2B4 IS A CHECKPOINT MOLECULE FOR iNKT CELL ANTI-TUMOR RESPONSE

By

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ABSTRACT

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Invariant natural killer T (iNKT) cells are robust cytotoxic effectors and immune modulators, which makes them ideal candidates for cancer immunotherapy. However, the use of iNKTs for cellular therapy against cancer has been limited due to their transient response in pre-clinical trials. Although TCR-CD1d interactions are generally required for iNKT cell cytotoxicity, the receptors and signaling mechanisms that co-operate with the TCR to promote maximal anti-tumor responses are poorly understood. Therefore, elucidating the mechanisms that regulate anti-tumor responses is critical for the development of effective iNKT-based therapies. Our efforts have shown that 2B4, a SLAM receptor, when expressed on iNKTs reduces their cytotoxic response against lymphoma cells. Surprisingly, 2B4 is not expressed on resting iNKTs but gets rapidly upregulated via stimulation through the TCR. 2B4 has two isoforms, which are splice variants of each other, of which the inhibitory long form is predominantly expressed in activated iNKTs. Our data show that 2B4 is a checkpoint molecule and has an inhibitory role in iNKT cell cytotoxicity. Indeed, when we overexpressed 2B4 in an iNKT cell hybridoma, the killing capacity of the iNKT cell line was abrogated. Moreover, 2B4 can be converted to a potent activating receptor by swapping its intracellular domains with proline motifs, which drastically augments tumor cell lysis. Taken together, this study highlights the important role of 2B4 in iNKT cell cytolysis and broadens the knowledge of immunoregulatory receptors in iNKT cells for future applications in cancer therapy.

I dedicate this thesis to all who suffer from cancer, whether human or animal.

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Guru Brahma Gurur Vishnu, Guru Devo Maheshwaraha

Guru Saakshat Para Brahma, Tasmai Sree Gurave Namaha

(English translation- The Guru is the representation of the Lord that creates and sustains knowledge as well as destroys the weeds of ignorance. I salute my Guru!)

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KEY TO ABBREVIATIONS

αGalCer	α-galactosylceramide
β-ManCer	β-mannosylceramide
γδT	Gamma-delta T cells
Ag	Antigen
ANOVA	Analysis of variance
APC	Antigen presenting cell
Bcl-xL	B-cell lymphoma-extra large
Bcl-2	B-cell lymphoma 2
BLI	Bioluminescent imaging
BTLA-4	B- and T- lymphocyte attenuator
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DN	Double negative (CD4- CD8-)
DP	Double positive (CD4+ CD8+)
EAE	Experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
ECD	Extracellular domain
EL4-Luc	Luciferase expressing EL4 cells
E:T	Effector: Target ratio
FACS	Fluorescence activated cell sorting

GATA-3	GATA binding factor-3
GPI	Glycophosphatidylinositol
GVHD	Graft-versus-host disease
h2B4	Human 2B4
Her2	Human epidermal receptor growth factor 2
IFN	Interferon
Ig	Immunoglobulin
iGb3	Isoglobotrihexosylcermide
IL	Interleukin
iNKT	Invariant Natural Killer T cell
iTCR	Invariant T cell receptor
ITSM	Immunoreceptor tyrosine-based switch motifs
Jα	Joining α chain
LAK	Lymphokine-activated killer
LAG-3	Lymphocyte-activation gene 3
LAT	Linker for activation of T cells
MHC	Major histocompatibility complex
NK	Natural killer
NKG	Natural killer cell receptor
NKT	Natural Killer T cell
NOD	Non obese diabetic
OCH	(2S,3S,4R)-1-O-(alpha-D-Galactopyranosyl)-N-tetracosanoyl-2-amino-1,3,4-nonanetriol)
OVA	Ovalbumin
p53	Tumor protein p53

PBMC	Peripheral blood mononuclear cells
PD-1	Programmed cell death protein-1
PD-L1	Programmed death ligand-1
PLZF	Promyelocytic leukemia zinc finger
qPCR	Quantitative real-time polymerase chain reaction
RORγt	Retinoic acid receptor related orphan nuclear receptor gamma
SAP	SLAM-associated protein
scFv	Single chain variable fragment
SD	Standard Deviation
SEM	Standard error of mean
SFR	SLAM family receptor
SH2	Src homology 2
SHP	Src homology 2-containing protein tyrosine phosphatase
SHIP	Src homology 2-containing inositol 5-phosphatase
SLAM	Signalling lymphocyte activation molecule
SLE	Systemic Lupus Erythematosus
T1D	Type-1 Diabetes
TAM	Tumor associated macrophages
T-bet	T-box expressed in T cells
TCR	T cell receptor
Tet	Tetramer
Tg	Transgenic
TGF	Transforming growth factor
TIGIT	T cell immunoreceptor with immunoglobulin and ITIM domains
Tim-3	T cell immunoglobulin and mucin domain-containing protein 3

