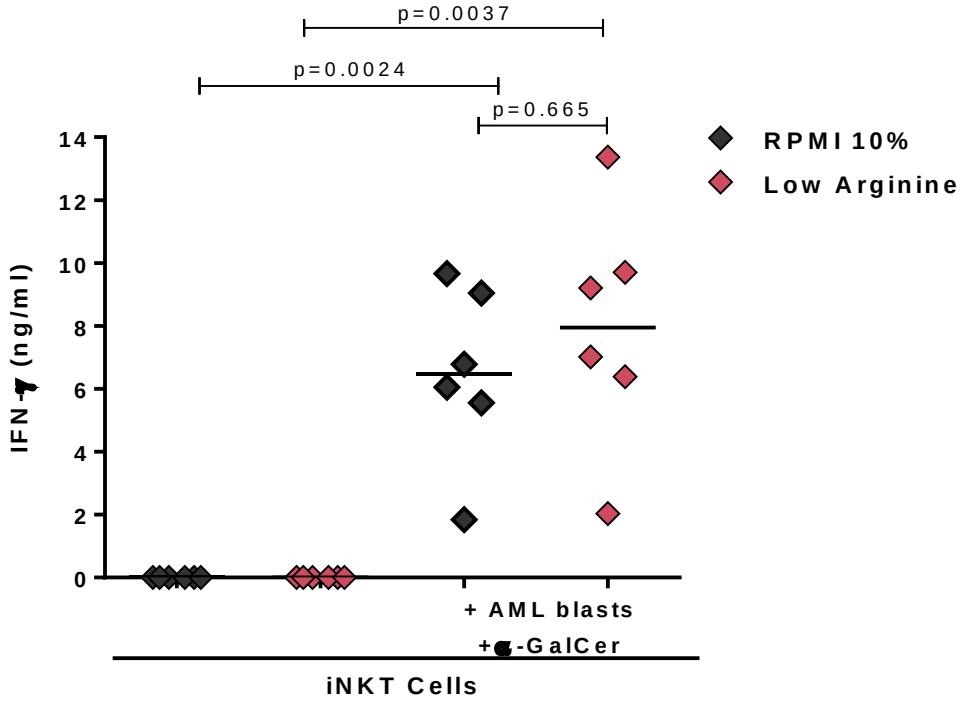


Figure 4.23: Low arginine AML microenvironment does not alter cytotoxic function of iNKT cells. AML patient pulsed with α-GalCer (100ng/ml) were co-cultured with iNKT cells (E:T ratio-0.25:1) for 72 hours in complete and low arginine conditions. Supernatants were harvested and IFN-γ release was measured by ELISA. Increase in INF-γ observed in in co-cultures of α-GalCer-loaded AML blasts with iNKT cells was not impaired by arginine low conditions. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Significance considered when $p<0.05$.

To establish if iNKT cell proliferation previously observed following antigen stimulation (Fig. 4.6) was not affected by low arginine conditions, patient AML blasts in the presence or absence of α-GalCer were co-cultured with iNKT cells in RPMI10% and low arginine media for 72 hours. Flow cytometry was used to measure the frequency of alive NKT cells (Fig. 4.24). Following iNKT-AML co-culture, iNKT cell frequency increased in RPMI10% which was further enhanced in the presence of α-GalCer (p=0.0247). In low arginine conditions, iNKT cell proliferation significantly increased following co-culture with AML blasts in the presence of α-GalCer (p=0.0299). No significant difference in iNKT cells proliferation was observed following co-culture with AML blasts in the presence (p=0.8316) or absence of α-GalCer (p=0.7051) between the RPMI10% and low arginine conditions, thus confirming iNKT cell activation and proliferation are not affect by low arginine condition in AML antigen mediated stimulation.

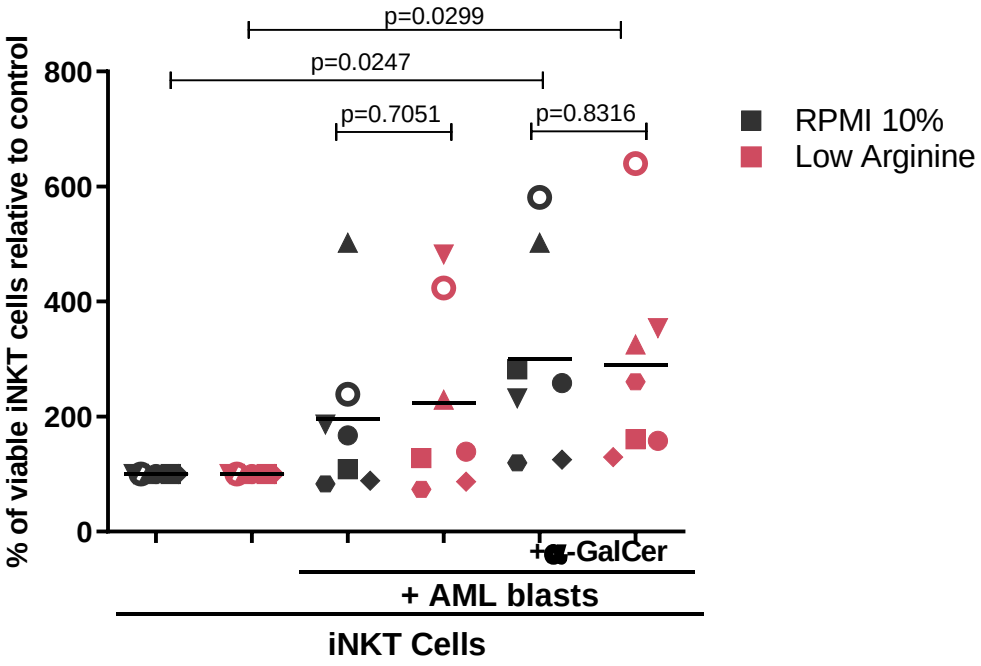


Figure 4.24: α-GalCer presentation by patient AML blasts drives iNKT cell proliferation, which is not affected by low arginine conditions.

iNKT cells were co-cultured with patient AML blasts in the presence of absence of α-GalCer for 72 hours in RPMI10% and low arginine conditions (n=7). iNKT cells frequency is enhanced following co-cultures with α-GalCer-loaded AML blasts both complete and low arginine conditions equally. E:T ratio used was 0.25: 1. iNKT cells were identified by gating on cells positively stained with fluorescent-labelled CD1d tetramer. Horizontal line across samples represents the grand mean. Statistics performed by paired t-test. Statistical significance considered when p<0.05.

The data so far indicates that the low arginine conditions did not suppress AML-iNKT interactions, we determined if it could inhibit the cytotoxic potential of iNKT cells. Patient AML blast viability was measured by flow cytometry using PI staining following 72 hour co-cultures of iNKT cells in the presence or absence of α-GalCer in RPM10% and low arginine media. The cytotoxic response of iNKT cells was not affected under low arginine conditions. AML blasts were equally killed following co-culture with α-GalCer activated iNKT cells in low arginine and complete conditions with no significant difference observed in the two experimental conditions (*p*=0.777) (Fig. 4.25). Thus, concurring that low arginine microenvironment does not alter or supress the functionality of activated iNKT cells.

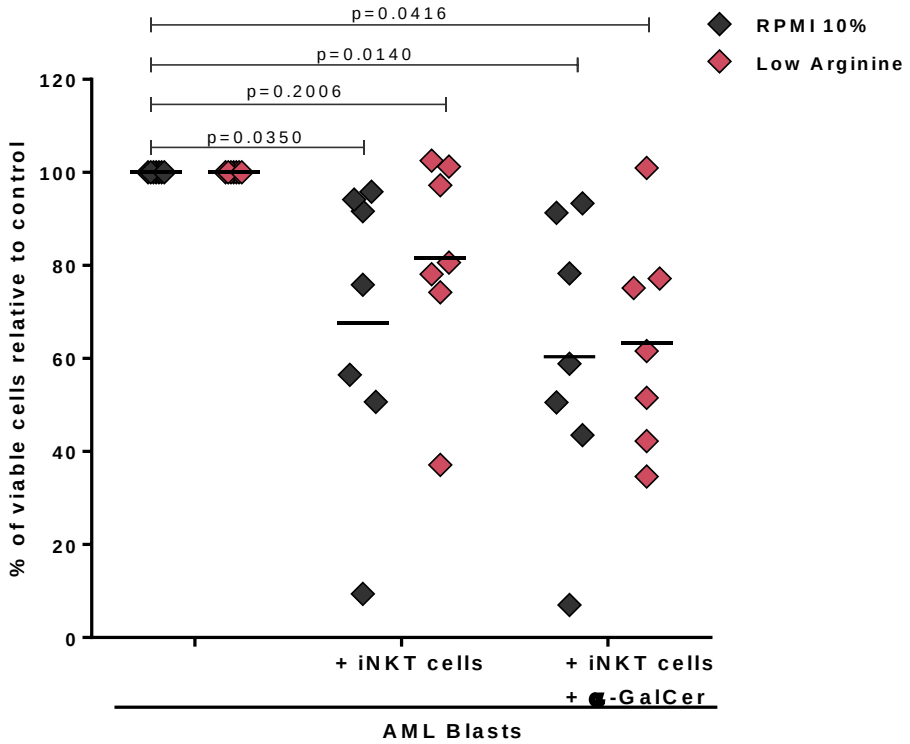


Figure 4.25: Low arginine AML microenvironment does not alter the cytotoxic function of iNKT cells.

AML patient blasts in the presence or absence of α-GalCer (100ng/ml) were co-cultured with iNKT cells (n=7) for 72 hours in complete and low arginine conditions. PI staining was used to determine blast viability by flow cytometry. α-GalCer-activated iNKT cells kill AML blasts in both complete and low arginine conditions equally. E:T ratio used was 0.25: 1. iNKT cells were identified by gating on cells positively stained with fluorescent-labelled CD1d tetramer. Horizontal line across samples represents the grand mean. Statistics performed by paired.t-test. Statistical significance considered when $p<0.05$.

iNKT cell expansion reduces AML blasts in vivo.

In order to confirm that iNKT cell proliferation and activation, driven in an antigen dependent manner, were unaffected by the low arginine conditions, as previously described (Fig, 4.25) *in vitro*, we used an immunocompetent AML murine model (MLL-AF9). Previously it was demonstrated that patient AML blasts orchestrate an immunosuppressive environment owing to their ability to expressing arginine depleting enzyme ARG2. Here, ELISA of serum of naïve and AML bearing mice (MLL-AF9) was used to establish arginine titres in the TME of mice. Statistically significantly (*p*=0.0137) lower levels of arginine were measured in AML bearing mice (on average 192µM) compared to naïve mice (on average 337µM) demonstrating that AML blasts also induce low arginine microenvironment *in vivo* (Fig. 4.26).

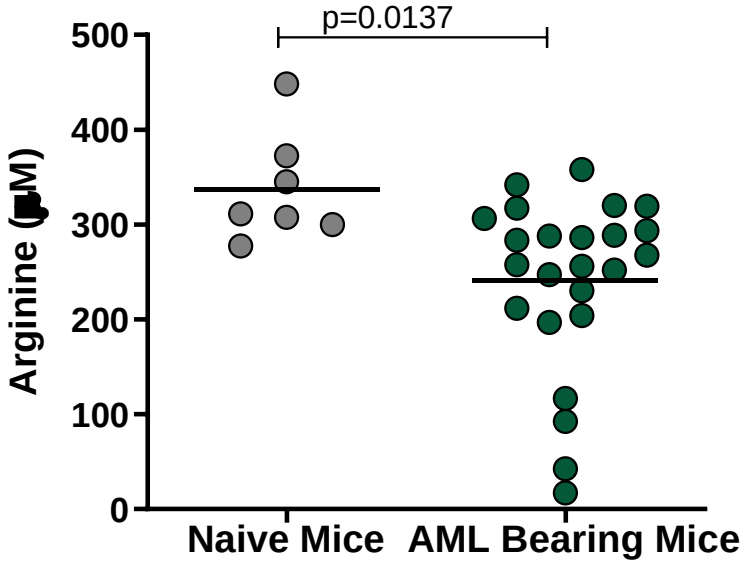


Figure 4.26: Low levels of arginine in AML bearing mice
Arginine levels were measured by ELISA in serum of naïve mice (n=7) SJL-Ptprca Pepcb/BoyJ and MLL-AF9 mice (n=24). AML bearing mice have significantly lower levels of arginine compared to naïve mice. Horizontal line across samples represents the grand mean. Statistics performed unpaired t-test. Statistical significance considered when $p<0.05$.

To investigate the possibility of iNKT cells being able to detect and target murine leukaemia tumour cells, CD1d expression was measured on blasts in the BM, blood, spleen, and liver AML bearing mice after 17 days of tumour engraftment. Flow cytometry analysis showed CD1d expression on alive AML blasts (CD45.2 positive) from harvested organs, on average CD1d expression was: 69.2% in BM, 65.25% in blood 50.18% in spleen and 49.04 % in liver (Fig. 4.27). Positive CD1d expression measured on AML blasts suggests potential binding with iNKT cells via a CD1d dependent manner.

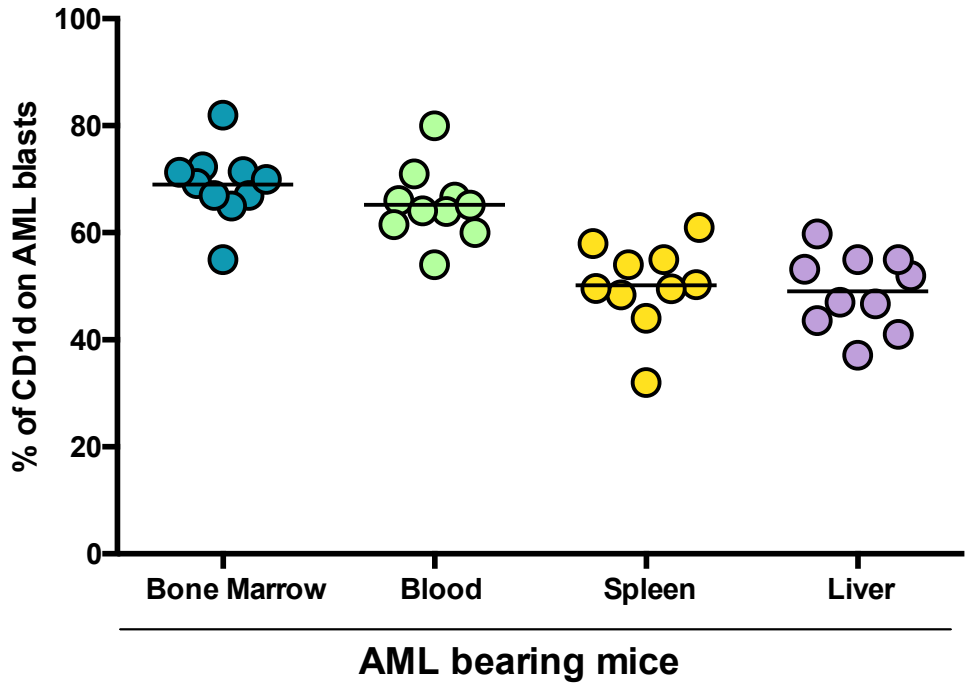


Figure 4.27: CD1d is expressed in AML blasts from MLL-AF9 mice.
CD1d expression on alive AML blasts (CD45.2 positive) was detected in AML bearing mice in the liver, blood, spleen and bone marrow as measured by flow cytometry. Horizontal line across samples represents the grand mean.

Taking this into account we determined if iNKT cells can expand in AML bearing mice following α-GalCer injection. AML bearing mice either received two doses of α-GalCer (2μg/mouse) intravenously, on day 5 and 10 or two doses of vehicle (PBS). To determine iNKT cell activation, 24 hours after second dose blood was collected and a multi-bead immunoassay was used to measure the release of an array of cytokines. Significant increase in IFN-γ (p=0.008) and TNF-α (p=0.008) in the

serum of AML bearing mice treated with α-GalCer was measured comparing AML mice treated with vehicle (Fig. 4.28).

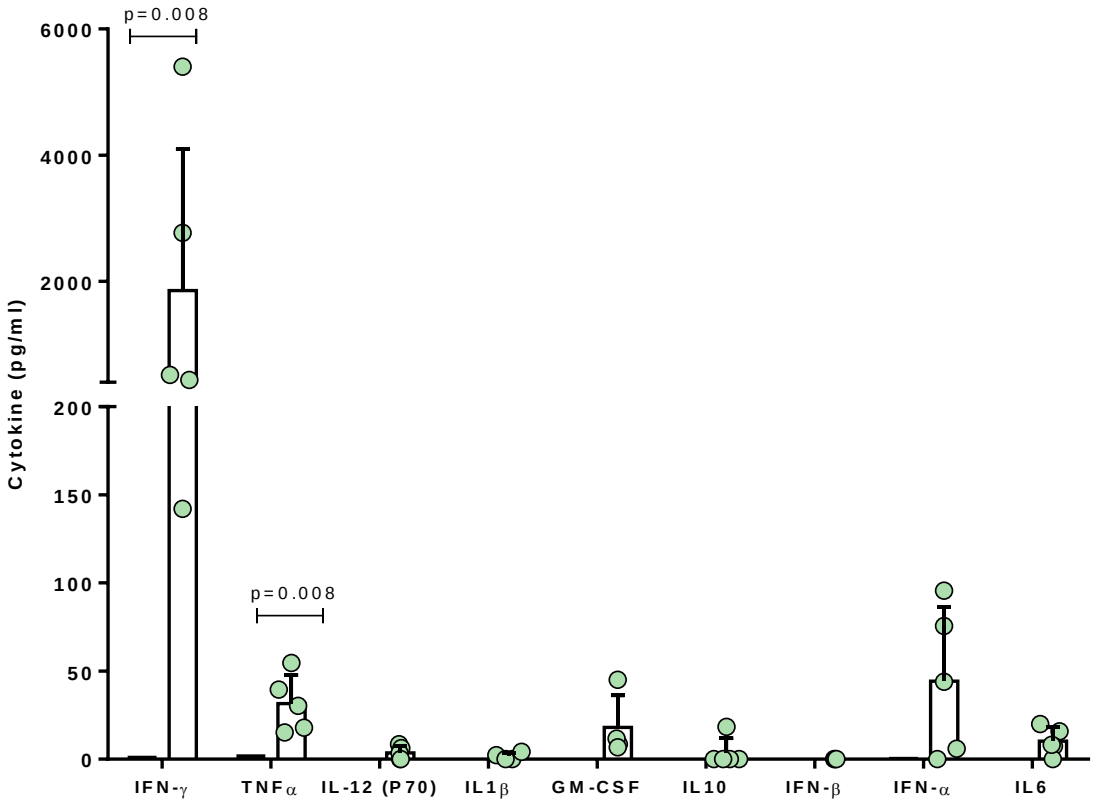


Figure 4.28: α-GalCer treatment in in MLL-AF9 mice induces significant release of IFN-γ and TNF-α in serum.
AML bearing mice within two doses of α-GalCer (2μg/mouse) or vehicle on day 5 and 10. 24 hours after the second dose, blood was collected, and cytokine release was measured in the serum using a multiplex assay by using fluorescence–encoded beads. α-GalCer treatment enhances levels of IFN-γ, and TNF-α. Statistics performed unpaired t-test. Statistical significance considered when $p<0.05$.

On day 17 mice were sacrificed and levels of iNKT cells were measured on harvested organs. Frequency of iNKT cells was measured on viable CD3 cells in the BM, blood and spleen ranged on average between 0.96%-2% and in the liver 9%. Following α-GalCer treatment a significant increase in iNKT cell frequency was observed compared to the vehicle. Average percentage of iNKT levels on viable CD3 cells in the BM, spleen, blood and liver were 6.07%, 7.78%, 10.95% and 23.9% respectively (Fig. 4.29).