TME	Tumor microenvironment
Tregs	Regulatory T cells
Vα, Vβ	Variable chain α, variable chain β
vTCR	Variable T cell receptor
XLP	X-linked lymphoproliferative disease

CHAPTER 1- INTRODUCTION

The balance between health and disease in the human body is precariously maintained by multiple organ systems working together. In a disease like cancer, this balance is disrupted on multiple levels, including in the immune system which is primarily tasked with the destruction and clearing of cells that are harmful to the organism. Cancer persists as the leading cause of death world-wide[1]. Blood cancers account for around 6.2% of new cases diagnosed annually, all over the world[2, 3]. Chemotherapy has long been used as the first line of treatment for various leukemias and lymphomas and has shown to be effective for numerous patients[4, 5]. Nonetheless, chemotherapy and radiation do not always ensure complete remission in many patients[6, 7, 8]. Hence, the need for newer, more efficient therapies against cancers arose and was answered, in part, by the development of immunotherapies that harness the immune system to destroy cancer cells[9, 10]. While modern immunotherapies focus mostly on T and NK cells as anti-cancer agents, a less well studied subset of T cells, Natural Killer T (NKT) cells, are promising candidates for cancer immunotherapy.

Natural Killer T cells

Natural killer T cells were first discovered in the late 1980s and early 1990s when multiple independent groups found a population of cells expressing both the $\alpha\beta$ T cell receptor and the NK cell marker NK1.1, i.e. $\alpha\beta$TCR+ NK1.1+ cells[11, 12, 13, 14, 15]. These unique T cells have intermediate levels of TCR expression and a propensity to express the Vβ8.2 chain[16]. Further studies revealed that this population of cells produces IFNγ and IL-4 upon stimulation of their TCR[16, 17, 18]. NKT cells were also considered unique since they recognize antigen through the MHC Class I like molecule CD1d[19]. NKT cells

are predominantly present in the thymus, spleen, and liver and to a lesser extent in the lymph nodes of mice[20]. Natural Killer T cells are a heterogeneous group and were further divided into Type I- invariant Natural Killer T cells (iNKT) and Type II NKT cells (Figure 1).

Invariant Natural Killer T (iNKT) cells

Invariant NKTs get their name from their specific αβ T cell receptor that contains an invariant α chain, in which a single Vα sequence is fused to a single Jα sequence, and which is paired with a β chain containing only a small subset of Vβ segments[21]. Thus, in mice, Vα14 pairs with Jα18 while in humans Vα24 pairs with Jα18[21]. Vβ pairing in mice is limited to Vβ8.2, Vβ7 and Vβ2 and in humans to Vβ11[20, 22]. This specific α,β pairing allows iNKT cells to recognize glycolipid antigens presented by a non-polymorphic MHC class I like molecule, CD1d. The most potent glycolipid antigen recognized by iNKT cells is α-galactosylceramide (αGalCer, αGC), a glycosphingolipid isolated from the marine algae *Agelas mauritianus*[23]. During the early days of research into murine iNKT cells, it was found that they exist as either CD4+ or CD4-CD8- (DN) cells[11, 20, 24]. One of the key characteristics of iNKT cells is their ability to rapidly produce copious amounts of cytokines like IL-4 and IFNγ within 2 hours of activation[18, 25, 26]. As a result of the cytokine secretion, iNKTs can activate other killer cells like NK[27, 28] and CD8+ T cells[29]. Additionally, iNKTs can also activate and cause maturation of dendritic cells (DCs) along with upregulation of CD80 and CD86, which acts as costimulatory molecules for other immune cells[26, 30]. Upon activation, DCs further produce IL-12[26] and present antigen through MHC-I and MHC-II, which contributes to the cross-priming of other immune cells[30, 31, 32]. After its potent

transactivation of other immune cells, iNKTs rapidly downregulate their TCR and 3-4 days post-activation undergo apoptosis[33, 34, 35]. Invariant NKT numbers in the body are back to normal by 6-9 days post-activation[34].

In humans, iNKT numbers are variable based on age, genetics, gender, and health conditions but in general, they make up 0.01-0.1% of the lymphocytes in the blood. Females have a slightly increased frequency of iNKT cells as compared to males[36]. Apart from their αGC reactivity, human iNKT cells can also be identified based on their Vα24 Vβ11 chains and are CD56+ and CD161+[24]. Human iNKTs were found to be either CD4+, CD8+ or DN[36, 37, 38], with CD4+ and DN being more prevalent than CD8+ cells[36]. IL-4 is mainly produced by CD4+ iNKTs whereas IFNγ is predominantly produced by DN iNKTs[24].