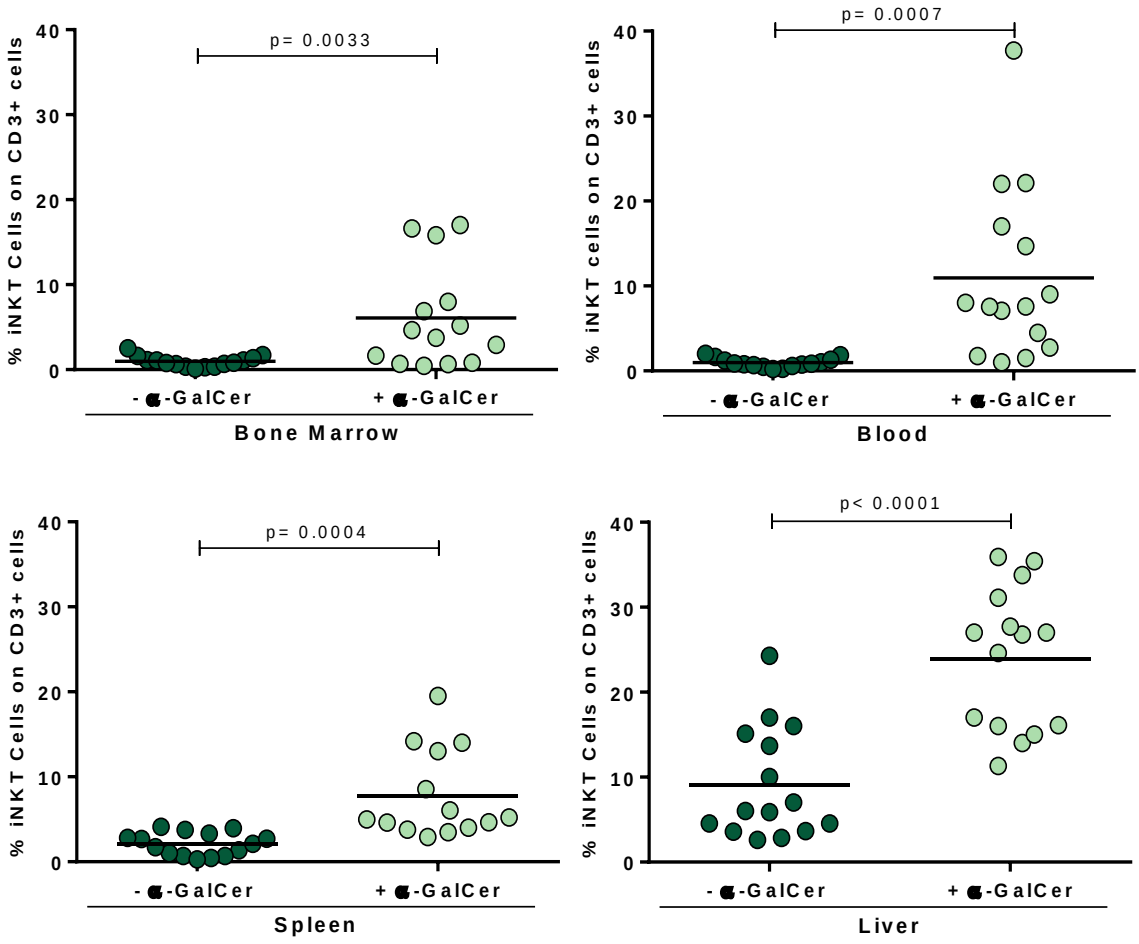


Figure 4.29: iNKT cells in MLL-AF9 mice proliferate following α-GalCer treatment.
AML bearing mice were given either two doses on day 5 and 10 of α-GalCer (2μg/mouse) or vehicle and sacrificed on day 17. Blood collected was lysed and cells from the bone marrow, spleen and liver were harvested, iNKT cell frequency was measured on alive cells via flow cytometry using fluorescent-labelled CD1d-tetramer. α-GalCer treated mice significantly increase the frequency of iNKT cells in the blood, bone marrow spleen and liver. Horizontal line across samples represents the grand mean. Statistics performed unpaired t-test. Statistical significance considered when

To determine if tumour regression was modulated by α-GalCer activated iNKT cells in AML bearing mice, the frequency of AML blasts (CD45.2) were measured. Activation of iNKT cells with α-GalCer led to a significant reduction in tumour burden. Lower numbers of viable AML blasts were measured in the α-GalCer treated group compared to control group in the BM, blood, spleen and liver. For example: mice receiving vehicle in the BM had 55% AML blasts while the α-GalCer group had 12% (Fig. 4.30).

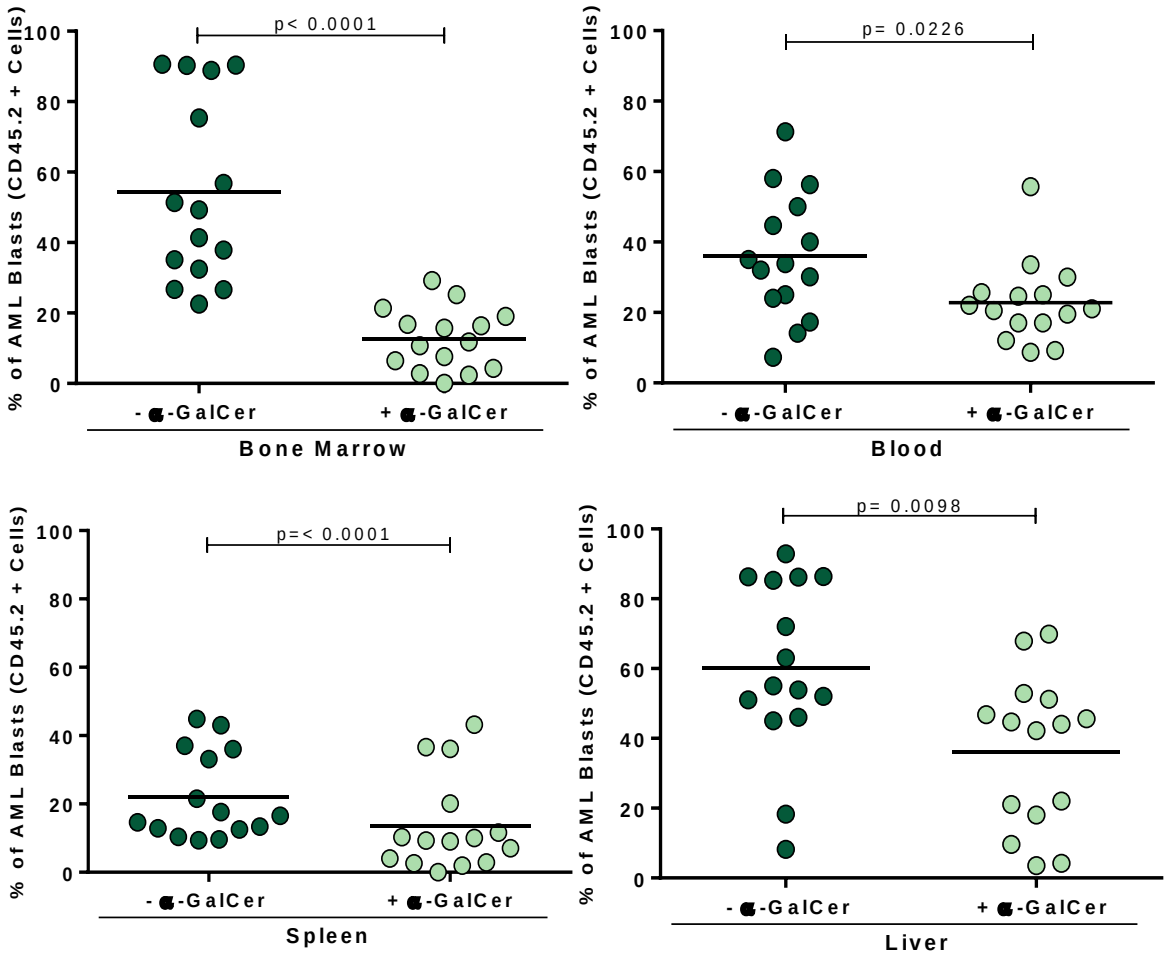


Figure 4.30: iNKT cell activation with a-GalCer leads to a reduction in leukaemia burden.
AML bearing mice were given either two doses on day 5 and 10 of α-GalCer (2μg/mouse) or vehicle and sacrificed on day 17. Blood was lysed and cells from the bone marrow, spleen and liver were harvested, and frequency of AML blast was measured via flow cytometry. α-GalCer injection in AML bearing mice significantly decreased the percentage of AML blasts in the blood, bone marrow and spleen and liver. Blast expression identified as CD45.2 positive was measured using flow cytometry. Horizontal line across samples represents the grand mean. Statistics performed unpaired t-test. Statistical significance considered when p<0.05.

Discussion

Having demonstrated that iNKT cells are functional within the AML microenvironment their ability to influence the AML tumour burden was investigated. Increase in IFN-γ levels, following co-cultures of iNKT cells with patient AML blasts, suggests iNKT cells interact with AML blasts. Given that no other APCs were added to the co-cultures this indicates that AML blasts can mediate antigen specific iNKT cells activation by presenting natural lipids. The ability of AML cells to induce iNKT cell activation by presenting specific antigens was further highlighted in the presence of potent antigen α-GalCer, since IFN-γ concentrations were further increased following AML-iNKT cells co-cultures.

A significant increase in basal ECAR after co-incubation with α-GalCer-loaded AML blasts, observed compared to cells alone, reconfirmed that iNKT cells can be activated within the AML microenvironment. This reliance on glycolysis to meet the ATP demand in the early phase of stimulation is consistent with the glycolytic switch reported during naïve T cell activation [249].

Activated iNKT cells following co-culture with AML cells, can upregulate or maintain the expression of different phenotypic markers. iNKT cells are constitutively expressing receptors which are typically found on natural killer cells such as CD161[257] and retained in the presence of with AML cells with and without of α-GalCer. Additionally, costimulatory molecule CD154 (CD40L) is also constitutively expressed on naïve cells and in activated iNKT cells [250-252]. iNKT cells express inducible cytokine receptor IL-2 receptor chain CD25 (IL-2RA) upon activation following co-culture with THP1 in the presence compared with absence α-GalCer [250, 251, 253]. Upon activation upregulation of activation markers CD69 and CD38 on iNKT cells was additionally observed. CD69 is involved in lymphocyte proliferation [252, 254] and is upregulated following co-cultures with iNKT cells both in the absence or presence of α-GalCer, while upregulation is CD38 is α-GalCer induced. In concurrence with other studies, upregulation expression of FASL on iNKT cells following α-GalCer mediated activation is demonstrated [250, 255]. Thus, the upregulation of activation and functional markers

on iNKT cells following co-culture with AML cells suggests that AML blasts can function as APCs without modifying iNKT cell phenotype. Additionally, activated iNKT cells can enhance the immune response by inducing activation and range of immune responses.

Moreover, iNKT cell activation mediated by co-culture of α-GalCer pulsed AML blasts trigger the release of cytokines: IL-2, IL-5, IL-6, IL-10 and GMCSF. The broad range of cytokines released by activated iNKT cells. This positions iNKT cells at a critical role in the immune system enabling them to jump-start and modulate adaptive immune system, by their ability to transactivate other immune cells such as NK, $CD8^+$ cells, DCs and B cells [200]. The indirect response iNKT cell activation has on AML blasts via other immune cells should be explored further. Additionally, α-GalCer not only activated iNKT cells when cultured with AML blasts, but also enhanced their proliferation and thus magnifying their role within the immune system.

iNKT cells, in addition to driving a range of immune responses through their capacity to release cytokines, they can also function as effectors in several contexts due to their cytotoxic potential. This chapter demonstrates that α-GalCer in addition to activating and enhancing iNKT cells it also cues mechanism of cytoxicity against patient AML blasts. iNKT cells elicit a direct cytotoxic response, reducing AML burden. The granzyme/perforin pathway has been described as the main mechanism applied by iNKT cells to kill cancer cells in myelomonocytic leukaemia [256, 257], and T-cell lymphoma [265]. Upregulation of Granzyme B in iNKT cell co-cultures with α-GalCer pulsed AML blasts suggests iNKT cells kill AML blasts through granzyme B, inducing apoptosis. Our findings demonstrate late apoptosis by upregulation of double positive Annexin V/ PI cells in AML blasts. Cleaved PARP, caspase 3 and caspase 9 in AML blasts α-GalCer pulsed following co-culture with activated iNKT cells, further confirmed that activated iNKT cells mediate apoptosis against AML. This is not surprising as granzyme B has been described as the most potent the pro-apoptotic granzyme, inducing apoptosis at low concentrations; due to its ability in mediating cell death by caspase- dependent and caspase-

independent manner [227, 259, 260]. PARP but not caspase 9 cleavage was also observed following on AML cells following co-culture with iNKT cells. This suggests AML blasts express and present a natural ligand, activating iNKT cells enabling them to cells mediate apoptosis against AML blasts in the absence of α-GalCer. However, apoptosis may be achieved via a different pathway such as caspase 7 but further experiments are required to understanding what the natural ligand is and mechanisms mediating a cytotoxic response absence of α-GalCer. CD107α degranulation is also observed on activated iNKT cells correlating with the increase in killing response and IFN-γ release. The capacity of activated iNKT cells to detect and kill AML blasts stresses their therapeutic potential against AML.

Our findings showing surface CD1d expression on AML blasts suggests that AML blasts mediate their interaction with iNKT cells through CD1d restriction. This was confirmed when no activation of iNKT cells (IFN-γ release) was observed, in co-cultures with CD1d negative AML cell lines (HL60, K562 and KG-1a) in the presence or absence α-GalCer. The use of anti-CD1d blocking mAb on α-GalCer pulsed AML blasts co-incubated with iNKT cells, verified that CD1d is necessary for AML-iTCR interaction since killing by iNKT cells was inhibited. Moreover, the data revealed that iNKT cells can kill AML tumour cells in a CD1d dependant manner in the absence of α-GalCer, indicating that the leukemic cells express a natural CD1d-bound ligand for iNKT cells, which is yet to be identified.

Recognising that iNKT cells can directly kill AML blasts in a CD1d dependent manner, CD1d distribution and expression can prove essential in regulating downstream immune responses mediated by iNKT cells such as cytokine release and antibody production. Thus, we hypothesised that the efficacy of antileukemic response mediated by iNKT cells can also be enhanced by increasing CD1d expression in AML blasts with low CD1d levels, as this could facilitate their recognition by iNKT cells. It has been reported that ATRA, an active vitamin A metabolite, increases CD1d expression [248]. Hence, the potential using ATRA to increase CD1d expression on AML blasts was explored. ATRA did

upregulate CD1d expression on AML blasts, similar response was reported in chronic lymphocytic leukaemia [268] however iNKT cell- mediated cytotoxicity against AML blasts was not enhanced in response to ATRA. Potentially longer incubations with ATRA or higher concentrations could have driven a more robust /persistent increase in CD1d expression on AML blasts, enabling interactions with iNKT cells and consequently AML lysis. However, ATRA at higher concentrations could not be explored due as it was toxic response on AML blasts. Interestingly, Chen et al showed that α-GalCer stimulation on spleen cells showed that ATRA reduced α-GalCer-mediated expression of IFN-γ in NKT cells, while increasing IL-4, rebalancing the ratio of Th2 to Th1 cytokines [248], which opposite to the desired response. Thus, using ATRA may not be an ideal method to enhance iNKT cell cytotoxicity against AML blasts. A possible alternative, which could be investigated further could be AM580, a retinobenzoic acid derivative that was created as ATRA receptor alpha (RARα) agonist since significant increase of CD107α by $CD8^+$ NKT cells was observed in the presence of α-GalCer loaded CLL cells in response to AM580 [268]. Therefore, it could potentially also be useful in AML.

AML patient blasts and cell lines express CD40 suggesting they are capable in interacting with iNKT cells via CD40-CD40L. Moreover, in addition to CD1d-iTCR binding, CD40-CD40L interactions between AML blasts and iNKT cells proved to be necessary for iNKT cell to elicit a cytotoxic response against AML blasts since killing was reversed by blocking these interactions. The anti-tumour response of CD40-CD40L interactions were also observed in melanoma, where iNKT cell interactions with neutrophils where not only CD1d but also CD40 dependent which in turn activated iNKT cells and decreased immunosuppressive release of IL-10 [217].

Tumour cells have been shown to use plethora strategies to escape immune recognition for example they can hinder antigen presentation by limiting expression of APCs on tumour cells or APC [242]. Thus, low arginine media was used to emulate the low arginine conditions in AML TME and explore iNKT cell function following co-cultures with AML blasts in the presence of α-GalCer. α-GalCer

presentation by CD1d positive AML blasts led to iNKT cell proliferation and activation (increase in IFN-γ) in low arginine conditions similarly to complete conditions. Importantly, iNKT cells retained their cytotoxic response against α-GalCer pulsed AML blasts and were not affected by low arginine conditions demonstrating that iNKT cells, in contrast to conventional T cells remained functional and were not suppressed by low arginine AML microenvironment. Identifying the capability of iNKT cells not only to withstand the low arginine microenvironment but also simultaneously elicit a strong anti-leukemic response opens exciting avenues for immunotherapy.

In vivo animal models represent a powerful tool providing a greater insight in the functional characterization of pathological mechanisms implicated in human disease, allowing for designs to be approved for new therapeutic strategies. An established AML murine model, MLL-AF9 was used to explore the anti-leukemic responses of iNKT cells *in vivo*. Similar to AML patients, arginine levels were supressed in the serum of AML bearing mice compared to naïve mice. Moreover, AML blasts in the BM, blood, liver, and spleen express CD1d indicating that AML have the capacity to mediate CD1d-iTCR interactions with iNKT cells. Thus, α-GalCer was administered to AML bearing mice to explore tolerance and determine if this potent antigen can be used as treatment against AML. α-GalCer increases iNKT cell frequency in the BM, blood, liver, and spleen compared to the untreated group, confirming iNKT cell proliferation in the presence of α-GalCer-loaded AML blasts shown *in vitro.* Additionally, raised IFN-γ concentrations in serum of α-GalCer treated mice suggest that iNKT cells are activated thus the immunocompromised TME does not affect iNKT cell functionality. Furthermore, in α-GalCer treated mice a significant decrease in AML blasts within the BM, blood, spleen, and liver was observed, identifying a clear correlation between increased frequency of iNKT cells and decrease of tumour burden. Hence establishing that upregulation of activated iNKT cells can mediate a cytotoxic response against AML blasts, demonstrating that α-GalCer can optimise iNKT cell activation, which mediate anti-AML response which can have therapeutic benefit to patients.

Chapter5

Results

Given that arginine can modulate a range of other amino acids, expression of L-type amino acid transporter 1(LAT-1) on iNKT cells was explored. LAT-1 encoded by the solute carrier transporter 7a5 (SLC7A5) is involved in the uptake of large neutral amino acids (e.g leucine, isoleucine, valine) [269]. Therefore, iNKT cells were co-incubated with THP1 cells for 16, 24 and 48 hours. iNKT cells were positively selected *via* MACS and expression of LAT-1 was measured by western blot (Fig.5.1). iNKT cell co-cultures with THP1 cells led to upregulation of the LAT-1 compared to iNKT cells alone (Fig.5.1).

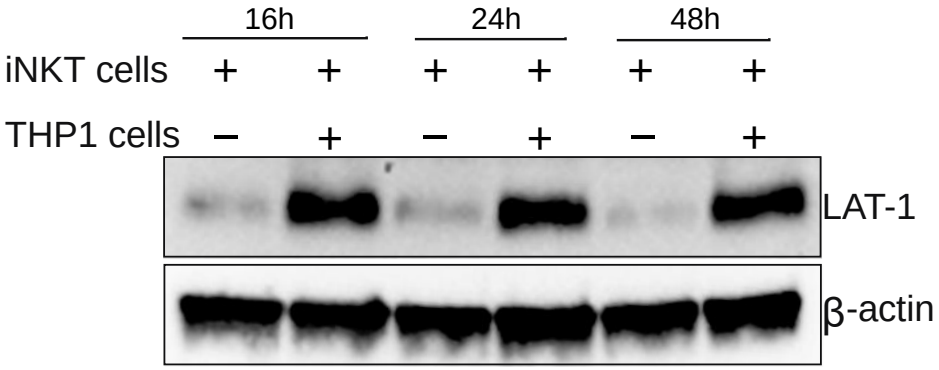


Figure 5.1: AML- iNKT cell co-culture drives LAT-1 upregulation.
AML cell line THP1 was co-culture with iNKT cells for 16, 24 and 48 hours. iNKT cells were positively isolated and lysed. Up-regulation of LAT-1 in iNKT cells following co-culture with THP1 cells was detected by western blot compared to iNKT cells alone. β-actin shown as a loading control (n=3).

ICS was used to re-confirm LAT-1 upregulation in iNKT cells following co-culture with AML cells previously observed by immunoblotting (Fig. 5.1) iNKT cells co-cultured with AML cells lines (THP1, U937 and NOMO) and patient derived AML blasts with iNKT cells in the presence or absence of α-GalCer for 72 hours (Fig. 5.2). Increase in LAT-1 expression on iNKT cells following the co-cultures with AML blasts was also observed compared to iNKT cells alone (Fig. 5.2).