Type II NKT cells

Type II NKT cells, like Type I NKTs, are also NK1.1+ and TCRαβ+ and also recognize antigens through the MHC Class-I-like molecule CD1d. Unlike iNKT cells, however, Type II NKT cells recognize a broad array of antigens like sulfatides[39], lyophosphatidylcholine[40], β-glucosylceramides and glucosylsphingosine[41]. Type II NKT cells differ from iNKTs in their recognition of antigens since they have a more diverse Vα and Vβ pairing[42]. Since Type II NKT cells recognize different antigens and have a more variable αβ TCR repertoire, it is difficult to create tools to identify them. So far, they can only be identified negatively as the population of NK1.1+TCRβ+ cells that is unresponsive to αGC[43]. Type II NKT cells develop in the thymus and in the

periphery are present in the spleen and liver[39]. They have canonically been known to be counter-regulatory to iNKTs wherein the activation of Type II NKTs has been shown to anergize iNKTs[44, 45, 46, 47, 48]. This is surprising since like iNKTs, Type II NKT cells also produce IFNγ[44, 49] and IL-4[49]. Recent studies indicate that Type II NKT cells can also control tumor progression upon activation through IFNγ production[50, 51]. Currently, there is no consensus about Type II NKTs being pro- or anti-tumor and by that extension, agonists, or antagonists of iNKT cell functions.

Like in mice, human Type II NKT cells are difficult to identify since they are also unresponsive to αGC. A study done in a hepatitis C model showed that the human liver has more Type II NKT cells than the mouse liver and they produce more Th1 cytokines than Th2 cytokines[52]. The same group also found that the human bone marrow has a predominance of Type II NKT cells[53], prompting many researchers to believe that Type II NKTs are more abundant than iNKTs in humans[43]. In the human bone marrow, Type II NKTs were found to produce Th2 cytokines whereas iNKTs produced both Th1 and Th2 cytokines[53]. Human Type II NKTs also recognize sulfatide, lysosulfatide and sphingolipid antigens[54], similar to what is seen in mice. Studies in human myelomas have shown that Type II NKT cells have a regulatory function and are possibly pro-tumorigenic[40]. While a lot is known about iNKTs, Type II NKT cells have proven to be a more elusive and difficult to study in both mice and humans.

Thymic Development of iNKT cells

Like conventional T cells, iNKTs also develop in the thymus and undergo positive and negative selection[55]. The precursors of iNKT cells are CD8+CD4+ (double positive, DP) cortical thymocytes[56]. These DP thymocytes express CD1d and are selectively recognized by the invariant TCR of the iNKTs[57]. The presence of CD1d is critical for positive selection since mice lacking CD1d failed to develop iNKTs[56, 57, 58]. In addition, the CD1d on the DP thymocyte needs to be loaded with an iNKT-specific antigen, which is most likely a self-antigen[20]. Initially, isoglobotrihexosylceramide (iGb3), which is a weak glycolipid antigen and has a structure similar to αGC, was considered to be the self-antigen loaded in the CD1d molecule[59, 60, 61, 62]. However, a subsequent study showed that iNKT cells can develop in mice that lack iGb3[63] and another study showed that iGb3 is not present in thymic tissue and hence cannot be the antigen that iNKTs are selected on [64]. Follow-up investigations showed a lack of consensus in the field[65, 66, 67] until an elegant study demonstrated that iNKTs are selected off of endogenous α-linked monoglycosylceramides, mainly α-galactosylceramides and α-glucosylceramides[68]. Invariant NKT cells that recognize the endogenous lipid antigens presented through the CD1d are positively selected[69]. On the other hand, if the premature iNKT cell binds with higher affinity to the self-antigen on which it is selected, it is eliminated through negative selection[70].

Linear versus functional diversity models of iNKT cell ontogeny

Following lineage commitment and antigen recognition, immature iNKT cells undergo a series of maturation stages. The norm in the field for a long time was to follow the sequential lineage development model. Recently, the lineage diversification model has

been proposed and studied extensively[71, 72, 73, 74]. While the two models appear mutually exclusive, they occur in tandem during iNKT cell development. According to the sequential lineage development model (Figure 2), iNKT cells are $CD24^{hi}$ $CD44^{lo}$ and NK1.1- at Stage 0. As they mature, they downregulate their CD24 ($CD24^{lo}$ $CD44^{lo}$ NK1.1-) in Stage 1, upregulate their CD44 ($CD24^{lo}$ $CD44^{hi}$ NK1.1-) in Stage 2 and consequently their NK1.1 ($CD24^{lo}$ $CD44^{hi}$ NK1.1+) in Stage 3. As they mature, iNKT cells acquire their memory phenotype that allows them to respond rapidly to antigen upon presentation. Stage 3 iNKT cells are considered mature and migrate to peripheral organs like the spleen and the liver. As iNKTs mature, they either downregulate both CD4 and CD8 to become double negative (DN) or downregulate only their CD8 to remain CD4+[75]. Simultaneously, they acquire their effector functions based on the predominance of various transcription factors as explained by the lineage diversification model.

A key regulator of iNKT cell development and function is promyelocytic leukemia zinc finger (PLZF)[71], a zinc finger transcription factor that also controls lineage differentiation[76]. PLZF, a.k.a. Zinc finger and BTB domain-containing protein 16 (UniProt No. Q05516, human, and Q3UQ17, mouse), is a member of the Krueppel C2H2-type zinc-finger protein family and in conjunction with transcription factors T-bet, GATA-3, and RORγt defines NKT1, NKT2, and NKT17 populations, respectively[73]. Like Th1, Th2 and Th17 cells, NKT1 cells predominantly secrete IFNγ, NKT2 cells generate IL-4, and NKT17 are IL-17 producers[73]. Irrespective of the eventual lineage fate of the iNKTs, they all originate from PLZF+ cells. A deficiency of PLZF in engineered mice caused a dramatic reduction in iNKT cells in the thymus and in peripheral organs[71]. PLZF is robustly expressed in Stage 1 iNKTs, after which its

expression is gradually reduced to the lowest levels in Stage 3. Consistent with this pattern of expression, amongst the three iNKT cell lineages NKT2 cells have the highest expression of PLZF and were found to be Stage 2 cells. GATA-3, the principal transcription factor responsible for NKT2 development is responsible for the secretion of IL-4 upon activation. A lack of GATA3 completely abrogates IL-4 and IL-13 production owing to altered TCR signaling. While NKT 2 cells have a robust expression of PLZF, NKT17 cells express RORγt and an intermediate level of PLZF, and are also terminally differentiated at Stage 2, like the NKT2 cells. The ability of NKT17 cells to secrete IL-17 is due to the presence of RORγt [77, 78]. Like PLZF expression, RORγt is expressed at the double positive stage and is not detectable in Stage 3 iNKTs[79, 80]. NKT1 cells, on the other hand, were found to be Stage 3 iNKTs[73]. They express the lowest levels of PLZF among the iNKT subtypes and are T-bet positive. In line with this observation, T-bet has been shown to regulate the terminal maturation of iNKTs[81]. T-bet is required for IFNγ production in iNKTs as well as their expression of NK cell markers and cytotoxicity[81]. Interestingly, while NKT1 cells are Stage 3 mature iNKT cells, NKT 2 and NKT 17 cells were found to attain functional maturity at stage 2 of development[73]. Furthermore, even though NKT2 cells are found in stage 2, they are terminally differentiated and do not further mature to NKT1[73]. It is important to note that even though iNKTs are divided into lineages based on their cytokine secretion, the fundamental property of an iNKT cell being able to secrete both Th1 and Th2 cytokines is not altered. NKT1 can still produce small amounts of Th2 cytokines upon activation, a property which sets iNKTs apart from conventional Th1 cells[73]. The distribution of iNKT cells is in line with the effects of the cytokines secreted by them. NKT1 cells are found abundantly in the liver and to a lesser extent in the spleen, NKT2 primarily in the spleen, and NKT17 in the lymph nodes and lungs. The Th1 cytokines

and robust cytotoxicity noted with NKT1 cells is valuable in the liver, which is exposed to various antigens and pathogens from the gut. Conversely, the steady Th2 cytokine secretion from the NKT2 in the spleen has been shown to stimulate B cells to produce IgE, causing conditioning of macrophages and skewing of CD8+ T cells to a memory phenotype[73, 82].

iNKT cells in health and disease

The presence of different iNKT cell lineages and their cytokines produced leads to their context-dependent role in the progression of various diseases. In Type 1 diabetes (T1D), iNKT cells play a desirable as well as a detrimental role in controlling disease progression. Early studies found that iNKT deficient Non Obese Diabetic (NOD) mice had higher chances of developing T1D as compared to iNKT sufficient-NOD mice[83]. Moreover, stimulating iNKTs in NOD mice with αGC resulted in a diabetic disease of lesser severity[83] suggesting a protective role of iNKT cells in T1D. Further studies showed that the protection against diabetes by iNKTs was through the copious production of IL-4 and reduced secretion of IFNγ[84, 85]. More recent studies have shown that iNKT cells can also exert a pathogenic role in the onset of T1D in NOD mice through increased secretion of IL-17[86]. A similar context-dependent role of iNKTs in disease sequalae is also encountered in asthma. Initial studies in the field showed that the lack of iNKT cells in an OVA-induced asthma model was favorable for the asthma outcome in terms of airway remodeling, Th2 inflammation, and OVA-specific IgE antibody production. In addition, the adoptive transfer of IL-4 and IL-13 producing iNKT cells showed their pathogenic role in asthma by restoring the severity of disease[87, 88]. While in these studies iNKT cells were not stimulated by a glycolipid antigen, it was

theorized that endogenous lipid antigens in OVA-challenged mice were responsible for their activation[87]. Interestingly, when OVA-sensitized mice were injected with αGC before challenge, iNKT cells showed a protective response against asthma rather than a pathogenic one[89]. This protection was mediated through the secretion of IFNγ, a Th1 cytokine to counter the Th2 predominance that is usually observed in this type of asthma[89]. While it is known that asthma is a complex disease and can have various subtypes depending on the cytokines involved with disease progression, we can see that iNKT cells can be detrimental or desirable depending on the overall context of the disease.