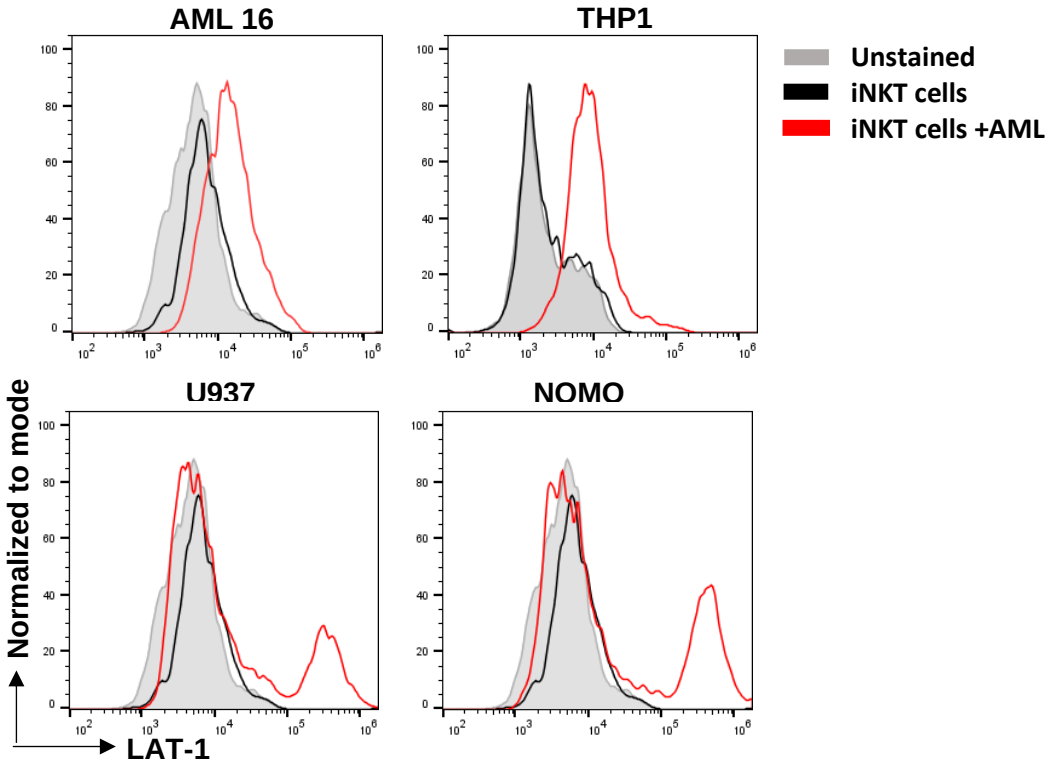
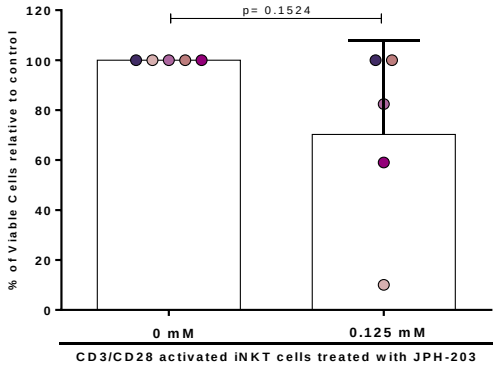


Figure 5.2 Activated iNKT cells upregulate LAT-1 expression.
LAT-1 expression on alive iNKT cells by ICS following co-culture with AML blasts derived from patient and cell lines THP1, U937 and NOMO (E: T ratio used 0.25:1) for 72 hours. LAT-1 upregulation on iNKT cells is observed following o-culture with AML blasts. iNKT cells were identified using fluorescent-labelled CD1d tetramer.

To define the critical role of amino acid uptake *via* LAT-1 in iNKT cell metabolism, JPH203 a tyrosine analogue which inhibits LAT-1 selectively was used to inhibit amino acid uptake [263]. CFSE labelled iNKT cells derived from five different healthy donors were activated with anti-CD3/CD28 and cultured in the presence of JPH-203 (0.125mM) for 96 hours. iNKT cell proliferation following LAT-1 inhibition was evaluated by flow cytometry. JPH-203 did not alter iNKT cell viability as no difference was observed in the presence or absence of JPH-203 inhibition (Fig. 5.3a). However, the presence of JPH-203 significantly decreased iNKT cell frequency (average from 100% to 27.71%)(p=0.08) in all treated donors (Fig 5.3b).

a.



b.

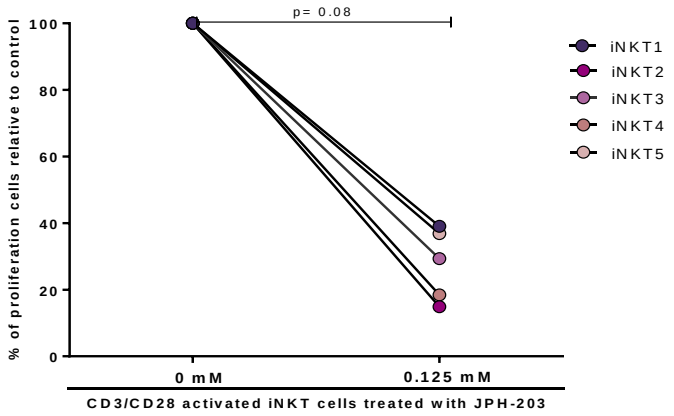


Figure 5.3 LAT-1 inhibition impairs iNKT cell activation induced IFN-γ release following stimulation with anti-CD3/CD28 antibodies and supress viability and proliferation.
JPH203 (0.125mM) was added to iNKT cells in the presence or absence of anti-CD3 (3μg/ml)/ anti-CD28 (2 μg/ml) antibodies for 96 hours. a) Viability (n=5) and b) proliferation (n=5) of CFSE labelled iNKT cells was determined by flow cytometry. JPH-203 suppressed stimulated iNKT cell proliferation and IFN-γ release. Viability was not affected. Data presented as mean ±SEM. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

Given that LAT-1 is an important amino acid transporter, involved in the cellular uptake of citrulline in T cells [271], the effect of citrulline depletion on iNKT cell activation was investigated. Citrulline can be used as vehicle for arginine synthesis. The enzyme ASS-1 catalyses the ATP-dependent condensation of citrulline and aspartate to produce arginine succinate which is then metabolized to arginine and fumarate by arginosuccinate (ASL)[272]. AML cell lines (THP1, U937 and NOMO) were co-cultured with iNKT cells in the presence or absence of α-GalCer in citrulline free conditions or RPMI10% for 72 hours. Supernatants were harvested and IFN-γ was measured by ELISA. Similar to JPH-203 inhibition, iNKT cells in citrulline depleted conditions supressed antigen-induce IFN-γ release in all AML cell lines (Fig. 5.4).

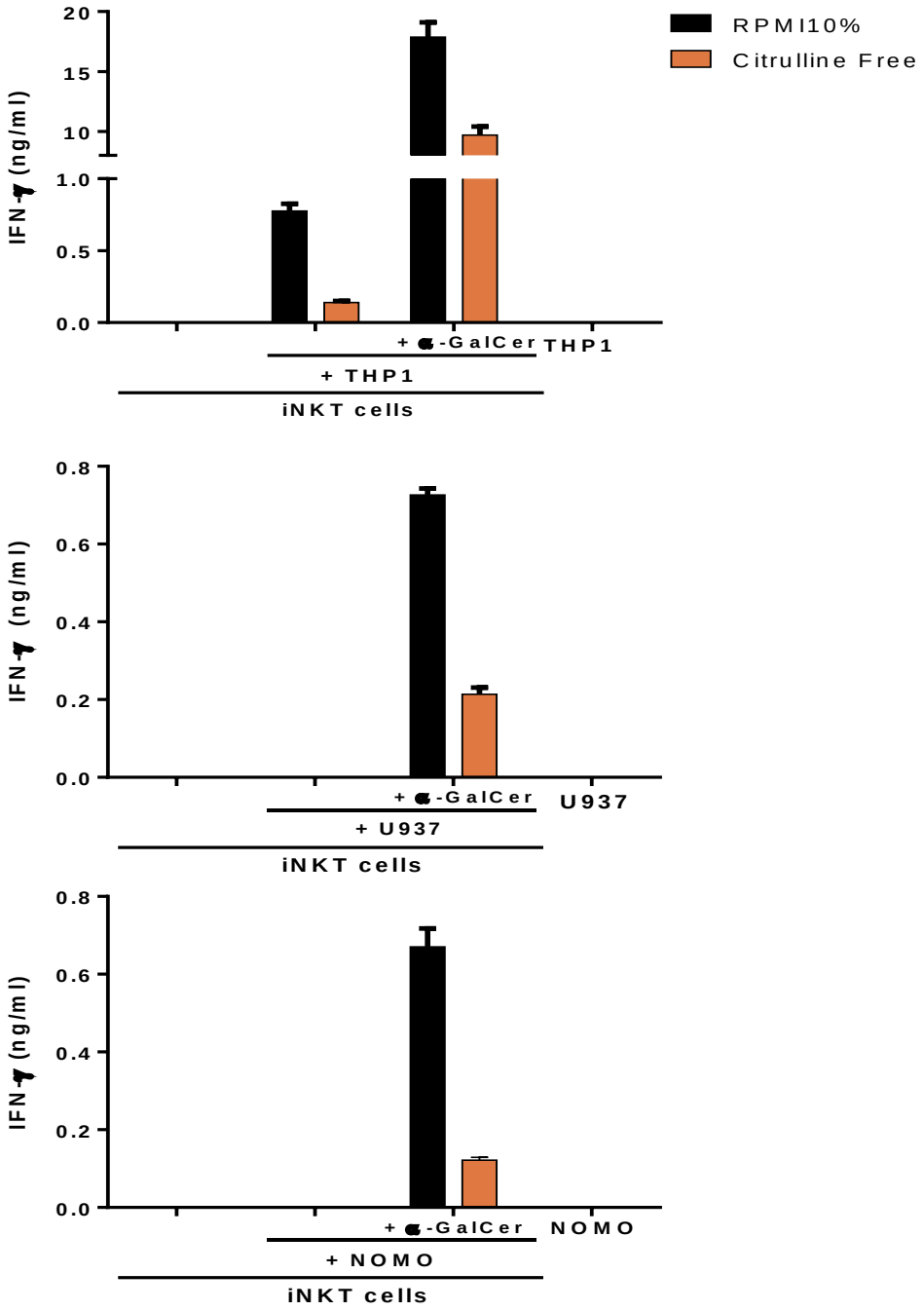


Figure 5.4: IFN-γ release induced by activated iNKT cells via αGalCer in co-cultures with AML blasts is inhibited in the absence of citrulline.

AML cell lines THP1, U937 and NOMO were pulsed in the presence or absence of α-GalCer (100ng/ml) and were co-cultured with iNKT cells (E: T ratio used 0.25:1) for 72 hours in complete and citrulline free conditions. Supernatants were harvested and IFN-γ concentration was measured by ELISA (n=2). IFN-γ release is inhibited α-GalCer activated iNKT cells cultured in citrulline free media. Data presented as mean ±SEM.

Given that citrulline is a source of arginine, changes in expression of enzymes involved in the arginine metabolic pathway on iNKT cells following the interaction with AML cells were investigated. Expression of enzymes synthesising arginine (ASS-1 and/or ASL), arginine catabolic enzymes (arginase 1 (ARG1) and ARG2) and OTC which catalysis ornithine to citrulline [273] was determined by western blot on iNKT cells following positive selection from the co-culture with THP1 cell at 16, 24 and 48 hours. Enzymes Low expression of ARG1, ARG2, and OTC was detected, while ASL was constitutively expressed on iNKT cells alone and in co-cultures with THP1 cells. However, a greater increase in expression of ASS-1 which is the rate limiting enzyme required for intracellular re-synthesis of arginine from citrulline was observed on iNKT cells following co-culture with THP1 cells (Fig. 5.5)

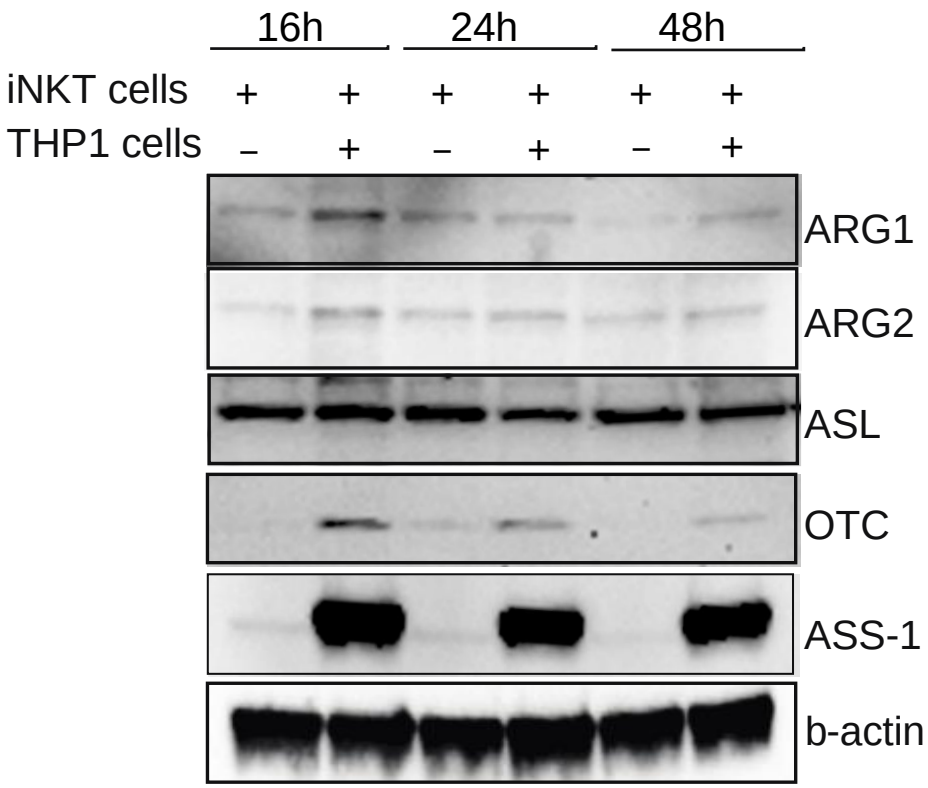


Figure 5.5: AML- iNKT cell interaction drives LAT-1 upregulation.
AML cell line THP1 was co-culture with iNKT cells for 16, 24 and 48 hours. iNKT cells were positively isolated by MACS and lysed. Up-regulation of LAT-1 iNKT cells following THP1 co-culture was detected by western blot. β-actin shown as a loading control (n=3).

To further confirm ASS-1 upregulation in iNKT cells following co-culture with AML cells, ICS was used. AML blasts derived from patients were co-cultured with iNKT cells in the presence or absence of α-GalCer for 48 hours. Flow cytometry analysis showed an increase of ASS-1 in iNKT cells activated by AML blasts (Fig 5.6).

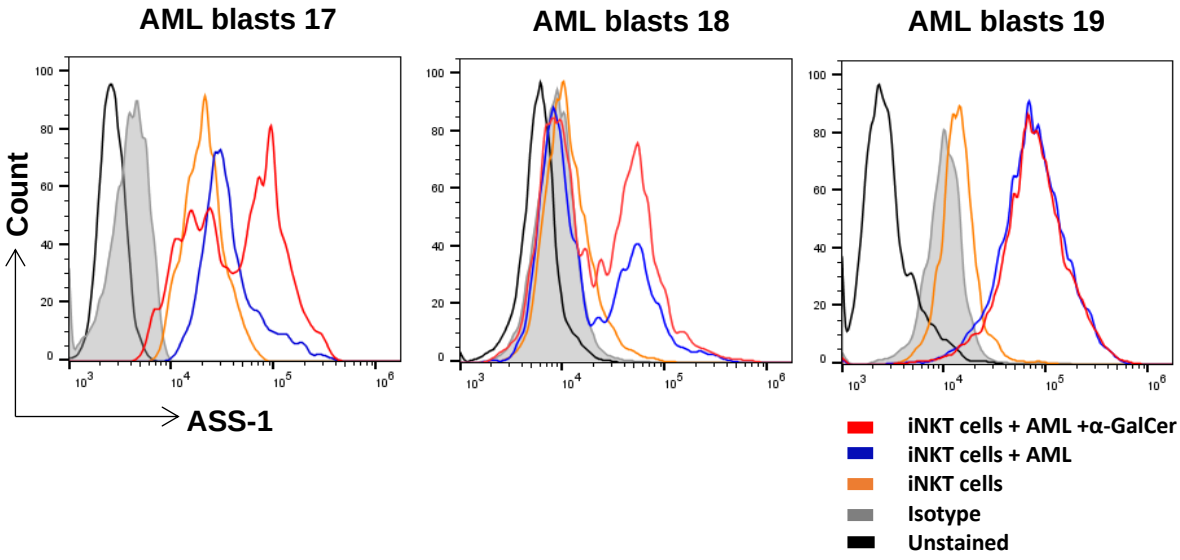


Figure 5.6: iNKT cells have increased ASS-1 expression following co-culture with AML blasts. Patient AML blasts (n=3) were co-cultured with iNKT cells (E:T ratio 1:0.025) in the presence or absence of α-GalCer for 48 hours. iNKT cells were identified using α-GalCer –loaded CD1d tetramer and ASS-1 expression was determined by ICS. ASS-1 expression is upregulated following co-cultures with AML blasts both in the presence and absence of α-GalCer.

Having shown that iNKT cells increase their expression of ASS-1 following co-cultures with AML blasts *in vitro*, increase in the capacity of arginine re-synthesis *in vivo via* ASS-1 upregulation was investigated (Fig 5.7). C57BL/6J mice were injected with BCT-100 from day 0 to 5 as previously described (chapter 3), to mimic low arginine conditions in the AML microenviroment, α-GalCer was administered on day 3. On day 5 thymus was harvested and ASS-1 expression was determined by ICS.

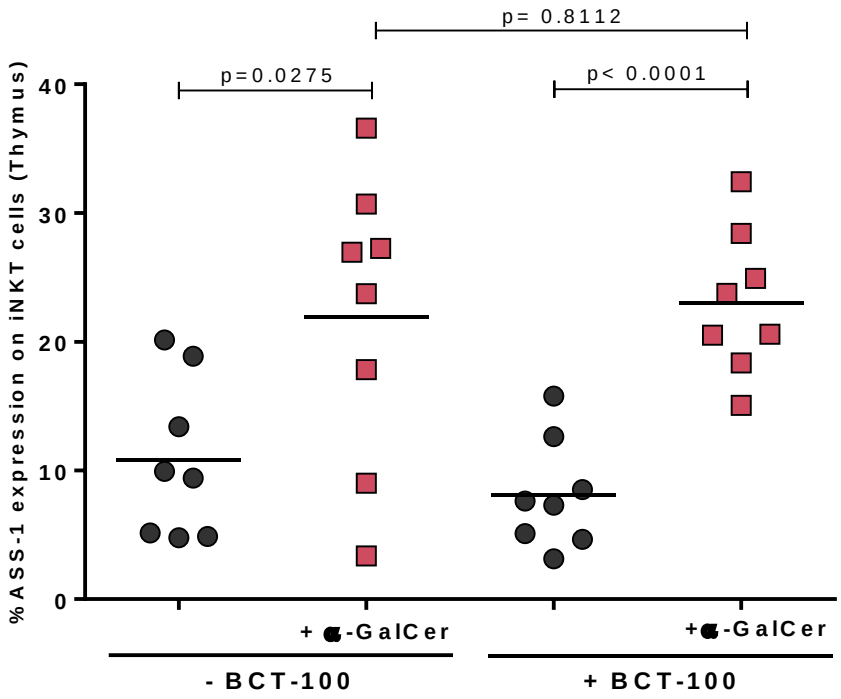


Figure 5.7: ASS-1 expression is upregulated in α-GalCer treated mice.
C57BL/6 mice (n=8) received a recombinant arginase BCT-100 (5mg/Kg) from day 0 to day 5 every 24 hours or vehicle. On day 3 both groups of mice were intravenously administered α-GalCer (2µg/mouse) or vehicle. On day 5 mice were culled and cells from thymus were harvested and ASS-1 expression was determined by ICS. iNKT cells were identified using florescent labelled CD1d tetramer. ASS-1 expression increased in α-GalCer treated mice both in the absence and presence of BCT-100. Horizontal line across samples represents the grand mean. Statistics performed by unpaired t-test. Statistical significance considered when $p<0.05$.

A significant upregulation of ASS-1 expression on iNKT cells in mice receiving α-GalCer compared to the untreated group (*p*=0.0275). ASS-1 expression was not affected by low arginine conditions, no significant difference ASS-1 expression was observed in mice receiving BCT-100 compared to mice which did not (*p*=0.8112) (Fig.5.7).

To determine if an increase in ASS-1 protein expression correlated to an increase in ASS-1 activity, an ASS-1 activity assay was carried out on iNKT cells lysate by measuring citrulline levels over time. For this assay, iNKT cells were co-cultured AML cell lines (THP1, U937, NOMO) in the presence or absence of α-GalCer for 48 hours. iNKT cells were positively selected and lysed ASS activity was determined using a colorimetric assay measuring citrulline hydrolysis and normalised to live cell numbers (Fig. 5.8). Corresponding to ASS-1 expression, significant increase in ASS-1 activity was also observed on activated iNKT cells was observed (Fig. 5.8).

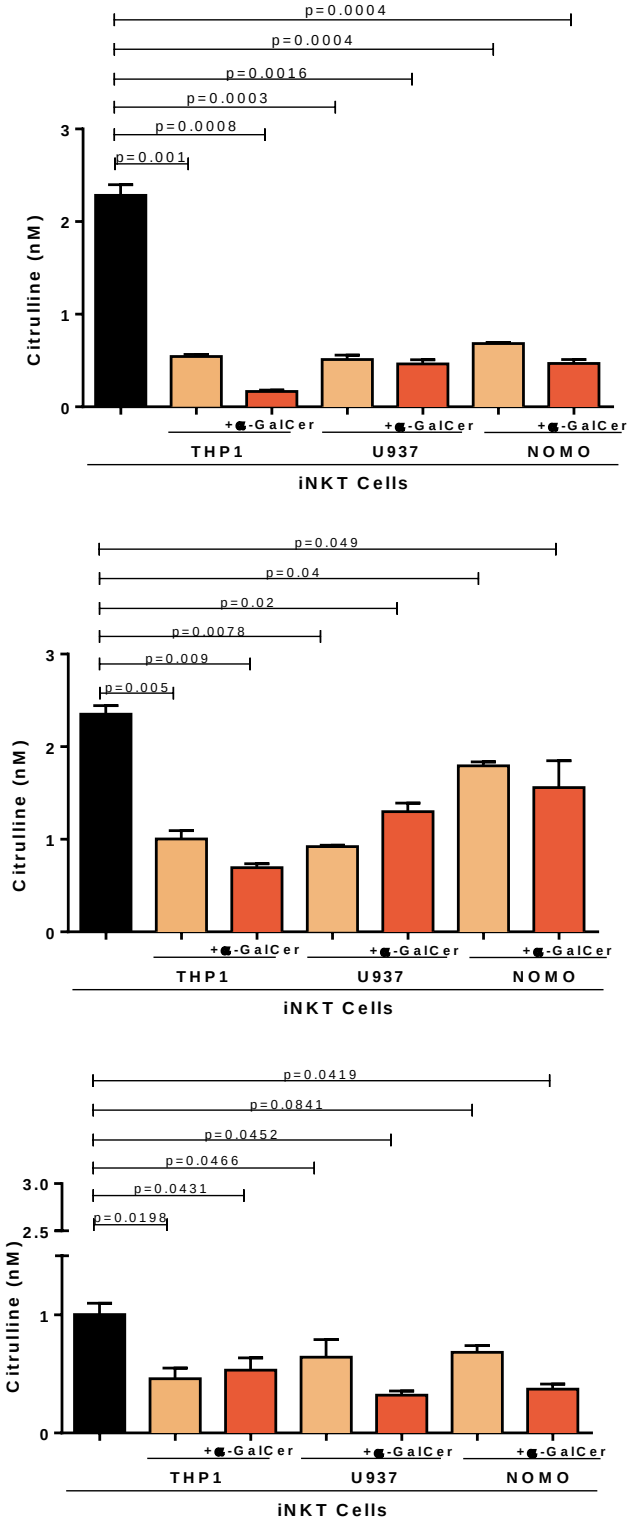


Figure 5.8: iNKT cells have increased ASS-1 expression and enzyme activity following co-culture with AML blasts.

AML cell lines THP1, U937 and NOMO cells were co-cultured with iNKT cells (1:0.025) in the presence or absence of α-GalCer for 48 hours. iNKT cells were positive selected by MACS. ASS-1 enzyme activity was determined on lysates by citrulline depletion using a colorimetric assay on enriched iNKT cells (n=3) assay was normalised to number of alive cells. Data represents mean ±SEM. Statistics performed by paired t-test. Statistical significance considered when $p<0.05$.

iNKT cells rescue antigen-specific T cell proliferation in myeloid immunosuppressive microenvironments.

Having demonstrated that iNKT cells are functional within the low arginine conditions found in the AML microenvironment we investigated their effect on T cell function *in vitro* and *in vivo*. To explore the capability of iNKT cells to overcome AML-induced suppression of T cell proliferation, iNKT cells were firstly co-cultured with patient AML blasts in the presence or absence α-GalCer (100ng/ml). After 8 hours the cells were washed and healthy T cells together with irradiated DCs were added to cultures for a further 96 hours. T cell proliferation was measured by ^{3}H-thymidine incorporation (Fig5.9)

Co-culture of allo-antigen driven T-cells with AML blasts led to a statistically significant reduction (average 27%) in T-cell proliferation. A statistically significant increase in T cell proliferation was observed (average 69%) in the presence of α-GalCer stimulated iNKT cells, restoring T cell proliferation (Fig. 5.9).

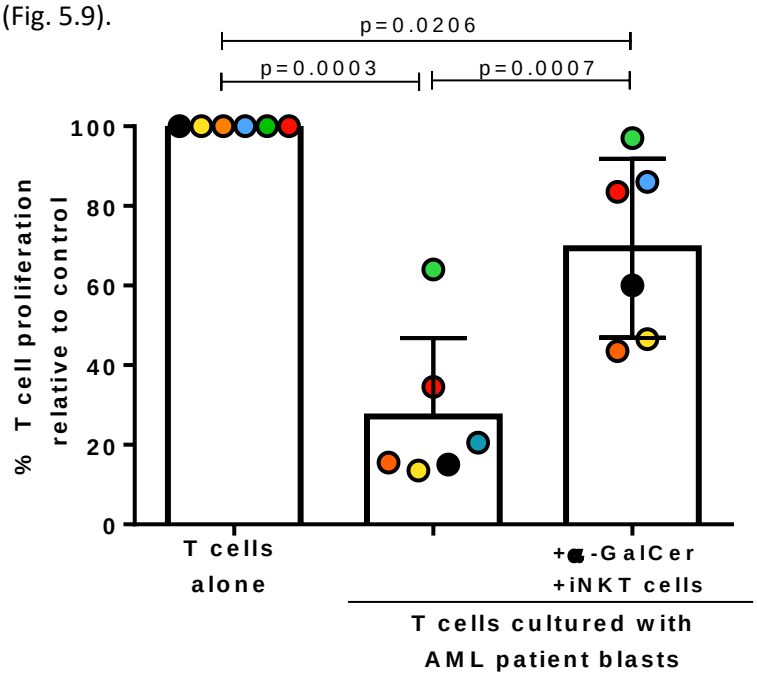


Figure 5.9: iNKT cells overcome AML-induced suppression of T cell proliferation.
AML blasts derived from patients (n=6) were firstly co-cultured with iNKT cells (1:0.25) in the presence of α-GalCer (100ng/ml) for 8 hours. Cells were washed and healthy T cells and allogenic irradiated DC (1:0.25) were added to cultures for 96 hours. T cell proliferation was measured by 3H-thymidine incorporation. Data represent mean ±SEM. α-GalCer activated iNKT cells, restore T cell proliferation, supressed by AML blasts. Statistics performed by unpaired t-test. Statistical significance considered when $p<0.05$.

To confirm that iNKT cell activation can restore T cells proliferation *in vivo*, the frequency of T cells (CD3 positive) on viable cells was measured (Fig.5.10), in naïve mice, AML bearing mice and AML bearing mice treated with α-Galcer as previously described (Fig 4.28-4.30).

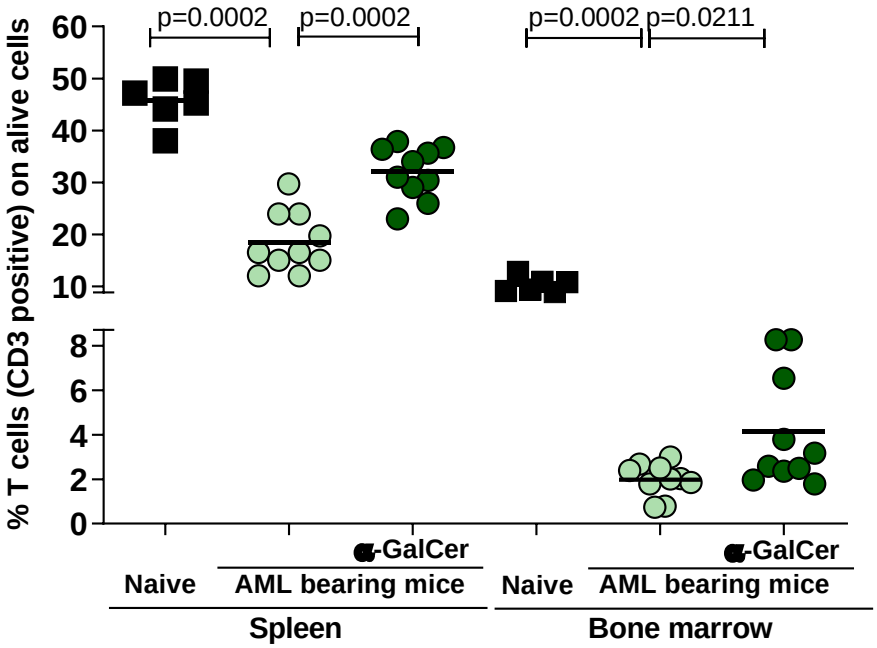


Figure 5.10: α-GalCer treatment enhances T cell expression in AML bearing mice.
T cells expression was measured on naïve (n=6), AML bearing mice (MLL-AF9) and AML bearing mice (n=10) were injected with α-GalCer (2µg/mouse) for 17 days. T cell expression was identified by flow cytometry using CD3 staining on alive cells. T cell expression in AML bearing mice increased in α-GalCer treated mice compared to untreated group. Horizontal line across samples represents grand mean. Statistics performed by unpaired t-test. Statistical significance considered when $p<0.05$.

A significant increase in T cell frequency was observed in the spleen (p=0.0002) and BM (p=0.ed to vehicle (Fig 5.10), within the BM, of AML bearing mice T cell frequency in the vehicle group was 1.4% while in in the α-GalCer treated group T cell frequency is significant higher 4.9%. Similarly in the spleen T cells expression in α-GalCer treated group of AML bearing mice was 35.4% compared to vehicle 17.4%. We previously showed that iNKT cells frequency is increased in the α-GalCer treated group, suggesting iNKT cells can rescue T cell function.

Discussion

This study demonstrates that despite T cells being supressed by low arginine conditions in the AML microenvironment, iNKT cells have the capacity to exert effector functions and adapt to tolerate the low arginine conditions by using an alternative pathway to source arginine. Given that amino acid transporters are key in delivering amino acids for metabolic pathways and play a key role in nutrient uptake, the role of LAT-1 on iNKT cells was explored. LAT-1 is a neutral amino acid transporter for valine, methionine, isoleucine, leucine, phenylalanine, histidine, tryptophan, and tyrosine [269]. Upregulation of LAT-1 by western blot and ICS on iNKT cells following co-culture with AML cell lines suggest amino acid uptake in iNKT cells could be mediated by LAT-1. This was further confirmed by small molecule JPH-203, to inhibit LAT-1. iNKT proliferation and IFN-γ release was supressed in the presence of JPH-203 following co-culture with AML blasts. LAT-1 has been also associated with citrulline uptake in T cells [271].

Given that the intracellular availability of arginine depends on its uptake from extracellular sources by amino acid transporters and/or *de novo* synthesis from citrulline by enzymes of the urea cycle, suggests that citrulline could provide an alternative source of arginine to iNKT cells. Citrulline depletion supressed iNKT cell ability to release IFN-γ following co-culture with AML blasts suggesting citrulline is essential for mediating iNKT cell activation. This infers that iNKT cells following co culture with AML blasts upregulate LAT-1 could promote uptake of extracellular citrulline to produce intracellular arginine *de novo.* Extracellular levels of citrulline levels on AML patients by ELISA should be analysed in the future. However, Crump *et al* did show significant increase in citrulline in plasma of AML patients relative to healthy donors [267] suggesting citrulline could potentially be used by iNKT cells. LAT-1, an isoform of the L-type amino acid transporters, and requires a covalent association with the heavy chain of the 4F2 cell-surface antigen (CD98) for its functional expression [269]. Therefore, to establish that LAT-1 is functional, CD98 expression can be explored in the future to demonstrate heterodimerization.

Citrulline is a key precursor for arginine, and ASS catalyses the ATP-dependent condensation of citrulline (rate limiting step) and aspartate to produce arginine succinate which is metabolized to arginine and fumarate by ASL [272]. Thus, the expression of all enzymes involved in metabolic pathway on iNKT cells was determined. This study shows that iNKT cells following co-culture with AML cells express low levels of arginine catabolism enzymes ARG1, ARG2 or OTC, while ASL is constitutively expressed. Strong ASS-1 and LAT-1 upregulation on iNKT cells following co-culture with AML cells. Constitutive expression of ASL suggests that ASS-1 and not ASL is dependent for arginine synthesis. The discrepancy between iNKT cells and conventional T cell proliferation in the low arginine microenvironment could possibly reflect differences in the expression and function of the urea cycle enzymes. Since it has been reported that conventional T cells do not express ASS-1 [275]. In addition to protein expression, we find enhanced ASS activity on α-GalCer stimulated iNKT cells. This, further implies that citrulline transported by LAT-1 can be catabolised by ASS-1 on iNKT cells.

In vivo ASS-1 expression was equally upregulated in iNKT cells in mice treated with α-Galcer regardless if BCT-100 was administered to systemically deplete arginine or not. This suggests that low arginine conditions do not impair ASS-1 upregulation. However, it important to note that murine T cells, in contrast to human T cells, do express ASS-1, thus are able to use citrulline as arginine source for growth [269, 270]. Thus, caution must be taken when interpreting mice experiments involving arginine-deprivation in the context of human disease such as AML.

Furthermore, we highlight that in addition to AML blast killing, activated iNKT cells following co-cultures with α-GalCer loaded AML blasts also rescue function of supressed T cells by observing a significant increase in proliferation both in vivo and in vitro, reversing inhibitory response mediated by low arginine conditions found in the AML microenvironment. Justifying why iNKT cells have been described as “orchestrators” of the immune response as iNKT cells can indirectly enhance immunogenic activity of T cells which can also contribute to anti-tumour immune responses [198,

218]. Consequently, antigen specific iNKT cell activation could also augment T cell immunity by promoting effective T cell function and reversing hyporesponsiveness by initiating T cell activation, cell cycle progression, and cytokine production however this should be further investigated. Nevertheless, the unique ability of iNKT cells adapt and tolerate low arginine conditions, proliferate also induce anti-leukemic response upon α-GalCer presentation highlights their clinical relevance. These findings help explain how iNKT cells can also function to abolish ARGI positive MDSCs which suppress T cells in the setting of influenza [278].

Chapter 6 Discussion and Conclusion

Summary

In this study we describe new insights on immune regulation within the low arginine AML microenvironment and the unique role of iNKT cells in targeting AML.

- Serum analysis of AML patients showed elevated levels of acute phase protein SAA compared to healthy donors.
- SAA enhances AML viability, and contributes to metabolic deviancy in AML blasts by signalling to the upregulation in expression and activity of the arginine catabolic enzyme ARG2.
- iNKT cell frequency is not suppressed in AML patients compared to healthy donors. iNKT cells retain their function in the low arginine AML microenvironment as they can proliferate and become activated in low arginine conditions.
- iNKT cells detect and interact with AML patient blasts in a CD1d/CD40 dependent manner.
- α-GalCer mediated activation of iNKT cells results in iNKT cell expansion, release a range of cytokines, induction of a direct cytotoxicity against AML blasts by apoptosis, and restores T cell proliferation.
- iNKT cells co-culture with AML blasts increases expression of the amino acid transporter LAT-1 and the enzyme ASS-1 to contribute to *de novo* arginine synthesis from citrulline. iNKT cells thus tolerate and adapt to low arginine concentrations.

Limitations

Due to the nature of research, throughout this study several limitations did arise. For example, due to ethical reasons background information of AML patients i.e. subtype was not disclosed. Therefore, we could not correlate our data to subtype of tumour burden which could have potentially explained outliers observed. Moreover, it would have been ideal to use AML patient blasts in all experiments and not cell lines, nonetheless we do not have systematic access to patient samples and small volumes are often donated. Hence, in experiments where high number of cells were required i.e., western blots or aim was to explore effects of iNKT activation mediated by AML i.e. surface markers, metabolic and ASS activity, AML cell lines were used. THP1 cell line was mainly used as stronger iNKT cell activation was observed in co-cultures compared to other cell lines. Moreover, given that iNKT cells comprise of such a small population of the T lymphocytes, iNKT cells harvested from humans had to be expanded in order to obtain sufficient numbers to perform experiments. Therefore, a potential loss of function or repertoire could not be explored. Additionally, AML killing was also observed following co-culture with iNKT cells even in the absence of α-GalCer, suggesting AML blasts express and present a natural ligand activating iNKT cells. Due to time constraints identifying natural ligand and mechanism of killing were not explored. Though it would be useful to investigate this further in future studies. Restrictions were also encountered in by the animal facility we collaborated as blood from mice once and decided to take the sample at the end of the experiment. However, cytokines explored are released at earlier and different time points and if samples were taken earlier a different cytokine profile may have been observed. Also, by using ASS-1 knockout mice in future studies, the role of ASS-1 in iNKT cell activation could be confirmed in the future. Despite these limitations, we do believe that usefulness and validity of the research presented is not compromised.

Discussion

In the past ten years, advances in immunotherapy have revolutionised treatment strategies against cancer. Immunotherapy is now regarded as a promising additional to genotoxic radiation, cytotoxic chemotherapy, and targeted therapy [231, 272]. However, despite many new immunotherapies with demonstrable efficacy in preclinical settings, clinical success has been disappointing; displaying minimal or no clinical benefit in some cancers like AML. The poor clinical outcome can be mainly addressed by our limited understanding of the complexity of the highly immunosuppressive TME [280].

Tumour progression is associated with significant modifications in metabolic pathways within tumour cells with resulting changes to the metabolome of the TME [10]. Changes in the tumour metabolic phenotype involves alterations in arginine concentrations. For instance, the arginine concentrations in the core regions of solid tumours are approximately five times lower as compared with periphery tumour and this difference is the highest between all of the measured amino acids [281].

AML blasts modify the immune microenvironment the expression and release of ARG2 [75]. Increased activity of the arginase isoforms, ARG1 and ARG2 have also been reported in other tumours and their enhanced activity typically associates with advanced disease stage and worse clinical prognosis [275-277]. AML blasts depend on the semi-essential amino acid arginine for survival and proliferation since arginine metabolism is vital for cell cycle activity, protein synthesis, and additional cell functions.

Our findings revealed that AML blasts directly release SAA, improving our understanding about the TME in AML. SAA is an acute phase protein which increases over 1000- fold from basal levels during infection. It is considered to be a key modulator in amplifying the immune response. [285]. SAA has also been reported in human cancer cell lines and carcinoma metastases [279]. In addition to our results demonstrating elevated SAA levels in plasma from AML patients, other studies have also

reported increased SAA levels in both solid tumours and haematological malignancies such as in colon, breast, lung, pancreas, prostate, ovary, melanoma, Hodgkin's disease, and non-Hodgkin's disease [279, 280]. There is a critical demand for cancer biomarkers since tumour markers are also shared and expressed in healthy tissue or in other disease, making identification challenging. Raised SAA levels in conjunction with existing markers could be used as an additional diagnostic marker for AML aiding with clinical assessments.

It is widely acknowledged that the TME is greatly influenced by numerous inflammatory molecules, which are key mediators of the onset, development, and growth of tumours. This study shows that SAA can directly augment AML viability, supporting tumour growth. The pleiotropic activities of SAA have been ascribed to its capacity to bind to multiple cell surface receptors and induce several intracellular signalling pathways. FPR2 receptor was responsible for driving SAA signals on AML blasts that resulted in enhanced blast viability. Notably, FPR2 has a unique biochemical property of binding to structurally diverse chemoattractants, including pathogen-associated chemotactic molecular patterns (PAMPs) and damage-associated chemotactic molecular patterns (DAMPs)[285]. A study suggests in breast cancer, SAA potentially functions as an endogenous DAMP that binds to pattern recognition receptors (PRRs) such as TLRs on numerous cell types including tumour and consequently activating the NLRP3 inflammasome [246]. Active inflammasome in breast cancer, induces IL-1β secretion which in turn supports tumour growth and ultimately metastasis [246]. Interestingly, the NLRP3 inflammasome has also been implicated in the pathogenic phenotype of leukaemias and MDS [287]. Specifically, higher NLRP3 expression in the bone marrow mononuclear cells and PBMCs of newly diagnosed AML patients correlates with the enhanced expression of the aryl hydrocarbon receptor which is involved in the differentiation of Th cell subsets [281, 282]. This suggests that in AML, SAA release could potentially act as a DAMP, capable of activating the NLRP3 inflammasome contributing tumour development and progression. Further investigation is required to confirm this hypothesis.

Downstream of SAA signalling, several pathways culminate in the expression of cytokines, capable of modulating the immune response such as IL-1β, TNF-α and IL-6. Data presented in this thesis shows that high SAA levels released by AML blasts lead to increase in IL-1β production and release thus promoting an inflammatory environment. This is in agreement with other studies that showed SAA is capable of supporting the secretion of IL-1β, IL-1R antagonist and several other interleukins from lymphocytes, granulocytes, monocytes and macrophages [234, 283, 284]. This is perhaps not unexpected, since tumour development or progression is linked with chronic inflammation [244], wherein prolonged inflammatory cytokine exposure has the possibility to promote tumour cell survival growth. Previous studies have suggested a role for IL-1 in the expansion of AML [240, 285] Carey et al. also observed increase of IL-1β and IL-1 receptors levels in AML patients showing that IL-1 promoted AML cell growth [233]. Thus, suggests that SAA upregulation in AML blasts could potentially contribute to tumour growth via IL-1β release, however further research is required.