A unique property of iNKT cells is their non-polymorphic CD1d on their surface[21, 90]. That is, there is a high specificity of CD1d and TCR similarities between species and within species, which allows cross-recognition of iNKTs without them being considered 'foreign' in the allogeneic[91] and xenogeneic setting[92, 93]. This contributes to its attractiveness as an off-the-shelf therapy since MHC polymorphism is the main cause of graft-versus-host disease (GVHD) and hence requires autologous transplantation. Through multiple studies, it has been shown that iNKT cells have a protective role in GVHD and cause suppression of the graft rejection response through various mechanisms such as the expansion of regulatory T cells (Tregs) and polarization of the conventional T cells to a Th2 phenotype[94, 95]. Most of these findings were observed in mouse models but subsequent human studies have also shown an advantageous role for iNKT cells protecting against GVHD. In the initial human studies, a correlation between increased numbers of circulating iNKT cells in the blood of patients and positive transplantation outcomes was determined[96]. Later studies supported the findings for better outcomes with a higher number of iNKTs in the graft[97, 98]. While

iNKT cells have shown promise as a potential treatment option for various diseases, it is extremely desirable for treating the second most common cause of death of modern times - cancer.

iNKT cells and anti-tumor response

Invariant NKT cells have been shown to be very important in the context of cancer. Initial studies showed that human patients suffering from a variety of cancers had reduced numbers of circulating iNKTs[99, 100, 101]. When the few iNKT cells that were present in cancer patients were studied, it was found that their proliferation and IFNγ production was reduced[101, 102, 103, 104]. Additionally, an increase in the number of circulating iNKT cells was an indication of a better prognosis of cancer[105]. While the human studies pointed to an association of iNKTs having a role in anti-tumor functions, definitive proof was obtained through mouse studies. In heterozygous p53 tumor suppressor knockout mice that are more susceptible to developing tumors, a lack of iNKT cells makes them even more prone to forming tumors[106]. Similarly, iNKT deficient mice (*Cd1d*$^{-/-}$ and *J*α*18*$^{-/-}$) have a higher incidence of developing tumors than iNKT sufficient mice, when exposed to the chemical carcinogen methylcholanthrene[107]. A direct way of proving iNKT cell involvement in these cases of carcinogen-induced tumors was to reconstitute the deficient mice with iNKTs, which resulted in the prevention of the growth of tumors[108]. In accordance with these findings, injecting mice with the iNKT cell agonist αGC inhibited tumor formation in spontaneous tumors[109], carcinogen-induced tumors[108, 110] or adoptively transferred tumors[111, 112]. The anti-tumor activity of iNKTs is two-pronged since 1) they have robust cytotoxic capabilities themselves and 2) they can activate other killer immune cells to lyse tumor targets.

Indeed, iNKTs once activated secrete copious amounts of cytokines, which further activate CD8+ T and NK cells[27, 28, 113]. In addition, activated iNKT cells secrete IFNγ and upregulate the CD40 ligand, which activates DCs[113]. Activated DCs go on to produce IL-12, which further activates NK and CD8 T cells[30]. In addition to the transactivation of other immune effectors, iNKTs can also kill tumor targets directly. Invariant NKT cells constitutively express cytolytic proteins like perforin and granzyme[114, 115] and can upregulate death receptors like Fas-Ligand and TRAIL[115, 116, 117]. In addition to killing CD1d+ cancer cells directly[118, 119], iNKT cells can also kill tumor-associated macrophages (TAMs)[120, 121], which are often associated with perpetuating a suppressive tumor microenvironment (TME)[122, 123, 124]. Various studies done in mice have shown that iNKT cells can either be CD4+ or DN. Further studies into the function of each of these subsets have shown that DN iNKT cells are excellent at cytotoxicity and more desirable in a cancer setting as compared to CD4+ cells[125].

While iNKTs have canonically been studied using αGC, which is one of the most potent iNKT cell agonists, multiple studies have been performed using structural variants of αGC to modulate functional responses. Alpha-GC induces both a Th1 and Th2 cytokine response but a c-glycoside analogue of αGC (α-C-GalCer) was shown to skew the iNKT cell cytokine profile towards a purely Th1-type response[126, 127]. One study found that surprisingly this response was achieved even though the α-C-GalCer had a weak interaction between the CD1d and TCR[128]. Modifying the α-C-GalCer into a NU-αGalCer analog increased the TCR binding affinity while maintaining the Th1 cytokine production and the ability to control B16-melanoma growth in an *in vivo* mouse model[128]. Other similar modifications in the same location of αGC to form NC-

αGalCer, 4ClPhC-αGalCer, and PyrC-αGalCer also showed a similar Th1 cytokine bias and increased anti-tumor activity of iNKT cells[129, 130]. A slight exception to the α-anomeric modifications was the finding that β-mannosylceramide (β-ManCer) also had a significant pro-inflammatory cytokine secretion and anti-tumor activity. The surprising aspect of β-ManCer activity is that it functioned through the activation of TNF-α and NOS pathways to achieve tumor control[131, 132] instead of the canonical iNKT secretion of IFNγ and perforin. Considering that iNKT cells can secrete Th2 cytokines as well as Th1 cytokines, Th2 skewing antigens were also discovered. An αGC analog with a truncation in the sphingosine chain gives rise to an antigen known as OCH and a modification in the di-unsaturated N-acyl chain gave rise to a compound called C20:2. Both OCH[133, 134] and C20:2[135] are Th2 polarizing antigens that preferentially induce IL-4 when recognized by iNKT cells. The main mechanism by which OCH functioned was postulated to be by limiting the interaction between CD1d and TCR, hence causing lower activation due to lower potency of the antigen[128]. Nonetheless, while OCH is not considered favorable for an anti-tumor response, it can help in controlling autoimmune diseases like Experimental Autoimmune Encephalomyelitis (EAE) where the anti-inflammatory function of iNKT cells is more desirable[134].

Current iNKT cell-based therapies

Once it was established the iNKT cells have a potent response to αGC, initial studies carried out by injecting free αGC saw a strong anti-tumor response[118, 136, 137]. One of the drawbacks of this approach was that iNKT cells would frequently become anergic to subsequent αGC stimulation[138, 139]. One approach used to circumvent this limitation

was the use of αGC-loaded-Antigen Presenting Cells (APCs) *in vivo*, which differentiated the iNKTs into IFNγ producing cells and prolonged the response as compared to mice injected with free αGC [138]. Additionally, αGC-loaded dendritic cells induced increased anti-tumor activity against a B16 melanoma model compared to free αGC [138]. Using αGC-loaded dendritic cells in pre-clinical trials, iNKTs prolonged tumor control in a mouse model of pancreatic adenocarcinoma[140]. Furthermore, injections of αGC-loaded dendritic cells in human patients suffering from myelomas[141, 142] and carcinomas[142] were well tolerated with minimal adverse effects. A disadvantage of using APCs in therapy is that depending on the type of APC used (B cells vs DCs) iNKT cell responses will differ[143]. Moreover, to avoid GVHD we need to collect autologous APCs, which are cost-prohibitive and challenging to grow in culture. Newer techniques aimed at redirecting iNKT cells in a CD1d independent manner have been studied and include bispecific molecules as well as Chimeric Antigen Receptor (CAR)-iNKTs. One of the iNKT cell therapies to gain the most traction and success in clinical trials are CAR-iNKTs, which are designed akin to CAR-T cells. The most notable amongst these are CAR-iNKTs oriented toward the killing of neuroblastomas and B cell lymphomas, which are currently in Phase I clinical trials. The CARs produced against neuroblastomas have a single chain variable fragment (scFv) that binds to the GD2 antigen[144, 145] while the B cell lymphoma scFv is directed against CD19[146, 147]. These treatments are remarkable since they have been shown to have the best increase in survival and tumor clearance in mouse models. A shortcoming of CAR-T cells is that the success shown in a clinical setting is mainly against hematological tumors while success against solid tumors is limited. This is primarily because of the inability to identify tumor associated antigens in solid tumors and the high toxicity associated with the CAR-T cell treatments[148]. To circumvent these

limitation of CARs, bispecific fusion proteins have been designed, which have shown promise against both hematological and solid tumors[149, 150, 151]. The bispecific fusion protein is the combination of the CD1d molecule attached to the specific tumor antigen through a scFv. Our lab has previously studied a soluble bispecific CD1d-CD19 fusion protein, which is designed to direct iNKT cells to B cell malignancies in a tumor antigen-specific but CD1d-independent manner[149]. Similar studies have shown promising results in targeting solid tumors like breast cancers[150] and hepatocellular carcinomas[151] using Her2- CD1d[150] or CEA-CD1d[151] fusion proteins, respectively. Although CAR-iNKTs and bispecific fusion proteins are more novel and sophisticated approaches to engage iNKT cells for robust anti-tumor responses, they have significant drawbacks, including the need for identification of tumor associated antigens, immunoediting by the tumor cells, and the complicated *in vitro* manipulation of iNKT cells. These issues can be circumvented using monoclonal antibodies targeted towards the invariant TCR (iTCR) of iNKT cells without the involvement of CD1d-mediated antigen presentation. To that end, we have shown in human[152] and mouse[153] studies that the iTCR monoclonal antibodies mediated strong iNKT cell activation, cytokine production, and suppression of tumor growth. As a proof of concept, the iTCR monoclonal antibody has been tested in pre-clinical mouse models[152] and long-term studies[154]. Based on the ongoing clinical trials and pre-clinical models, iNKT cells show tremendous promise as a curative treatment modality for intractable cancers. To achieve this goal iNKT cell therapies would benefit greatly from a deeper understanding of the intracellular signaling involved in iNKT cell activation.