Furthermore, SAA was shown to be a key factor in driving upregulation of both intracellular ARG2 levels and arginase activity on AML blasts. The increase in ARG2 expression, corresponded with a reduction in extracellular arginine, and the creation of an immunosuppressive microenvironment. Arginine depletion has been associated with impaired T cells function and proliferation. Mussai et al reported that AML blasts create an immunosuppressive niche through elevated expression of ARG2 (and not ARG1), resulting in an arginine-low microenvironment that supressed T cell proliferation [75]. Data presented in this thesis re-confirmed that AML blasts reduce T cell proliferation but also shows AML patients have reduced T cell frequency compared to healthy donors. Other studies have shown that the rapid downregulate CD3ζ levels on T cells when cultured in arginine depleted culture media is a key mechanism for arginine mediated T cell suppression. CD3ζ is an essential component of the TCR complex, connecting antigen recognition with intracellular signalling to increased T cell expansion and activation [242, 286, 287]. Cancer patients have often been shown to have a lower

CD3ζ expression of in their T cells [293]. This suggests that ARG2 could be metabolic gatekeeper of T cells.

iNKT cells are a unique population of T cells that been shown to bridge the innate and adaptive immunity. Despite the low numbers in humans, the ability of iNKT cells to mount potent anti-tumour responses has been the focus of numerous investigations. AML patients failing to replenish their iNKT cell levels following HSCT are associated with poor prognosis [240]. Similar with AML, poor prognosis associated to reduced iNKT cell numbers is also described in head and neck squamous cell carcinoma [217, 229], whilst elevated numbers of circulating or intra-tumour iNKT cells have been attributed to improved prognosis in colon cancer, neuroblastoma, prostate cancer and other haematologic malignancies [244-246]. Interestingly, iNKT cell frequency and function in AML was not suppressed compared to healthy controls, suggesting that unlike T cells which are influenced by the low arginine microenvironment created by AML, iNKT cells could serve as the main drivers of anti-AML immunity.

Reports described iNKT cells mediating an effective antitumour response by three mechanisms: direct tumour killing, via the chemotactic recruitment and activation of other innate-, and adaptive-immune cells, and finally by controlling immunosuppressive cells within the TME. Our findings reveal that iNKT cells can bind with AML blasts, the interaction is enhanced by α-GalCer driving increase in iNKT cell frequency and cytokines release. This can trigger a cascade of downstream events including the activation of other immune cells (DCs, NKs, $CD8^{+}$) and thus indirectly contribute to anti-tumour immunity [180].

Even though preliminary studies suggest that iNKT cells recognise leukaemia cells and directly kill them [257, 288] several questions about: the role of α-GalCer, mechanisms of cytotoxicity and CD1d restriction are yet to be completely answered. Additionally, most studies exploring AML-iNKT cell interactions using AML cell lines. Metelitsa et al. did use primary myeloid leukaemia blasts to demonstrate iNKT cell cytotoxicity nonetheless the blasts used were cryopreserved, and a very high

proportion of iNKT cells were used to target AML blasts (E:T ratio 10:1) [263]. To truly comprehend if iNKT cells have the potential to be exploited and used as immunotherapy to target AML it is paramount that in addition to AML cell lines, these questions are answered by using primary AML blasts at an E:T ratio (0.25:1) closer to physiological conditions. This study demonstrates that α-GalCer mediated activation of iNKT cells elicits a direct cytotoxic response, against AML blasts through granzyme B release. The granzyme/perforin pathway has been described as the main mechanism applied by iNKT cells to kill cancer cells in myelomonocytic leukaemia [263] and T-cell lymphoma [265].

In our study CD1d expression was identified on AML tumour cells which can mediate α-GalCer presentation to iNKT cells leading to their activation and proliferation. This was confirmed by using CD1d blocking antibodies and revealing CD1d negative AML blasts could not mount an anti-tumour response. Recognition and killing of CD1d positive tumours by iNKT cells have been described in literature. For example, in EL4 T cell lymphoma model, both *in vivo* and *in vitro* iNKT cells directly killed lymphoma cells via perforin in a CD1d dependent manner [220, 258]. Moreover, in the prostate murine model TRAMP CD1d positive cells, iNKT cells directly reduced tumour cells [216]. Additionally, CD1d transduced breast and glioma cell lines, are lysed by iNKT cells [290, 291]. Nonetheless, CD1d expression on AML blasts did vary between patients. Analysis of juvenile myelomonocytic leukaemias revealed that progenitors of monocyte-like leukaemia cells express CD1d after losing CD34 and before and after acquiring of CD14 and CD64. This indicates that CD1d could be stage-specific to differentiation antigen for myelomonocytic/monocytic leukaemias and explains why expression is not equal throughout all patient samples [263].

Aoki et al however showed that iNKT cell mediated degranulation can occur against AML cells, mainly in K562 and HL60 cell lines in a CD1d-independent manner although a much higher effector to target ratio (5:1) was required to achieve maximum cytotoxicity [134] However a much lower E:T ratio

(0.25:1) was used in our study trying to achieve more physiological conditions. Therefore, it is difficult to compare the two studies. Having established that iNKT cells directly kill AML blasts in a CD1d dependent manner, ATRA was used with aim to enhance CD1d expression on AML blasts and enhancing recognition by iNKT cells and sequentially boost the anti-tumour response. Even though CD1d expression did increase cytotoxic response of iNKT cells did not change.

Nonetheless, at least one rare example demonstrated by patient statistics, microarrays and immunohistochemistry increase of CD1d expression is positively correlated with tumour progression or relapse in clear cell renal carcinoma or renal cell carcinoma [292]. It is possible that suppressive type II iNKT cells are activated via CD1d. Overall though our evidence and other studies demonstrates how CD1d is vital in mounting iNKT-driven antitumour immune response including AML [293].

While the greatest iNKT cell activation and cytotoxicity occurred in the presence of α-GalCer-pulsed patient AML blasts, in the absence of α-GalCer weaker iNKT cell activation and cytotoxicity against patient AML blasts was observed This implies that the leukemic cells express a natural CD1d-bound ligand for iNKT cells, which is yet to be identified. Even though the subject of determining endogenous self-antigens mediating iNKT cell activation is controversial molecules within the glycosphingolipid and phospholipid families are the most probable candidates. Gangliosides have been shown to be overexpressed in malignant melanoma and tumours from neuroectodermal origin compared to normal tissue [302] and it is suggested that they can act as tumour antigens. For example, the disalylganglioside GD2 antigen expressed on the surface of: neuroblastoma, glioma, melanoma, and non-small cell lung cancer [303]. GD3 ganglioside has been labelled as a melanoma marker due to its over-expression [302]. Additionally, GM2 is over-expressed on a range of cancers, including malignant melanoma and neuroblastomas [304]. Studies have shown that some gangliosides can also be shed into the TME [305]. For example, GM2, GM3 and GD1a gangliosides have been shown to be shed by medulloblastoma cell lines [305]. Recognition of these overexpressed GSL and gangliosides

on the surface of tumour cells could enable differential recognition and killing of tumour cells by iNKT cells. In fact, GD3 expressed on melanoma has been shown to induce a CD1d-restricted iNKT cell response. Raised concentrations of sphingosine and ceramide derivatives have been identifies in the BM and blood of AML patients, further highlighting the potential of AML blasts expressing natural iNKT cell ligands. Attempts to use GD3 based vaccines to target melanoma show only a weak immune activation [294, 296, 298] . Similarly, the hypothetical natural ligand(s) were less agonistic for iNKT cell TCR and elicited only a weak cytotoxic response compared to α-GalCer. The identification of alternative natural ligands could provide an alternative approach to activate the immune system against AML.

iNKT cells, like conventional T cells are affected by the conditions of their activation. At least two signals are required for T cell activation and consequently, proliferation, increased survival, cytokine release and differentiation into effector cells. Signal 2 co-stimulatory molecules can positively or negatively regulate iNKT cell activation and function [158]. It is therefore, not surprising that signal 2 is also important in controlling antitumour immunity. Having shown that iNKT cells constitutively express CD40L and AML blasts express CD40 this suggests they are capable in mediating CD40-CD40L interactions. CD40-CD40L interactions proved to be essential for iNKT cell to elicit a cytotoxic response against AML blasts. Antibody mediated blocking of these CD40-CD40L interactions reversed AML blast killing by iNKT. The anti-tumour role of these CD40-CD40L interactions were also observed in melanoma, where iNKT cell interactions with neutrophils were both CD1d but also CD40 dependent which in turn activated iNKT cells and decreased immunosuppressive release of IL-10 [217] CD40-CD40L co-stimulatory interaction are therefore necessary for iNKT mediated anti-tumour response, against AML.

To establish if iNKT cells could be exploited and used as viable immunotherapy against AML, it is critical to determine the effect the TME has on iNKT cells since tumour cells have been shown to use

a plethora of strategies to escape immune recognition. For example, they can hinder antigen presentation by limiting expression of antigen on tumour cells or APC. This study for the first time shows iNKT cells (unlike T cells) are not affected by low arginine conditions created by AML and that they can expand upon stimulation. Moreover, in low arginine conditions iNKT cells activated by α-GalCer-loaded AML blasts proliferate and kill AML blasts. Hence these key effector functions should be utilized for immunotherapy to target AML.

In vivo experiments further revalidated findings observed *in vitro* using an immunocompetent AML murine model. Increase in iNKT cell frequency in α-GalCer treated AML bearing mice accompanied by raised IFN-γ levels indicated iNKT cells are activated, expand. Similarly, Crowe et al. reported an approximately a tenfold expansion of iNKT cell numbers in the mouse spleen after 2-3 days of 2 μg α-GalCer injection [307]. In the same study, a three and seven-fold increase in iNKT cell expansion was observed in the BM and liver respectively following α-GalCer injection [307]. This study showed a reduction in tumour burden in α-GalCer treated AML bearing mice and, confirms results from *in vivo* experiments presented in this thesis; wherein following α-GalCer treatment iNKT cells are activated, proliferate and significantly reduce AML blasts irrespective of the immunocompromised TME.

Identifying a population of immune cells (iNKT cells) which have the capacity to tolerate, the immunosuppressive TME, adapt, remain functional and simultaneously abrogate AML blasts are key characteristics for effective immunotherapy. This suggests iNKT cells are key in shaping an effective immune response and thus are suitable targets AML immunotherapy. This study shows that iNKT cells did express low levels of arginine catabolism enzymes ARG1 or ARG2, however upregulate amino acid uptake via LAT-1 and increase arginine resynthesis from citrulline by ASS-1. Additionally, supressed IFN-γ production by iNKT cells in the presence of α-GalCer loaded AML cells, in citrulline free conditions infers that α-GalCer-activated iNKT cells can use citrulline as an alternative source of

arginine. Crump et al show significant increase in citrulline in plasma of AML patients relative to healthy donors suggesting there is sufficient citrulline availability in AML plasma to be utilized by arginine-starved iNKT cells; further research is however required to confirm this [267]. Interestingly, in the study by Crump and colleagues, T cells were not able to use citrulline to synthesise arginine [267] and further emphasizing tolerance to low arginine conditions is a unique characteristic to iNKT cells.

Moreover, in response to amino acid starvation, increase in ASS expression has been shown to rely on ATF4 for transcriptional alterations [300, 301].Thus the role of ATF4 in driving ASS-1 upregulation by α-GalCer pulsed iNKT cells could be explored in future studies. Interestingly, in contrast to iNKT cells, it has been demonstrated that T cells cannot induce ASS-1 as they do not transcribe *ASS-1* gene [268, 302]. Moreover, our group has shown that CAR-T cells engineered to express *ASS-1* demonstrate an enhanced proliferation and *in vivo*, with a corresponding enhancement of their anti-tumour activity against AML blasts [311]. This implies that inducing ASS-1 expression can act as an effective mechanism to overcome arginine depleted microenvironment.

Crump et al demonstrate that THP1 cells grown under arginine free conditions depends on ASS-1 upregulation driven by binding of ATF4 and CEBPβ transcription factors to an intronic enhancer [267]. Unlike THP1, T cells grown in arginine free conditions did not induce ASS-1 expression due to the inability of ATF4 and CEBPβ to effectively bind to the same ASS-1 enhancer. The weak transcription of ASS-1 in T cells is therefore constrained by poor chromatin accessibility and supressed histone methylation [267]. In contrast to conventional T cells, AML blasts and activated iNKT cells uptake citrulline for arginine re-synthesis. It is highly probable that upon activation iNKT cells adopt a similar mechanism to AML blasts making them able to withstand and proliferate in low arginine conditions, however deeper investigations are necessary [267].

These findings can potentially help explain how iNKT cells function to abolish ARG1 positive MDSCs that suppress T cells in the setting of influenza. The capacity of iNKT cells to abolish the inhibitory activity of MDSCs, relied on CD1d and CD40 interactions [278], similar to AML. Interestingly, De Santo et al. demonstrated immunosuppressive activity of neutrophils induced by SAA was reversed by iNKT cells in melanoma [217]. Suggesting iNKT cells have the unique capacity to adapt to the immunosuppressive TME driven by SAA, identifying a new niche functionality for these relatively rare immune cells.

Finally, this study demonstrates that activated iNKT cells lead to a secondary enhancement of T cell immunity, restoring T cell proliferation which was supressed by AML blasts. Thus, deeper investigation is necessary to confirm T cell functionality. Therefore, iNKT cells have been shown to be important players in tumour immunosurveillance through their ability to mediate direct AML lysis and restore T cell function acting as promising candidates to be used for immunotherapy.

Therapeutic approaches

Over the past decades different approaches have been explored and developed to exploit iNKT cells in order to treat various types of cancers. These strategies could be adopted to treat AML.

α-GalCer

Multiple clinical trials have taken place exploring α-GalCer iNKT cell-based immunotherapy [304]. In an early phase I clinical trials, soluble α-GalCer was directly injected to cancer patients even though it did not have any serious complications and was well-tolerated no objective clinical response was observed [305]. Possible explanations for iNKT cell hyporesponsiveness in human trials could be associated with inefficient delivery of α-GalCer or induction of anergy. To circumvent these limitations and enhance anti-tumour responses mediated by iNKT cells a number of clinical involving loading of autologous α-GalCer-pulsed APCs were carried in patients with recurrent or advanced solid tumours such as head and neck squamous cell carcinoma and non-small cell lung cancer [314]. α-

GalCer-pulsed DCs were also tried in patients with advanced multiple myeloma. therapeutic use of, iNKT cells did not sufficiently expand and persist in the blood, nor was there any clinical improvement observed in patients. This strategy is more promising; however, some caveats did arise for instance variability in iNKT cell frequency and the challenging to attain a sufficient number of autologous monocyte-derived DCs (moDCs) from immune suppressed cancer patients [304, 306].

Adoptive transfer of iNKT cells

We show in this thesis that in iNKT cell frequency in AML patients is not significantly altered compared to healthy individuals. As such of autologous α-GalCer-pulsed APCs or of autologous α-GalCer-pulsed APCs could be used potentially could potentially be used as AML therapy. Adoptive transfer of ex vivo expanded iNKT cells in combination with α-GalCer is an effective treatment against CD1d positive ALL cells implanted in immunodeficient NOD/SCID mice [315]. Having demonstrated AML blasts also express CD1d, suggests adoptive transfer of activated iNKT cells could be considered as prospective approach to target AML. Though, not much is known about therapeutic use of ex vivo expanded iNKT cells against leukaemia in humans [236]. Clinical trials using this strategy in advanced melanoma and non-small cell lung cancer where promising, displaying enhanced iNKT cell expansion and increase IFN-γ concentrations in serum *in vivo* displaying safety and feasibility of treatment. Thus a strategy to be further explored with AML patients in the future [227].

CAR-iNKT cells

Most recently an exciting development in iNKT cell immunotherapy is the generation of chimeric antigen receptor expressing NKT (CAR-iNKT) cells [308, 309]. CAR based-immunotherapy in solid tumours and AML has had minimal clinical efficacy, partly due to the immunosuppressive TME, CAR iNKT cells are a promising therapeutic strategy given that they remain functional within the immunosuppressive AML microenvironment. Heczey et al show even though both GD2-specific CAR-T cells and CAR-iNKT cells were able to infiltrate the neuroblastoma tumours, the CAR-iNKT where more effective [308]. Additionally, severe graft-versus-host-disease (GVHD) was observed in NSG mice from CAR-T cells generated from an allogeneic donor but not evident in GD2- specific CAR-iNKT cells [308]. Using CAR-iNKT cells are greatly advantageous and promising as they allow for ‘‘off the shelf’’ therapy as they are not likely to mount allogeneic responses against host MHC. Recently, interim results from the first phase I trial with GD2- specific CAR-iNKT cells for children with relapsed or resistant neuroblastoma demonstrated CAR-iNKT cells can be expanded to clinical scale and safely applied to treat patients with cancer [318]. By engineering CD33-CARiNKT cells to target AML blasts could be most promising strategy to drive an anti-AML response as it can accomplish dual targeting by both CD1d and CD33. Their ability to proliferate, and naturally traffic to AML blasts, mediating an effective anti-tumour response would need to be explored since previously poor proliferation and short persistence of CAR-T cells lead to tumour relapse following transient transmission. A similar approach was used to B lineage malignancies with CAR19-iNKT cells [319]. Therefore, iNKT cells provide optimal platform for CAR-based immunotherapy in the future.

Concluding remarks

In conclusion this study shows SAA is released by AML blasts, enhancing AML growth and upregulated ARG2 expression. The resulting increase in ARG2 expression and activity, creates low arginine microenvironment which supress T cells. iNKT have a seemingly unique adaptation to the AML amino acid metabolic microenvironment, by increasing LAT-1 and ASS-1 expression. iNKT cells interact with AML blasts in a CD1d/Cd40 dependent manner and retain their functionality under lower arginine conditions. They can become activated and expand upon antigen stimulation by α-GalCer, releasing IFN-γ, and augment T cell proliferation. Additionally, α-GalCer activated iNKT cells directly induce a robust anti-leukaemic cytolytic response mediated by granzyme B against AML blasts. iNKT cells are an attractive target for immunotherapy. However, to date no clinical trials of iNKT cells against AML have taken place. Identifying the dynamic roles and functional states of iNKT cells is key for establishing successful therapies to target AML and the TME.

Appendix

Supplementary figures:

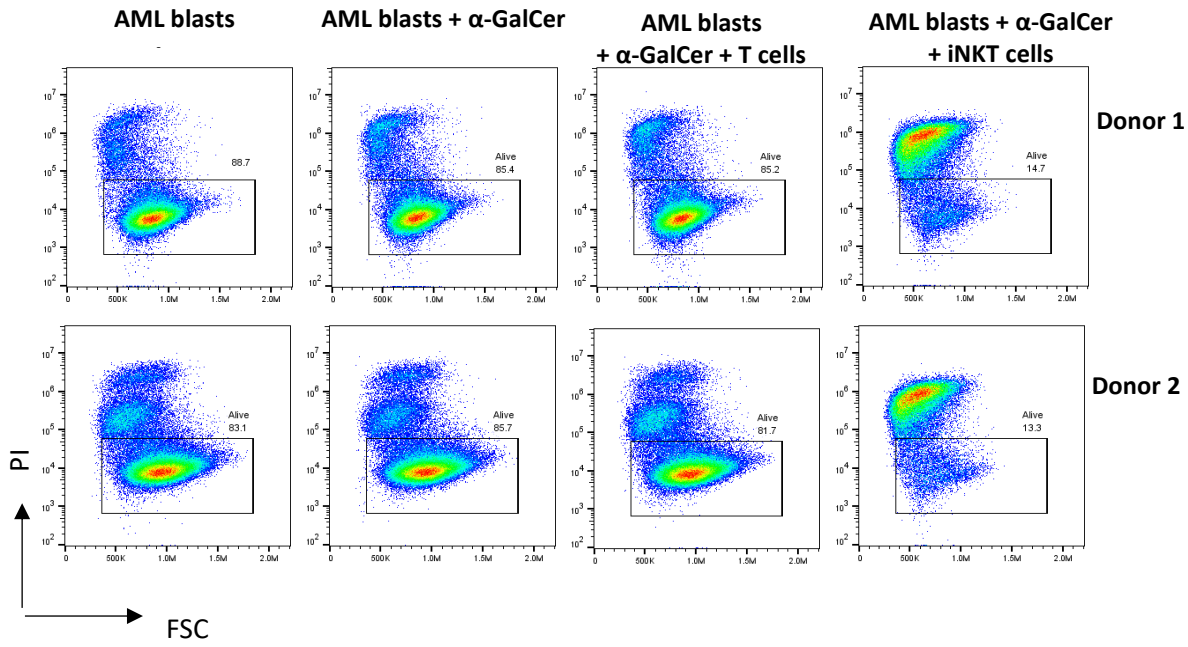


Figure 1: α-GalCer specifically stimulates iNKT cells.
AML blasts in the presence of α-GalCer were co-cultured with iNKT cells and T cells at effector: target ratio 0:25:1 for 72 hours. Viability of AML blasts was determined by flow cytometry using propidium iodide. In the presence of CD1d positive AML blasts, α-GalCer specifically drives iNKT cell but not T cells activation, kill AML blasts. α-GalCer alone did not alter AML viability

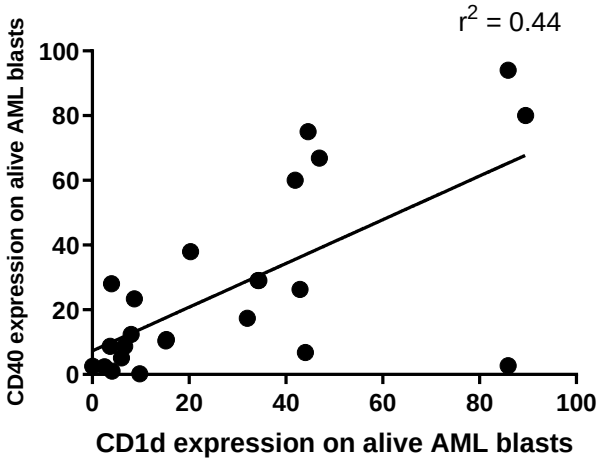


Figure 2: Correlation is observed between expression of CD1d and CD40 on alive AML blasts.
A positive correlation (0.66) between CD1d and CD40 expression was measured on patient alive AML blasts (CD33 positive)

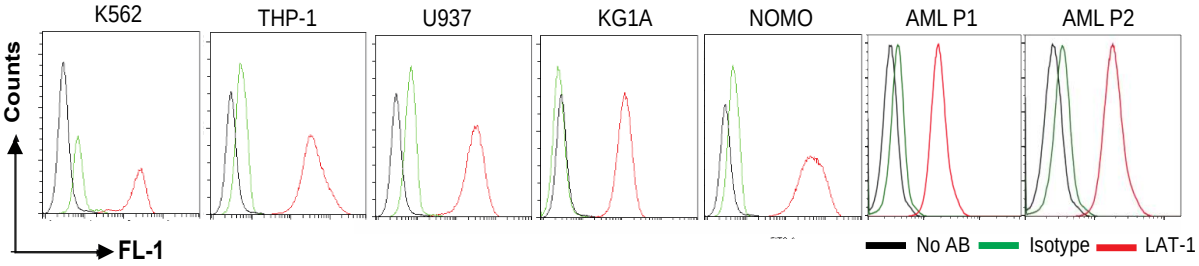


Figure 3: AML blasts express LAT-1
LAT-1 expression on alive AML cell lines and AML patient blasts determined by flow cytometry.

Publications

- **Victoria Stavrou**, Livingstone Fultang, Sarah Booth, *et al*. (2021) **Invariant NKT cells metabolically adapt to the acute myeloid leukaemia microenvironment** (under submission).

- Fultang L, Booth S, Yogev O, Martins da Costa B, Tubb V, Panetti S, **Stavrou V** et *al.* (2020) **Metabolic engineering against the arginine microenvironment enhances CAR-T cell proliferation and therapeutic activity**. Blood.

- Mussai, F., Wheat, R., Sarrou, E., Booth, S., **Stavrou, V**., Fultang, L *et al*. (2019). **Targeting the arginine metabolic brake enhances immunotherapy for leukaemia.** *International journal of cancer*,

References

1. Smith, M., et al., *Adult acute myeloid leukaemia.* Crit Rev Oncol Hematol, 2004. **50**(3): p. 197-222.
2. Juliusson, G. and R. Hough, *Leukemia.* Prog Tumor Res, 2016. **43**: p. 87-100.
3. Hehlmann, R., *Chronic Myeloid Leukemia in 2020.* Hemasphere, 2020. **4**(5): p. e468.
4. Winters, A.C. and K.M. Bernt, *MLL-Rearranged Leukemias-An Update on Science and Clinical Approaches.* Frontiers in pediatrics, 2017. **5**: p. 4-4.
5. Yamamoto, J.F. and M.T. Goodman, *Patterns of leukemia incidence in the United States by subtype and demographic characteristics, 1997–2002.* Cancer Causes & Control, 2008. **19**(4): p. 379-390.
6. De Kouchkovsky, I. and M. Abdul-Hay, *'Acute myeloid leukemia: a comprehensive review and 2016 update'.* Blood Cancer J, 2016. **6**(7): p. e441.
7. Ries, L.A.G., *Cancer Survival Among Adults: U.S. SEER Program, 1988-2001, Patient and Tumor Characteristics*. 2007: U.S. Department of Health and Human Services, National Institutes of Health, National Cancer Institute.
8. Shah, A., et al., *Survival and cure of acute myeloid leukaemia in England, 1971-2006: a population-based study.* Br J Haematol, 2013. **162**(4): p. 509-16.
9. Saultz, J.N. and R. Garzon, *Acute Myeloid Leukemia: A Concise Review.* Journal of Clinical Medicine, 2016. **5**(3): p. 33.
10. Lamble, A.J. and E.F. Lind, *Targeting the Immune Microenvironment in Acute Myeloid Leukemia: A Focus on T Cell Immunity.* Frontiers in oncology, 2018. **8**: p. 213-213.
11. Szotkowski, T., et al., *Secondary acute myeloid leukemia - a single center experience.* Neoplasma, 2010. **57**(2): p. 170-8.
12. Sill, H., et al., *Therapy-related myeloid neoplasms: pathobiology and clinical characteristics.* British journal of pharmacology, 2011. **162**(4): p. 792-805.
13. Fong, C.-t. and G.M. Brodeur, *Down's syndrome and leukemia: Epidemiology, genetics, cytogenetics and mechanisms of leukemogenesis.* Cancer Genetics and Cytogenetics, 1987. **28**(1): p. 55-76.
14. Pötzsch, C., T. Voigtländer, and M. Lübbert, *p53 Germline mutation in a patient with Li-Fraumeni Syndrome and three metachronous malignancies.* Journal of Cancer Research and Clinical Oncology, 2002. **128**(8): p. 456-460.
15. Preston, D.L., et al., *Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950-1987.* Radiat Res, 1994. **137**(2 Suppl): p. S68-97.
16. Savitz, D.A. and K.W. Andrews, *Review of epidemiologic evidence on benzene and lymphatic and hematopoietic cancers.* Am J Ind Med, 1997. **31**(3): p. 287-95.
17. Pogoda, J.M., et al., *Smoking and Risk of Acute Myeloid Leukemia: Results from a Los Angeles County Case-Control Study.* American Journal of Epidemiology, 2002. **155**(6): p. 546-553.
18. Chen, Y., et al., *Persistence of cytogenetic abnormalities at complete remission after induction in patients with acute myeloid leukemia: prognostic significance and the potential role of allogeneic stem-cell transplantation.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2011. **29**(18): p. 2507-2513.
19. Kumar, C.C., *Genetic abnormalities and challenges in the treatment of acute myeloid leukemia.* Genes & cancer, 2011. **2**(2): p. 95-107.
20. Döhner, H., D.J. Weisdorf, and C.D. Bloomfield, *Acute Myeloid Leukemia.* New England Journal of Medicine, 2015. **373**(12): p. 1136-1152.
21. Bacher, U., et al., *Mutations of the TET2 and CBL genes: novel molecular markers in myeloid malignancies.* Ann Hematol, 2010. **89**(7): p. 643-52.

22. Kelly, L.M. and D.G. Gilliland, *Genetics of myeloid leukemias.* Annu Rev Genomics Hum Genet, 2002. **3**: p. 179-98.
23. Takahashi, S., *Current findings for recurring mutations in acute myeloid leukemia.* Journal of hematology & oncology, 2011. **4**: p. 36-36.
24. Ley, T.J., et al., *Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia.* N Engl J Med, 2013. **368**(22): p. 2059-74.
25. Conway O'Brien, E., S. Prideaux, and T. Chevassut, *The Epigenetic Landscape of Acute Myeloid Leukemia.* Advances in Hematology, 2014. **2014**: p. 103175.
26. Shih, A.H., et al., *The role of mutations in epigenetic regulators in myeloid malignancies.* Nat Rev Cancer, 2012. **12**(9): p. 599-612.
27. Patel, J.P., et al., *Prognostic relevance of integrated genetic profiling in acute myeloid leukemia.* The New England journal of medicine, 2012. **366**(12): p. 1079-1089.
28. Im, A.P., et al., *DNMT3A and IDH mutations in acute myeloid leukemia and other myeloid malignancies: associations with prognosis and potential treatment strategies.* Leukemia, 2014. **28**(9): p. 1774-1783.
29. Park, S.H., et al., *Incidences and Prognostic Impact of c-KIT, WT1, CEBPA, and CBL Mutations, and Mutations Associated With Epigenetic Modification in Core Binding Factor Acute Myeloid Leukemia: A Multicenter Study in a Korean Population.* Annals of laboratory medicine, 2015. **35**(3): p. 288-297.
30. Goldman, S.L., et al., *Epigenetic Modifications in Acute Myeloid Leukemia: Prognosis, Treatment, and Heterogeneity.* Frontiers in genetics, 2019. **10**: p. 133-133.
31. Allis, C.D. and T. Jenuwein, *The molecular hallmarks of epigenetic control.* Nat Rev Genet, 2016. **17**(8): p. 487-500.
32. Lindsley, R.C., et al., *Acute myeloid leukemia ontogeny is defined by distinct somatic mutations.* Blood, 2015. **125**(9): p. 1367-1376.
33. *Genomic and Epigenomic Landscapes of Adult De Novo Acute Myeloid Leukemia.* New England Journal of Medicine, 2013. **368**(22): p. 2059-2074.
34. Yu, J., et al., *Clinical implications of recurrent gene mutations in acute myeloid leukemia.* Experimental Hematology & Oncology, 2020. **9**(1): p. 4.
35. Spencer, D.H., et al., *CpG Island Hypermethylation Mediated by DNMT3A Is a Consequence of AML Progression.* Cell, 2017. **168**(5): p. 801-816.e13.
36. Lagunas-Rangel, F.A., et al., *Acute Myeloid Leukemia-Genetic Alterations and Their Clinical Prognosis.* International journal of hematology-oncology and stem cell research, 2017. **11**(4): p. 328-339.
37. Pourrajab, F., et al., *Genetic Characterization and Risk Stratification of Acute Myeloid Leukemia.* Cancer management and research, 2020. **12**: p. 2231-2253.
38. Singh, A.A., et al., *Multi-omics profiling reveals a distinctive epigenome signature for high-risk acute promyelocytic leukemia.* Oncotarget, 2018. **9**(39): p. 25647-25660.
39. Vardiman, J.W., N.L. Harris, and R.D. Brunning, *The World Health Organization (WHO) classification of the myeloid neoplasms.* Blood, 2002. **100**(7): p. 2292-2302.
40. Arber, D.A., et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia.* Blood, 2016. **127**(20): p. 2391-405.
41. Vardiman, J.W., et al., *The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes.* Blood, 2009. **114**(5): p. 937-51.
42. Estey, E.H., *Acute myeloid leukemia: 2019 update on risk-stratification and management.* American Journal of Hematology, 2018. **93**(10): p. 1267-1291.
43. Campos, L., et al., *Surface marker expression in adult acute myeloid leukaemia: correlations with initial characteristics, morphology and response to therapy.* Br J Haematol, 1989. **72**(2): p. 161-6.

44. Wolach, O. and R.M. Stone, *How I treat mixed-phenotype acute leukemia.* Blood, 2015. **125**(16): p. 2477-85.
45. Hanahan, D. and Lisa M. Coussens, *Accessories to the Crime: Functions of Cells Recruited to the Tumor Microenvironment.* Cancer Cell, 2012. **21**(3): p. 309-322.
46. Chevrier, S., et al., *An Immune Atlas of Clear Cell Renal Cell Carcinoma.* Cell, 2017. **169**(4): p. 736-749.e18.
47. Jiménez-Sánchez, A., et al., *Heterogeneous Tumor-Immune Microenvironments among Differentially Growing Metastases in an Ovarian Cancer Patient.* Cell, 2017. **170**(5): p. 927-938.e20.
48. Kozako, T., et al., *PD-1/PD-L1 expression in human T-cell leukemia virus type 1 carriers and adult T-cell leukemia/lymphoma patients.* Leukemia, 2009. **23**(2): p. 375-382.
49. Christiansson, L., et al., *Increased level of myeloid-derived suppressor cells, programmed death receptor ligand 1/programmed death receptor 1, and soluble CD25 in Sokal high risk chronic myeloid leukemia.* PLoS One, 2013. **8**(1): p. e55818.
50. Shimauchi, T., et al., *Augmented expression of programmed death-1 in both neoplastic and non-neoplastic CD4+ T-cells in adult T-cell leukemia/lymphoma.* Int J Cancer, 2007. **121**(12): p. 2585-90.
51. Riches, J.C., et al., *T cells from CLL patients exhibit features of T-cell exhaustion but retain capacity for cytokine production.* Blood, 2013. **121**(9): p. 1612-21.
52. Gassner, F.J., et al., *Chemotherapy-induced augmentation of T cells expressing inhibitory receptors is reversed by treatment with lenalidomide in chronic lymphocytic leukemia.* Haematologica, 2014. **99**(5): p. 67-9.
53. Khong, H.T. and N.P. Restifo, *Natural selection of tumor variants in the generation of "tumor escape" phenotypes.* Nature Immunology, 2002. **3**(11): p. 999-1005.
54. Zhou, Q., et al., *Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia.* Blood, 2011. **117**(17): p. 4501-4510.
55. Zhou, P., et al., *In vivo discovery of immunotherapy targets in the tumour microenvironment.* Nature, 2014. **506**(7486): p. 52-57.
56. Vereecque, R., et al., *γ-Ray irradiation induces B7.1 expression in myeloid leukaemic cells.* British Journal of Haematology, 2000. **108**(4): p. 825-831.
57. Williams, P., et al., *The distribution of T-cell subsets and the expression of immune checkpoint receptors and ligands in patients with newly diagnosed and relapsed acute myeloid leukemia.* Cancer, 2019. **125**(9): p. 1470-1481.
58. Zhang, L., T.F. Gajewski, and J. Kline, *PD-1/PD-L1 interactions inhibit antitumor immune responses in a murine acute myeloid leukemia model.* Blood, 2009. **114**(8): p. 1545-52.
59. Epperly, R., S. Gottschalk, and M.P. Velasquez, *A Bump in the Road: How the Hostile AML Microenvironment Affects CAR T Cell Therapy.* Frontiers in Oncology, 2020. **10**(262).
60. Jiang, X., et al., *Immune checkpoint: The novel target for antitumor therapy.* Genes & diseases, 2019. **8**(1): p. 25-37.
61. Teague, R.M. and J. Kline, *Immune evasion in acute myeloid leukemia: current concepts and future directions.* Journal for ImmunoTherapy of Cancer, 2013. **1**(1): p. 13.
62. Han, Y., et al., *Acute Myeloid Leukemia Cells Express ICOS Ligand to Promote the Expansion of Regulatory T Cells.* Frontiers in Immunology, 2018. **9**(2227).
63. Zhou, Q., et al., *Depletion of endogenous tumor-associated regulatory T cells improves the efficacy of adoptive cytotoxic T-cell immunotherapy in murine acute myeloid leukemia.* Blood, 2009. **114**(18): p. 3793-802.
64. Menter, T., et al., *Beneficial role of increased FOXP3(+) regulatory T-cells in acute myeloid leukaemia therapy response.* Br J Haematol, 2018. **182**(4): p. 581-583.

65. Fisher, S.A., et al., *Increased regulatory T cell graft content is associated with improved outcome in haematopoietic stem cell transplantation: a systematic review.* Br J Haematol, 2017. **176**(3): p. 448-463.
66. Que, Y., et al., *Study on the Immune Escape Mechanism of Acute Myeloid Leukemia With DNMT3A Mutation.* Frontiers in Immunology, 2021. **12**(1912).
67. Orleans-Lindsay, J.K., et al., *Acute myeloid leukaemia cells secrete a soluble factor that inhibits T and NK cell proliferation but not cytolytic function--implications for the adoptive immunotherapy of leukaemia.* Clin Exp Immunol, 2001. **126**(3): p. 403-11.
68. Al-Matary, Y.S., et al., *Acute myeloid leukemia cells polarize macrophages towards a leukemia supporting state in a Growth factor independence 1 dependent manner.* Haematologica, 2016. **101**(10): p. 1216-1227.
69. Lv, M., K. Wang, and X.-j. Huang, *Myeloid-derived suppressor cells in hematological malignancies: friends or foes.* Journal of Hematology & Oncology, 2019. **12**(1): p. 105.
70. Corzo, C.A., et al., *Mechanism Regulating Reactive Oxygen Species in Tumor-Induced Myeloid-Derived Suppressor Cells.* The Journal of Immunology, 2009. **182**(9): p. 5693-5701.
71. Nagaraj, S., et al., *Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer.* Nat Med, 2007. **13**(7): p. 828-35.
72. Pyzer, A.R., et al., *MUC1-mediated induction of myeloid-derived suppressor cells in patients with acute myeloid leukemia.* Blood, 2017. **129**(13): p. 1791-1801.
73. Kittang, A.O., et al., *Expansion of myeloid derived suppressor cells correlates with number of T regulatory cells and disease progression in myelodysplastic syndrome.* Oncoimmunology, 2015. **5**(2): p. e1062208-e1062208.
74. Mussai, F., et al., *Acute myeloid leukemia creates an arginase-dependent immunosuppressive microenvironment.* Blood, 2013. **122**(5): p. 749-758.
75. Kantarjian, H., et al., *Acute myeloid leukemia: current progress and future directions.* Blood Cancer Journal, 2021. **11**(2): p. 41.
76. Dombret, H. and C. Gardin, *An update of current treatments for adult acute myeloid leukemia.* Blood, 2016. **127**(1): p. 53-61.
77. Chen, K.T.J., et al., *Recent Treatment Advances and the Role of Nanotechnology, Combination Products, and Immunotherapy in Changing the Therapeutic Landscape of Acute Myeloid Leukemia.* Pharmaceutical research, 2019. **36**(9): p. 125-125.
78. Levis, M., *Midostaurin approved for FLT3-mutated AML.* Blood, 2017. **129**(26): p. 3403-3406.
79. Dugan, J. and D. Pollyea, *Enasidenib for the treatment of acute myeloid leukemia.* Expert Rev Clin Pharmacol, 2018. **11**(8): p. 755-760.
80. Lancet, J.E., et al., *Final results of a phase III randomized trial of CPX-351 versus 7+3 in older patients with newly diagnosed high risk (secondary) AML.* Journal of Clinical Oncology, 2016. **34**(15_suppl): p. 7000-7000.
81. Sweeney, C. and P. Vyas, *The Graft-Versus-Leukemia Effect in AML.* Frontiers in Oncology, 2019. **9**(1217).
82. Lichtenegger, F.S., et al., *Recent developments in immunotherapy of acute myeloid leukemia.* Journal of hematology & oncology, 2017. **10**(1): p. 142-142.
83. Hargadon, K.M., C.E. Johnson, and C.J. Williams, *Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors.* International Immunopharmacology, 2018. **62**: p. 29-39.
84. Feldman, E.J., et al., *Phase III randomized multicenter study of a humanized anti-CD33 monoclonal antibody, lintuzumab, in combination with chemotherapy, versus chemotherapy alone in patients with refractory or first-relapsed acute myeloid leukemia.* J Clin Oncol, 2005. **23**(18): p. 4110-6.
85. Zhou, Y. and E. Song, *Immunotherapy for acute myeloid leukemia: the dawn of a new era?* Annals of Blood, 2020. **5**.