Role of SAP and Fyn in iNKT cell development and anti-tumor response

There are many molecules involved in the development of iNKT cells, but the most critical ones are associated with the SAP and Fyn axis. Fyn, a proto-oncogene product and Src family tyrosine kinase, was the earliest adaptor protein studied in conventional T cell and iNKT cell development. While Fyn was found to be dispensable for conventional T cell development[155, 156], studies from *Fyn*$^{-/-}$ mice found that these mice had reduced numbers of iNKTs in the thymus and the periphery[157]. This suggested a lineage-specific role of Fyn in iNKT cell development. The SH3 domain of Fyn binds to the SH2 domain of SLAM-associated protein (SAP), a.k.a. SH2 domain–containing protein 1A (SH2D1A) [158]. Specifically, the amino acid arginine at position 78 (R78) on the SAP molecule is the most crucial for Fyn binding since mutation of the R78 residue on SAP completely abrogated the binding of Fyn to SAP[159]. SAP is encoded by the *Sh2d1a* gene. In humans, germline mutation in the *SH2D1A* gene causes X-linked lymphoproliferative (XLP) disease, which is characterized by abnormal immune responses to pathogens as well as the development of lymphomas and agammaglobulinemia [160, 161, 162]. The effect of SAP deficiency is also lineage-specific since it does not cause developmental defects in NK and T cells while it is critical for iNKT cell development. Mutations or absence of the *SH2D1A* gene causes an almost complete lack of iNKTs in mice and humans[160, 161, 162]. While the exact stage of the developmental block in *Sap*$^{-/-}$ mice is not known, introducing a Vα14 transgene in *Sap*$^{-/-}$ mice allowed the detection of a few iNKTs, which had low levels of NK1.1 and high CD24 expression that is compatible with immature cells (Stage 1)[163]. Consistent with this finding, overexpression of pro-survival genes that are necessary for positive selection of iNKTs like Bcl-xL and Bcl-2, did not rescue iNKT development in *Sap-/-* mice indicating that the block in iNKT development occurs after positive selection[163].

On the other hand, research into the block in development in *Fyn*$^{-/-}$ mice produced conflicting results. In Fyn-deficient Vα14 Tg+ mice, the iNKT cell numbers were restored[157]. This suggested that Fyn is required before the rearrangement of the TCR in iNKTs. Subsequent studies, though, showed that *Fyn*$^{-/-}$ mice have reduced numbers of mature iNKTs but had comparable levels of immature cells seen in Fyn sufficient mice. This was suggestive of Fyn's role in development after positive selection[164]. In terms of lineage differentiation, the absence of SAP in SAP-deficient Vα14 Tg^{+} mice caused the failure of the iNKT cells to secrete Th2 cytokines like IL-4 and IL-13. Indeed, GATA3 levels in these cells were significantly reduced, which suggests that SAP is required for GATA3 expression and, consequently, IL-4 production by iNKTs. Surprisingly in these SAP-deficient Vα14 Tg^{+} mice, there was IFNγ production, albeit through lower numbers of IFNγ producing cells[165]. Strikingly, these mice also showed a skewing of the iNKT cell lineage to IL-17 producing RORγt+ cells, which suggests that in the absence of SAP there is a preferential expression of NKT17 cells[165]. While the lack of SAP leads to defects in cytokine secretion, it can be argued that this is due to immature iNKTs and not due to the participation of SAP in cytokine secretion. A study performed recently disproved this hypothesis since it showed that a majority of the iNKTs in SAP-deficient Vα14 Tg^{+} mice are CD44^{hi} and hence mature[165], suggesting that SAP does play a role in modulating iNKT cell cytokine production. In contrast, other groups found that conditional deletion of SAP after iNKT cell development does not alter its cytokine production and transactivation *in vivo*[166, 167]. Hence, these studies proved that SAP does not play a role in cytokine production if it is absent after iNKT cell development and maturation is complete. Since Fyn is located downstream of SAP it can be assumed that Fyn also probably does not play a role in cytokine production. Indeed, *Fyn*$^{-/-}$ iNKTs or cells that are mutated at the

region of Fyn and SAP binding do not show a reduction in cytokine production[168, 169]. Collectively, these studies highlight a critical role for SAP and Fyn in iNKT cell development and function. However, until recently the role of SLAM, which is the upstream partner of SAP and Fyn, was not clearly defined.

Role of SLAM family receptors in iNKT cell development

The SLAM family of receptors (SLAM*f)* consists of 7 well-characterized members and 2 newer members that are considered non-classical SLAM*f* receptors. Of the seven members, SLAM*f*1 (CD150), SLAM*f*3 (Ly-9), SLAM*f*5 (CD84), SLAM*f*6 (Ly108), SLAM*f*7 (CRACC) are homotypic receptors[170, 171, 172]. SLAM*f*2 (CD48) and SLAM*f*4 (2B4) are heterotypic receptors and bind to each other[173]. SLAM*f* receptors are distributed on various hematopoietic cells and function as immunoreceptors that can augment or hamper immune cell responses. To analyze the role of SLAM*f* receptors in NKT cell development, a total SLAM*f* receptor knockout mouse (lacking all seven SLAM*f* receptors) was studied[174]. A drastic defect in iNKT cell numbers in the thymus and periphery was observed in the total SLAM*f* receptor knockout mouse (SFR-deficient)[174, 175]. This indicated that the SLAM*f* receptors had an important role in iNKT cell development. To delineate which member of the SLAM family was responsible for the defect noted in iNKT cell development, studies with individual knockouts or two or more SFR knockout mice were carried out. In line with the expression on the iNKT cell precursor in the thymus, lack of SLAM*f*1 and SLAM*f*6 together had a profound effect on the cell numbers[164, 174]. Analysis of their individual functions using bone marrow chimeras of SLAM*f*1 deficient and SLAM*f*6 deficient mice showed that a lack of SLAM*f*6 caused a 40-50% greater reduction in iNKT cell numbers than observed with a lack of SLAM*f*1[164, 175]. Consistent with these observations, overexpression of