86. Appelbaum, F.R. and I.D. Bernstein, *Gemtuzumab ozogamicin for acute myeloid leukemia.* Blood, 2017. **130**(22): p. 2373-2376.
87. Daver, N., et al., *T-cell-based immunotherapy of acute myeloid leukemia: current concepts and future developments.* Leukemia, 2021. **35**(7): p. 1843-1863.
88. Chen, L. and D.B. Flies, *Molecular mechanisms of T cell co-stimulation and co-inhibition.* Nature reviews. Immunology, 2013. **13**(4): p. 227-242.
89. Barrett, A.J. and K. Le Blanc, *Immunotherapy prospects for acute myeloid leukaemia.* Clinical and experimental immunology, 2010. **161**(2): p. 223-232.
90. Maslak, P.G., et al., *Phase 2 trial of a multivalent WT1 peptide vaccine (galinpepimut-S) in acute myeloid leukemia.* Blood Adv, 2018. **2**(3): p. 224-234.
91. van de Loosdrecht, A.A., et al., *A novel allogeneic off-the-shelf dendritic cell vaccine for post-remission treatment of elderly patients with acute myeloid leukemia.* Cancer immunology, immunotherapy : CII, 2018. **67**(10): p. 1505-1518.
92. Toubal, A., et al., *Mucosal-associated invariant T cells and disease.* Nature Reviews Immunology, 2019. **19**(10): p. 643-657.
93. Keller, A.N., et al., *MAIT cells and MR1-antigen recognition.* Curr Opin Immunol, 2017. **46**: p. 66-74.
94. Godfrey, D.I., et al., *The burgeoning family of unconventional T cells.* Nature Immunology, 2015. **16**(11): p. 1114-1123.
95. Bendelac, A., P.B. Savage, and L. Teyton, *The biology of NKT cells*, in *Annual Review of Immunology*. 2007, Annual Reviews: Palo Alto. p. 297-336.
96. Salio, M., et al., *Biology of CD1- and MR1-Restricted T Cells.* Annual Review of Immunology, 2014. **32**(1): p. 323-366.
97. Bendelac, A., R.D. Hunziker, and O. Lantz, *Increased interleukin 4 and immunoglobulin E production in transgenic mice overexpressing NK1T cells.* Journal of Experimental Medicine, 1996. **184**(4): p. 1285-1293.
98. Godfrey, D.I., S. Stankovic, and A.G. Baxter, *Raising the NKT cell family.* Nature Immunology, 2010. **11**(3): p. 197-206.
99. Halder, R.C., et al., *Type IINKT cell-mediated anergy induction in type INKT cells prevents inflammatory liver disease.* Journal of Clinical Investigation, 2007. **117**(8): p. 2302-2312.
100. Exley, M.A., et al., *Cutting Edge: A Major Fraction of Human Bone Marrow Lymphocytes Are Th2-Like CD1d-Reactive T Cells That Can Suppress Mixed Lymphocyte Responses.* The Journal of Immunology, 2001. **167**(10): p. 5531-5534.
101. Dasgupta, S. and V. Kumar, *Type II NKT cells: a distinct CD1d-restricted immune regulatory NKT cell subset.* Immunogenetics, 2016. **68**(8): p. 665-676.
102. Tiwary, S., J.A. Berzofsky, and M. Terabe, *Altered Lipid Tumor Environment and Its Potential Effects on NKT Cell Function in Tumor Immunity.* Frontiers in Immunology, 2019. **10**(2187).
103. Brutkiewicz, R.R., et al., *TAP-INDEPENDENT, BETA(2)-MICROGLOBULIN-DEPENDENT SURFACE EXPRESSION OF FUNCTIONAL-MOUSE CD1.1.* Journal of Experimental Medicine, 1995. **182**(6): p. 1913-1919.
104. Bendelac, A., et al., *CD1 RECOGNITION BY MOUSE NK1(+) T-LYMPHOCYTES.* Science, 1995. **268**(5212): p. 863-865.
105. Mendiratta, S.K., et al., *CD1d1 mutant mice are deficient in natural T cells that promptly produce IL-4.* Immunity, 1997. **6**(4): p. 469-477.
106. Calabi, F. and C. Milstein, *A novel family of human major histocompatibility complex-related genes not mapping to chromosome 6.* Nature, 1986. **323**(6088): p. 540-3.
107. Calabi, F., et al., *Two classes of CD1 genes.* Eur J Immunol, 1989. **19**(2): p. 285-92.
108. de la Salle, H., et al., *Assistance of Microbial Glycolipid Antigen Processing by CD1e.* Science, 2005. **310**(5752): p. 1321-1324.

109. Park, S.-H., J.H. Roark, and A. Bendelac, *Tissue-Specific Recognition of Mouse CD1 Molecules.* The Journal of Immunology, 1998. **160**(7): p. 3128-3134.
110. Koch, M., et al., *The crystal structure of human CD1d with and without alpha-galactosylceramide.* Nature Immunology, 2005. **6**(8): p. 819-826.
111. Kawano, T., et al., *CD1d-restricted and TCR-mediated activation of V(alpha)14 NKT cells by glycosylceramides.* Science, 1997. **278**(5343): p. 1626-1629.
112. Barral, D.C. and M.B. Brenner, *CD1 antigen presentation: how it works.* Nature Reviews Immunology, 2007. **7**(12): p. 929-941.
113. Joyce, S., E. Girardi, and D.M. Zajonc, *NKT cell ligand recognition logic: molecular basis for a synaptic duet and transmission of inflammatory effectors.* J Immunol, 2011. **187**(3): p. 1081-9.
114. Girardi, E. and D.M. Zajonc, *Molecular basis of lipid antigen presentation by CD1d and recognition by natural killer T cells.* Immunological reviews, 2012. **250**(1): p. 167-179.
115. Girardi, E., et al., *Unique Interplay between Sugar and Lipid in Determining the Antigenic Potency of Bacterial Antigens for NKT Cells.* PLOS Biology, 2011. **9**(11): p. e1001189.
116. Scharf, L., et al., *The 2.5 Å structure of CD1c in complex with a mycobacterial lipid reveals an open groove ideally suited for diverse antigen presentation.* Immunity, 2010. **33**(6): p. 853-62.
117. Li, Y., et al., *The Vα14 invariant natural killer T cell TCR forces microbial glycolipids and CD1d into a conserved binding mode.* Journal of Experimental Medicine, 2010. **207**(11): p. 2383-2393.
118. Borg, N.A., et al., *CD1d–lipid-antigen recognition by the semi-invariant NKT T-cell receptor.* Nature, 2007. **448**(7149): p. 44-49.
119. De Libero, G. and L. Mori, *Novel insights into lipid antigen presentation.* Trends Immunol, 2012. **33**(3): p. 103-11.
120. Freigang, S., et al., *Fatty acid amide hydrolase shapes NKT cell responses by influencing the serum transport of lipid antigen in mice.* J Clin Invest, 2010. **120**(6): p. 1873-84.
121. van den Elzen, P., et al., *Apolipoprotein-mediated pathways of lipid antigen presentation.* Nature, 2005. **437**(7060): p. 906-10.
122. Roy, K.C., et al., *Involvement of secretory and endosomal compartments in presentation of an exogenous self-glycolipid to type II NKT cells.* J Immunol, 2008. **180**(5): p. 2942-50.
123. Zhou, D., et al., *Editing of CD1d-bound lipid antigens by endosomal lipid transfer proteins.* Science, 2004. **303**(5657): p. 523-7.
124. León, L., et al., *Saposins utilize two strategies for lipid transfer and CD1 antigen presentation.* Proc Natl Acad Sci U S A, 2012. **109**(12): p. 4357-64.
125. Yuan, W., et al., *Saposin B is the dominant saposin that facilitates lipid binding to human CD1d molecules.* Proc Natl Acad Sci U S A, 2007. **104**(13): p. 5551-6.
126. Winau, F., et al., *Saposin C is required for lipid presentation by human CD1b.* Nat Immunol, 2004. **5**(2): p. 169-74.
127. Dougan, S.K., et al., *Microsomal triglyceride transfer protein lipidation and control of CD1d on antigen-presenting cells.* The Journal of experimental medicine, 2005. **202**(4): p. 529-539.
128. León, L., et al., *Saposins utilize two strategies for lipid transfer and CD1 antigen presentation.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(12): p. 4357-4364.
129. Brigl, M. and M.B. Brenner, *CD1: Antigen Presentation and T Cell Function.* Annual Review of Immunology, 2004. **22**(1): p. 817-890.
130. Godfrey, D.I. and S.P. Berzins, *Control points in NKT-cell development.* Nature Reviews Immunology, 2007. **7**(7): p. 505-518.
131. Scott-Browne, J.P., et al., *Germline-encoded recognition of diverse glycolipids by natural killer T cells.* Nature Immunology, 2007. **8**(10): p. 1105-1113.

132. Morita, M., et al., *STRUCTURE-ACTIVITY RELATIONSHIP OF ALPHA-GALACTOSYLCERAMIDES AGAINST B16-BEARING MICE.* Journal of Medicinal Chemistry, 1995. **38**(12): p. 2176-2187.
133. Kinjo, Y., et al., *Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria.* Nat Immunol, 2011. **12**(10): p. 966-74.
134. Chang, Y.J., et al., *Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity.* J Clin Invest, 2011. **121**(1): p. 57-69.
135. Tangri, S., et al., *Presentation of peptide antigens by mouse CD1 requires endosomal localization and protein antigen processing.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14314-9.
136. Castaño, A.R., et al., *Peptide binding and presentation by mouse CD1.* Science, 1995. **269**(5221): p. 223-6.
137. Liu, Y., et al., *Endogenous collagen peptide activation of CD1d-restricted NKT cells ameliorates tissue-specific inflammation in mice.* J Clin Invest, 2011. **121**(1): p. 249-64.
138. Chang, Y.J., et al., *Influenza infection in suckling mice expands an NKT cell subset that protects against airway hyperreactivity.* Journal of Clinical Investigation, 2011. **121**(1): p. 57-69.
139. Fischer, K., et al., *Mycobacterial phosphatidylinositol mannoside is a natural antigen for old-restricted T cells.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(29): p. 10685-10690.
140. Wang, J., et al., *Lipid binding orientation within CD1d affects recognition of Borrelia burgorferi antigens by NKT cells.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1535-40.
141. Birkholz, A.M. and M. Kronenberg, *Antigen specificity of invariant natural killer T-cells.* Biomedical Journal, 2015. **38**(6): p. 470-483.
142. Tilly, K., P.A. Rosa, and P.E. Stewart, *Biology of infection with Borrelia burgdorferi.* Infectious disease clinics of North America, 2008. **22**(2): p. 217-v.
143. Brennan, P.J., et al., *Structural determination of lipid antigens captured at the CD1d–T-cell receptor interface.* Proceedings of the National Academy of Sciences, 2017. **114**(31): p. 8348-8353.
144. Zajonc, D.M. and E. Girardi, *Recognition of Microbial Glycolipids by Natural Killer T Cells.* Front Immunol, 2015. **6**: p. 400.
145. Kinjo, Y., et al., *Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria.* Nature Immunology, 2011. **12**(10): p. 966-974.
146. Ito, Y., et al., *Helicobacter pylori cholesteryl α-glucosides contribute to its pathogenicity and immune response by natural killer T cells.* PLoS One, 2013. **8**(12): p. e78191.
147. Matsuda, J.L., et al., *Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers.* Journal of Experimental Medicine, 2000. **192**(5): p. 741-753.
148. Wun, K.S., et al., *A molecular basis for the exquisite CD1d-restricted antigen specificity and functional responses of natural killer T cells.* Immunity, 2011. **34**(3): p. 327-39.
149. Benlagha, K., et al., *A thymic precursor to the NK T cell lineage.* Science, 2002. **296**(5567): p. 553-5.
150. Pellicci, D.G., et al., *A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage.* J Exp Med, 2002. **195**(7): p. 835-44.
151. Watarai, H., et al., *Development and function of invariant natural killer T cells producing T(h)2- and T(h)17-cytokines.* PLoS Biol, 2012. **10**(2): p. e1001255.
152. Doisne, J.M., et al., *Skin and peripheral lymph node invariant NKT cells are mainly retinoic acid receptor-related orphan receptor (gamma)t+ and respond preferentially under inflammatory conditions.* J Immunol, 2009. **183**(3): p. 2142-9.
153. Georgiev, H., et al., *Distinct gene expression patterns correlate with developmental and functional traits of iNKT subsets.* Nat Commun, 2016. **7**: p. 13116.

154. Krovi, S.H. and L. Gapin, *Invariant Natural Killer T Cell Subsets—More Than Just Developmental Intermediates.* Frontiers in Immunology, 2018. **9**(1393).
155. Hogquist, K. and H. Georgiev, *Recent advances in iNKT cell development.* F1000Research, 2020. **9**: p. F1000 Faculty Rev-127.
156. Shissler, S.C., M.S. Lee, and T.J. Webb, *Mixed Signals: Co-Stimulation in Invariant Natural Killer T Cell-Mediated Cancer Immunotherapy.* Frontiers in immunology, 2017. **8**: p. 1447-1447.
157. Chung, Y., et al., *A Critical Role of Costimulation during Intrathymic Development of Invariant NK T Cells.* The Journal of Immunology, 2008. **180**(4): p. 2276-2283.
158. Seiler, M.P., et al., *Elevated and sustained expression of the transcription factors Egr1 and Egr2 controls NKT lineage differentiation in response to TCR signaling.* Nature Immunology, 2012. **13**(3): p. 264-271.
159. Wang, H. and K.A. Hogquist, *How Lipid-Specific T Cells Become Effectors: The Differentiation of iNKT Subsets.* Frontiers in Immunology, 2018. **9**(1450).
160. Michel, M.L., et al., *Critical role of ROR-γt in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation.* Proc Natl Acad Sci U S A, 2008. **105**(50): p. 19845-50.
161. Lee, Y.J., et al., *Steady-state production of IL-4 modulates immunity in mouse strains and is determined by lineage diversity of iNKT cells.* Nature Immunology, 2013. **14**(11): p. 1146-1154.
162. Engel, I., et al., *Innate-like functions of natural killer T cell subsets result from highly divergent gene programs.* Nat Immunol, 2016. **17**(6): p. 728-39.
163. Lee, Y.J., et al., *Lineage-Specific Effector Signatures of Invariant NKT Cells Are Shared amongst γδ T, Innate Lymphoid, and Th Cells.* J Immunol, 2016. **197**(4): p. 1460-70.
164. Crowe, N.Y., et al., *Differential antitumor immunity mediated by NKT cell subsets in vivo.* J Exp Med, 2005. **202**(9): p. 1279-88.
165. Dashtsoodol, N., et al., *Alternative pathway for the development of V(α)14(+) NKT cells directly from CD4(-)CD8(-) thymocytes that bypasses the CD4(+)CD8(+) stage.* Nat Immunol, 2017. **18**(3): p. 274-282.
166. Kumar, A., et al., *Natural Killer T Cells: An Ecological Evolutionary Developmental Biology Perspective.* Frontiers in Immunology, 2017. **8**(1858).
167. Terashima , A., et al., *A novel subset of mouse NKT cells bearing the IL-17 receptor B responds to IL-25 and contributes to airway hyperreactivity.* Journal of Experimental Medicine, 2008. **205**(12): p. 2727-2733.
168. Lynch, L., et al., *Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of Treg cells and macrophages in adipose tissue.* Nature Immunology, 2015. **16**(1): p. 85-95.
169. Sag, D., et al., *IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset.* J Clin Invest, 2014. **124**(9): p. 3725-40.
170. Tuttle, K.D., et al., *TCR signal strength controls thymic differentiation of iNKT cell subsets.* Nature Communications, 2018. **9**(1): p. 2650.
171. Zhao, M., et al., *Altered thymic differentiation and modulation of arthritis by invariant NKT cells expressing mutant ZAP70.* Nature Communications, 2018. **9**(1): p. 2627.
172. De Santo, C., et al., *Invariant NKT cells reduce the immunosuppressive activity of influenza A virus-induced myeloid-derived suppressor cells in mice and humans.* Journal of Clinical Investigation, 2008. **118**(12): p. 4036-4048.
173. Barral, P., et al., *B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(24): p. 8345-8350.
174. Zhou, D.P., et al., *Lysosomal glycosphingolipid recognition by NKT cells.* Science, 2004. **306**(5702): p. 1786-1789.

175. Brennan, P.J., et al., *Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals.* Nat Immunol, 2011. **12**(12): p. 1202-11.
176. Paget, C., et al., *TLR9-mediated dendritic cell activation uncovers mammalian ganglioside species with specific ceramide backbones that activate invariant natural killer T cells.* PLoS biology, 2019. **17**(3): p. e3000169-e3000169.
177. O'Keeffe, J., M. Podbielska, and E.L. Hogan, *Invariant natural killer T cells and their ligands: focus on multiple sclerosis.* Immunology, 2015. **145**(4): p. 468-475.
178. Brennan, P.J., M. Brigl, and M.B. Brenner, *Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions.* Nature Reviews Immunology, 2013. **13**(2): p. 101-117.
179. Long, J., et al., *Improvement of HBsAg gene-modified dendritic cell-based vaccine efficacy by optimizing immunization method or the application of β-glucosylceramide.* Immunol Invest, 2013. **42**(2): p. 137-55.
180. López-Sagaseta, J., et al., *The molecular basis for recognition of CD1d/α-galactosylceramide by a human non-Vα24 T cell receptor.* PLoS biology, 2012. **10**(10): p. e1001412-e1001412.
181. El Haj, M., et al., *Potential role of NKT regulatory cell ligands for the treatment of immune mediated colitis.* World journal of gastroenterology, 2007. **13**(44): p. 5799-5804.
182. Fox, L.M., et al., *Recognition of lyso-phospholipids by human natural killer T lymphocytes.* PLoS biology, 2009. **7**(10): p. e1000228-e1000228.
183. Brennan, P.J., et al., *Activation of iNKT cells by a distinct constituent of the endogenous glucosylceramide fraction.* Proceedings of the National Academy of Sciences, 2014. **111**(37): p. 13433-13438.
184. Kougias, P., et al., *Lysophosphatidylcholine and secretory phospholipase A2 in vascular disease: mediators of endothelial dysfunction and atherosclerosis.* Med Sci Monit, 2006. **12**(1): p. Ra5-16.
185. Wu , D.Y., et al., *Cross-presentation of Disialoganglioside GD3 to Natural Killer T Cells.* Journal of Experimental Medicine, 2003. **198**(1): p. 173-181.
186. Gumperz, J.E., et al., *Murine CD1d-Restricted T Cell Recognition of Cellular Lipids.* Immunity, 2000. **12**(2): p. 211-221.
187. Brutkiewicz, R.R., *CD1d Ligands: The Good, the Bad, and the Ugly.* The Journal of Immunology, 2006. **177**(2): p. 769-775.
188. Mallevaey, T. and T. Selvanantham, *Strategy of lipid recognition by invariant natural killer T cells: 'one for all and all for one'.* Immunology, 2012. **136**(3): p. 273-282.
189. Sullivan, B.A., et al., *Mechanisms for Glycolipid Antigen-Driven Cytokine Polarization by Vα14i NKT Cells.* The Journal of Immunology, 2010. **184**(1): p. 141-153.
190. Venkataswamy, M.M. and S.A. Porcelli, *Lipid and glycolipid antigens of CD1d-restricted natural killer T cells.* Semin Immunol, 2010. **22**(2): p. 68-78.
191. Chang, Y.J., et al., *Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids.* Proc Natl Acad Sci U S A, 2007. **104**(25): p. 10299-304.
192. Li, X., et al., *Design of a potent CD1d-binding NKT cell ligand as a vaccine adjuvant.* Proc Natl Acad Sci U S A, 2010. **107**(29): p. 13010-5.
193. Schmieg, J., et al., *Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide.* J Exp Med, 2003. **198**(11): p. 1631-41.
194. Hammond, K.J.L., et al., *NKT cells are phenotypically and functionally diverse.* European Journal of Immunology, 1999. **29**(11): p. 3768-3781.
195. Kim, C.H., B. Johnston, and E.C. Butcher, *Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity.* Blood, 2002. **100**(1): p. 11-16.

196. Germanov, E., et al., *Critical role for the chemokine receptor CXCR6 in homeostasis and activation of CD1d-restricted NKT cells.* Journal of Immunology, 2008. **181**(1): p. 81-91.
197. Lee, W.Y., et al., *An intravascular immune response to Borrelia burgdorferi involves Kupffer cells and iNKT cells.* Nature Immunology, 2010. **11**(4): p. 295-U36.
198. Matsuda, J.L., et al., *CD1d-restricted iNKT cells, the 'Swiss-Army knife' of the immune system.* Current Opinion in Immunology, 2008. **20**(3): p. 358-368.
199. Kok, W.L., et al., *Pivotal Advance: Invariant NKT cells reduce accumulation of inflammatory monocytes in the lungs and decrease immune-pathology during severe influenza A virus infection.* Journal of Leukocyte Biology, 2012. **91**(3): p. 357-368.
200. Moreno, M., et al., *Differential indirect activation of human invariant natural killer T cells by Toll-like receptor agonists.* Immunotherapy, 2009. **1**(4): p. 557-70.
201. Barral, P., et al., *The location of splenic NKT cells favours their rapid activation by blood-borne antigen.* The EMBO journal, 2012. **31**(10): p. 2378-2390.
202. Carnaud, C., et al., *Cutting Edge: Cross-Talk Between Cells of the Innate Immune System: NKT Cells Rapidly Activate NK Cells.* The Journal of Immunology, 1999. **163**(9): p. 4647-4650.
203. Paget, C., et al., *Activation of Invariant NKT Cells by Toll-like Receptor 9-Stimulated Dendritic Cells Requires Type I Interferon and Charged Glycosphingolipids.* Immunity, 2007. **27**(4): p. 597-609.
204. Brigl, M., et al., *Innate and cytokine-driven signals, rather than microbial antigens, dominate in natural killer T cell activation during microbial infection.* The Journal of experimental medicine, 2011. **208**(6): p. 1163-1177.
205. Fujii, S., et al., *The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation.* J Exp Med, 2004. **199**(12): p. 1607-18.
206. Doherty, D.G., et al., *Activation and Regulation of B Cell Responses by Invariant Natural Killer T Cells.* Frontiers in immunology, 2018. **9**: p. 1360-1360.
207. Leadbetter, E.A., et al., *NK T cells provide lipid antigen-specific cognate help for B cells.* Proceedings of the National Academy of Sciences, 2008. **105**(24): p. 8339-8344.
208. Morita, M., et al., *Structure-Activity Relationship of .alpha.-Galactosylceramides against B16-Bearing Mice.* Journal of Medicinal Chemistry, 1995. **38**(12): p. 2176-2187.
209. Shissler, S.C., et al., *Immunotherapeutic strategies targeting natural killer T cell responses in cancer.* Immunogenetics, 2016. **68**(8): p. 623-638.
210. Crowe, N.Y., M.J. Smyth, and D.I. Godfrey, *A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas.* The Journal of experimental medicine, 2002. **196**(1): p. 119-127.
211. Kammertoens, T., et al., *B-cells and IL-4 promote methylcholanthrene-induced carcinogenesis but there is no evidence for a role of T/NKT-cells and their effector molecules (Fas-ligand, TNF-α, perforin).* International Journal of Cancer, 2012. **131**(7): p. 1499-1508.
212. Swann, J.B., et al., *Type I natural killer T cells suppress tumors caused by p53 loss in mice.* Blood, 2009. **113**(25): p. 6382-5.
213. Bellone, M., et al., *iNKT cells control mouse spontaneous carcinoma independently of tumor-specific cytotoxic T cells.* PLoS One, 2010. **5**(1): p. e8646.
214. De Santo, C., et al., *Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A.* Nature Immunology, 2010. **11**(11): p. 1039-1046.
215. Nair, S. and M.V. Dhodapkar, *Natural Killer T Cells in Cancer Immunotherapy.* Frontiers in Immunology, 2017. **8**(1178).
216. Wolf, B.J., J.E. Choi, and M.A. Exley, *Novel Approaches to Exploiting Invariant NKT Cells in Cancer Immunotherapy.* Frontiers in immunology, 2018. **9**: p. 384-384.

217. Molling, J.W., et al., *Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma.* J Clin Oncol, 2007. **25**(7): p. 862-8.
218. McEwen-Smith, R.M., M. Salio, and V. Cerundolo, *The regulatory role of invariant NKT cells in tumor immunity.* Cancer immunology research, 2015. **3**(5): p. 425-435.
219. Dhodapkar, M.V., et al., *A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma.* The Journal of experimental medicine, 2003. **197**(12): p. 1667-1676.
220. Gorini, F., et al., *Invariant NKT cells contribute to chronic lymphocytic leukemia surveillance and prognosis.* Blood, 2017. **129**(26): p. 3440-3451.
221. Renukaradhya, G.J., et al., *Type I NKT cells protect (and type II NKT cells suppress) the host's innate antitumor immune response to a B-cell lymphoma.* Blood, 2008. **111**(12): p. 5637-45.
222. Renukaradhya, G.J., et al., *Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo.* International Journal of Cancer, 2006. **118**(12): p. 3045-3053.
223. Li, J., et al., *NKT Cell Responses to B Cell Lymphoma.* Medical Sciences, 2014. **2**(2): p. 82-97.
224. Lam, P.Y., M.D. Nissen, and S.R. Mattarollo, *Invariant Natural Killer T Cells in Immune Regulation of Blood Cancers: Harnessing Their Potential in Immunotherapies.* Frontiers in Immunology, 2017. **8**(1355).
225. Chan, A.C., et al., *Natural killer T cell defects in multiple myeloma and the impact of lenalidomide therapy.* Clinical & Experimental Immunology, 2014. **175**(1): p. 49-58.
226. Hayakawa, Y., et al., *IFN-γ–mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, α-galactosylceramide.* Blood, 2002. **100**(5): p. 1728-1733.
227. Díaz-Basabe, A., F. Strati, and F. Facciotti, *License to Kill: When iNKT Cells Are Granted the Use of Lethal Cytotoxicity.* International journal of molecular sciences, 2020. **21**(11): p. 3909.
228. de Lalla, C., et al., *Invariant NKT Cell Reconstitution in Pediatric Leukemia Patients Given HLA-Haploidentical Stem Cell Transplantation Defines Distinct CD4⁺ and CD4[−] Subset Dynamics and Correlates with Remission State.* The Journal of Immunology, 2011. **186**(7): p. 4490-4499.
229. Najera Chuc, A.E., et al., *Low number of invariant NKT cells is associated with poor survival in acute myeloid leukemia.* J Cancer Res Clin Oncol, 2012. **138**(8): p. 1427-32.
230. Bedard, M., M. Salio, and V. Cerundolo, *Harnessing the Power of Invariant Natural Killer T Cells in Cancer Immunotherapy.* Frontiers in Immunology, 2017. **8**(1829).
231. Bondhopadhyay, B., et al., *Cancer immunotherapy: a promising dawn in cancer research.* American journal of blood research, 2020. **10**(6): p. 375-385.
232. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-867.
233. Carey, A., et al., *Identification of Interleukin-1 by Functional Screening as a Key Mediator of Cellular Expansion and Disease Progression in Acute Myeloid Leukemia.* Cell Rep, 2017. **18**(13): p. 3204-3218.
234. Fourie, C., et al., *Serum amyloid A and inflammasome activation: A link to breast cancer progression?* Cytokine & Growth Factor Reviews, 2021. **59**: p. 62-70.
235. Craddock, C.F., et al., *Outcome of Azacitidine Therapy in Acute Myeloid Leukemia Is not Improved by Concurrent Vorinostat Therapy but Is Predicted by a Diagnostic Molecular Signature.* Clinical Cancer Research, 2017. **23**(21): p. 6430-6440.
236. Vettore, L., R.L. Westbrook, and D.A. Tennant, *New aspects of amino acid metabolism in cancer.* British Journal of Cancer, 2020. **122**(2): p. 150-156.
237. Fultang, L., et al., *Molecular basis and current strategies of therapeutic arginine depletion for cancer.* Int J Cancer, 2016. **139**(3): p. 501-9.

238. Jenkinson, C.P., W.W. Grody, and S.D. Cederbaum, *Comparative properties of arginases.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 1996. **114**(1): p. 107-132.
239. Mussai, F., et al., *Targeting the arginine metabolic brake enhances immunotherapy for leukaemia.* International Journal of Cancer, 2019. **145**(8): p. 2201-2208.
240. Turzanski, J., et al., *Interleukin-1beta maintains an apoptosis-resistant phenotype in the blast cells of acute myeloid leukaemia via multiple pathways.* Leukemia, 2004. **18**(10): p. 1662-70.
241. Munder, M., et al., *Suppression of T-cell functions by human granulocyte arginase.* Blood, 2006. **108**(5): p. 1627-34.
242. Grzywa, T.M., et al., *Myeloid Cell-Derived Arginase in Cancer Immune Response.* Frontiers in Immunology, 2020. **11**(938).
243. Norian, L.A., et al., *Tumor-Infiltrating Regulatory Dendritic Cells Inhibit CD8⁺ T Cell Function via l-Arginine Metabolism.* Cancer Research, 2009. **69**(7): p. 3086-3094.
244. Tachibana, T., et al., *Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas.* Clin Cancer Res, 2005. **11**(20): p. 7322-7.
245. Shaulov, A., et al., *Peripheral blood progenitor cell product contains Th1-biased noninvariant CD1d-reactive natural killer T cells: implications for posttransplant survival.* Experimental hematology, 2008. **36**(4): p. 464-472.
246. Metelitsa, L.S., et al., *Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2.* The Journal of experimental medicine, 2004. **199**(9): p. 1213-1221.
247. Muhammad Ali Tahir, S., et al., *Loss of IFN-γ Production by Invariant NK T Cells in Advanced Cancer.* The Journal of Immunology, 2001. **167**(7): p. 4046-4050.
248. Chen, Q. and A.C. Ross, *All-trans-retinoic acid and CD38 ligation differentially regulate CD1d expression and α-galactosylceramide-induced immune responses.* Immunobiology, 2015. **220**(1): p. 32-41.
249. Chang, C.H., et al., *Posttranscriptional control of T cell effector function by aerobic glycolysis.* Cell, 2013. **153**(6): p. 1239-51.
250. Krijgsman, D., M. Hokland, and P.J.K. Kuppen, *The Role of Natural Killer T Cells in Cancer—A Phenotypical and Functional Approach.* Frontiers in Immunology, 2018. **9**(367).
251. Chan, W.K., et al., *Multiplex and Genome-Wide Analyses Reveal Distinctive Properties of KIR⁺ and CD56⁺ T Cells in Human Blood.* The Journal of Immunology, 2013. **191**(4): p. 1625-1636.
252. Montoya, C.J., et al., *Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11.* Immunology, 2007. **122**(1): p. 1-14.
253. Sandberg, J.K., N. Bhardwaj, and D.F. Nixon, *Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Vα24 NKT cell compartment.* European Journal of Immunology, 2003. **33**(3): p. 588-596.
254. Kitayama, S., et al., *Cellular Adjuvant Properties, Direct Cytotoxicity of Re-differentiated Vα24 Invariant NKT-like Cells from Human Induced Pluripotent Stem Cells.* Stem cell reports, 2016. **6**(2): p. 213-227.
255. Liu, D., et al., *Medulloblastoma expresses CD1d and can be targeted for immunotherapy with NKT cells.* Clinical Immunology, 2013. **149**(1): p. 55-64.
256. Metelitsa, L.S., et al., *Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells.* Leukemia, 2003. **17**(6): p. 1068-1077.
257. Nicol, A., et al., *Human invariant Vα24+ natural killer T cells activated by α-galactosylceramide (KRN7000) have cytotoxic anti-tumour activity through mechanisms distinct from T cells and natural killer cells.* Immunology, 2000. **99**(2): p. 229-234.

258. Bassiri, H., et al., *iNKT Cell Cytotoxic Responses Control T-Lymphoma Growth In Vitro and In Vivo.* Cancer Immunology Research, 2014. **2**(1): p. 59-69.
259. Boivin, W.A., et al., *Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma.* Lab Invest, 2009. **89**(11): p. 1195-220.
260. Anthony, D.A., et al., *Functional dissection of the granzyme family: cell death and inflammation.* Immunol Rev, 2010. **235**(1): p. 73-92.
261. Ghnewa, Y.G., et al., *Retinoic acid induction of CD1d expression primes chronic lymphocytic leukemia B cells for killing by CD8+ invariant natural killer T cells.* Clinical Immunology, 2017. **183**: p. 91-98.
262. Scalise, M., et al., *The Human SLC7A5 (LAT1): The Intriguing Histidine/Large Neutral Amino Acid Transporter and Its Relevance to Human Health.* Frontiers in Chemistry, 2018. **6**(243).
263. Oda, K., et al., *L-type amino acid transporter 1 inhibitors inhibit tumor cell growth.* Cancer Sci, 2010. **101**(1): p. 173-9.
264. Werner, A., et al., *Reconstitution of T Cell Proliferation under Arginine Limitation: Activated Human T Cells Take Up Citrulline via L-Type Amino Acid Transporter 1 and Use It to Regenerate Arginine after Induction of Argininosuccinate Synthase Expression.* Frontiers in immunology, 2017. **8**: p. 864-864.
265. Kohlmeier, M., *Chapter 8 - Amino Acids and Nitrogen Compounds*, in *Nutrient Metabolism (Second Edition)*, M. Kohlmeier, Editor. 2015, Academic Press: San Diego. p. 265-477.
266. Morris, S.M., Jr, *Enzymes of Arginine Metabolism.* The Journal of Nutrition, 2004. **134**(10): p. 2743S-2747S.
267. Crump, N.T., et al., *Chromatin accessibility governs the differential response of cancer and T cells to arginine starvation.* Cell Reports, 2021. **35**(6): p. 109101.
268. Ohno, T., et al., *Elevated gene expression of argininosuccinate synthetase in peripheral lymphocytes from systemic lupus erythematosus (SLE) patients.* Autoimmunity, 1992. **13**(2): p. 127-32.
269. Fletcher, M., et al., *l-Arginine depletion blunts antitumor T-cell responses by inducing myeloid-derived suppressor cells.* Cancer Res, 2015. **75**(2): p. 275-83.
270. Tarasenko, T.N., J. Gomez-Rodriguez, and P.J. McGuire, *Impaired T cell function in argininosuccinate synthetase deficiency.* Journal of leukocyte biology, 2015. **97**(2): p. 273-278.
271. De Santo, C., et al., *Invariant NKT cells reduce the immunosuppressive activity of influenza A virus–induced myeloid-derived suppressor cells in mice and humans.* The Journal of Clinical Investigation, 2008. **118**(12): p. 4036-4048.
272. Kruger, S., et al., *Advances in cancer immunotherapy 2019 – latest trends.* Journal of Experimental & Clinical Cancer Research, 2019. **38**(1): p. 268.
273. Zhu, Y., et al., *Nanomedicines modulating tumor immunosuppressive cells to enhance cancer immunotherapy.* Acta pharmaceutica Sinica. B, 2020. **10**(11): p. 2054-2074.
274. Pan, M., et al., *Regional glutamine deficiency in tumours promotes dedifferentiation through inhibition of histone demethylation.* Nature Cell Biology, 2016. **18**(10): p. 1090-1101.
275. Bron, L., et al., *Prognostic value of arginase-II expression and regulatory T-cell infiltration in head and neck squamous cell carcinoma.* International Journal of Cancer, 2013. **132**(3): p. E85-E93.
276. Czystowska-Kuzmicz, M., et al., *Small extracellular vesicles containing arginase-1 suppress T-cell responses and promote tumor growth in ovarian carcinoma.* Nature Communications, 2019. **10**(1): p. 3000.
277. Ma, Z., et al., *Overexpression of Arginase-1 is an indicator of poor prognosis in patients with colorectal cancer.* Pathology - Research and Practice, 2019. **215**(6): p. 152383.

278. Mieke De, B., et al., *Structure and Expression of Different Serum Amyloid A (SAA) Variants and their Concentration-Dependent Functions During Host Insults.* Current Medicinal Chemistry, 2016. **23**(17): p. 1725-1755.
279. Malle, E., S. Sodin-Semrl, and A. Kovacevic, *Serum amyloid A: an acute-phase protein involved in tumour pathogenesis.* Cellular and molecular life sciences : CMLS, 2009. **66**(1): p. 9-26.
280. Weinstein, P.S., et al., *Acute-phase proteins or tumour markers: the role of SAA, SAP, CRP and CEA as indicators of metastasis in a broad spectrum of neoplastic diseases.* Scand J Immunol, 1984. **19**(3): p. 193-8.
281. Urwanisch, L., M. Luciano, and J. Horejs-Hoeck, *The NLRP3 Inflammasome and Its Role in the Pathogenicity of Leukemia.* International journal of molecular sciences, 2021. **22**(3): p. 1271.
282. Azizi, G., et al., *The Newly Identified T Helper 22 Cells Lodge in Leukemia.* International journal of hematology-oncology and stem cell research, 2015. **9**(3): p. 143-154.
283. Preciado-Patt, L., et al., *Serum amyloid A complexed with extracellular matrix induces the secretion of tumor necrosis factor-α by human T-lymphocytes.* Letters in Peptide Science, 1998. **5**(5): p. 349-355.
284. Furlaneto, C.J. and A. Campa, *A novel function of serum amyloid A: a potent stimulus for the release of tumor necrosis factor-alpha, interleukin-1beta, and interleukin-8 by human blood neutrophil.* Biochem Biophys Res Commun, 2000. **268**(2): p. 405-8.
285. Bruserud, Ø., *Effects of endogenous interleukin 1 on blast cells derived from acute myelogenous leukemia patients.* Leukemia Research, 1996. **20**(1): p. 65-73.
286. Irving, B.A. and A. Weiss, *The cytoplasmic domain of the T cell receptor zeta chain is sufficient to couple to receptor-associated signal transduction pathways.* Cell, 1991. **64**(5): p. 891-901.
287. Bronte, V. and P. Zanovello, *Regulation of immune responses by L-arginine metabolism.* Nature Reviews Immunology, 2005. **5**(8): p. 641-654.
288. Metelitsa, L.S., et al., *Human NKT Cells Mediate Antitumor Cytotoxicity Directly by Recognizing Target Cell CD1d with Bound Ligand or Indirectly by Producing IL-2 to Activate NK Cells.* The Journal of Immunology, 2001. **167**(6): p. 3114-3122.
289. Nieda, M., et al., *TRAIL expression by activated human CD4+Vα24NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells.* Blood, 2001. **97**(7): p. 2067-2074.
290. Hix, L.M., et al., *CD1d-Expressing Breast Cancer Cells Modulate NKT Cell-Mediated Antitumor Immunity in a Murine Model of Breast Cancer Metastasis.* PLOS ONE, 2011. **6**(6): p. e20702.
291. Dhodapkar, K.M., et al., *Invariant natural killer T cells are preserved in patients with glioma and exhibit antitumor lytic activity following dendritic cell-mediated expansion.* International Journal of Cancer, 2004. **109**(6): p. 893-899.
292. Chong, T.W., et al., *CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival.* J Clin Pathol, 2015. **68**(3): p. 200-5.
293. Terabe, M., et al., *A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumor immunosurveillance.* J Exp Med, 2005. **202**(12): p. 1627-33.
294. Tsuchida, T., et al., *Gangliosides of human melanoma.* Cancer, 1989. **63**(6): p. 1166-74.
295. Mujoo, K., et al., *Disialoganglioside GD2 on human neuroblastoma cells: target antigen for monoclonal antibody-mediated cytolysis and suppression of tumor growth.* Cancer Res, 1987. **47**(4): p. 1098-104.
296. Portoukalian, J., G. Zwingelstein, and J.F. Doré, *Lipid composition of human malignant melanoma tumors at various levels of malignant growth.* Eur J Biochem, 1979. **94**(1): p. 19-23.
297. Chang, F., R. Li, and S. Ladisch, *Shedding of gangliosides by human medulloblastoma cells.* Exp Cell Res, 1997. **234**(2): p. 341-6.

298. Chapman, P.B., et al., *Sequential Immunization of Melanoma Patients with GD3 Ganglioside Vaccine and Anti-Idiotypic Monoclonal Antibody That Mimics GD3 Ganglioside.* Clinical Cancer Research, 2004. **10**(14): p. 4717-4723.
299. Crowe, N.Y., et al., *Glycolipid Antigen Drives Rapid Expansion and Sustained Cytokine Production by NK T Cells.* The Journal of Immunology, 2003. **171**(8): p. 4020-4027.
300. Harding, H.P., et al., *An integrated stress response regulates amino acid metabolism and resistance to oxidative stress.* Mol Cell, 2003. **11**(3): p. 619-33.
301. Ye, J., et al., *The GCN2-ATF4 pathway is critical for tumour cell survival and proliferation in response to nutrient deprivation.* The EMBO Journal, 2010. **29**(12): p. 2082-2096.
302. Sugimura, K., et al., *Elevated argininosuccinate synthetase activity in adult T leukemia cell lines.* Leuk Res, 1990. **14**(10): p. 931-4.
303. Fultang, L., et al., *Metabolic engineering against the arginine microenvironment enhances CAR-T cell proliferation and therapeutic activity.* Blood, 2020. **136**(10): p. 1155-1160.
304. Zhang, Y., et al., *α-GalCer and iNKT Cell-Based Cancer Immunotherapy: Realizing the Therapeutic Potentials.* Frontiers in immunology, 2019. **10**: p. 1126-1126.
305. Giaccone, G., et al., *A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors.* Clin Cancer Res, 2002. **8**(12): p. 3702-9.
306. Waldowska, M., A. Bojarska-Junak, and J. Roliński, *A brief review of clinical trials involving manipulation of invariant NKT cells as a promising approach in future cancer therapies.* Central-European journal of immunology, 2017. **42**(2): p. 181-195.
307. Davide, B., et al., *Adoptive immunotherapy mediated by ex vivo expanded natural killer T cells against CD1d-expressing lymphoid neoplasms.* Haematologica, 2009. **94**(7): p. 967-974.
308. Heczey, A., et al., *Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy.* Blood, 2014. **124**(18): p. 2824-33.
309. Tian, G., et al., *CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo.* J Clin Invest, 2016. **126**(6): p. 2341-55.
310. Heczey, A., et al., *Anti-GD2 CAR-NKT cells in patients with relapsed or refractory neuroblastoma: an interim analysis.* Nature Medicine, 2020. **26**(11): p. 1686-1690.
311. Rotolo, A., et al., *Enhanced Anti-lymphoma Activity of CAR19-iNKT Cells Underpinned by Dual CD19 and CD1d Targeting.* Cancer Cell, 2018. **34**(4): p. 596-610.e11.