SLAM*f*6 restored iNKT cell development, which was not noted with overexpression of SLAM*f*1[175]. While SLAM*f*1 and SLAM*f*6 were considered the most important in terms of iNKT cell development, other research groups created triple knockout mice that lacked SLAM*f*1, SLAM*f*5 and SLAM*f*6[176, 177]. Interestingly, they found that the triple knockout mice had lower iNKT cell numbers than SLAM*f*6 deficient mice[176, 177]. While *Sap*$^{-/-}$ mice had virtually no detectable iNKTs, single[178], double[164], and triple[176] SFR knockout mice had a small but consistent presence of iNKT cells, which is suggestive of compensation and redundancy within the receptors. A combined deletion of SLAM*f*1, SLAM*f*5, SLAM*f*6 and SLAM*f*7 showed a similar defect of iNKT cells as the SLAM*f*1 and SLAM*f*6 deficient mouse but an additional deletion of SLAM*f*3 and SLAM*f*4 showed a further reduction in iNKT cell numbers[175]. This pointed to a role for both SLAM*f*3 and SLAM*f*4 in iNKT cell development. Surprisingly, another group found that SLAM*f*3 deficient mice have increased iNKT cell numbers and hence concluded that SLAM*f*3 is a negative regulator of iNKT cell development[179].

Analysis of the iNKTs from total SFR-deficient mice showed that they had fewer NKT1s and increased NKT2 and NKT17 cells[174]. Consistent with this finding, there was a preferential expression of Vβ7 chain, which is known to be associated with NKT2 cells[175]. Since PLZF is a known regulator of iNKT cell lineage differentiation, an analysis of its expression was carried out. Indeed, a reduction of PLZF in stage 0 and stage 1 iNKT cells was observed[174]. Considering that the majority of iNKTs in the total SFR-deficient mice were stage 0 cells, it was suggested a role for SFR's in iNKT cell development after positive selection. Interestingly, the very few stage 1, 2 and 3 iNKTs from these mice had increased proliferation but lower survival[174]. In line with this observation, a reduction of the pro-survival molecule Bcl-2 was found in the SFR-

deficient iNKTs, and overexpression of Bcl-2 was sufficient in restoring their numbers[175]. Another striking feature was that the TCR signal strength appeared markedly increased in mice lacking all the SFRs. The high TCR signal strength also contributed to the increased death of iNKTs in the total SFR-deficient mice and can explain the skewing of the iNKT cell lineage to a predominance of NKT 2 and NKT 17 cells, which are known to require a higher TCR signal strength for development[174, 180].

SFRs are extensively expressed on various hematopoietic cells and have important roles in various immune cells. Two members of the SFRs are different since they are heterotypic binders. One of these heterotypic receptors, CD48 or SLAM*f*2, has the unique property that it is embedded in the lipid raft region not like the other SLAM*f* receptor structures with a transmembrane domain but with a glycophosphatidylinositol (GPI) anchor[181, 182]. Interestingly, during EBV infection, B cells upregulate their CD48 expression[183]. Additionally, stimulation of B cells with IL-4 also causes an increase in surface CD48[184]. In asthma patients, an increase of CD48 was observed on the surface of eosinophils[185]. The increase in CD48 expression in various disease conditions facilitates the progression of the immune responses. CD48 cross-linking alone in B cells induces their proliferation and immunoglobulin secretion[186]. When combined with TCR stimulation, CD48 caused proliferation and activation of T cells[187]. Studies have shown that binding of iNKTs to CD48+ mast cells cause a release of IL-4, IL-13 and IFNγ by the iNKTs[188]. The mechanism by which CD48 mediates these effects is through its binding to its heterotypic partner, 2B4[189, 190].

2B4-SAP in NK and T cell anti-tumor and cytokine response

2B4, also known as SLAM*f*4 or CD244, shares its ligand CD48 with CD2 in mice. Analysis of the structure of the 2B4 receptor showed that it has structural homology to CD48 as well as to human CD2[191]. Follow-up studies showed that the affinity of 2B4-CD48 binding tenfold greater than that of CD2-CD48 binding[189]. Apart from binding affinity, 2B4 and CD2 also differ in their signaling domains (Figure 3). 2B4, like the other SLAM receptors, contains various tyrosine-rich motifs in its intra-cellular compartment, whereas CD2 has a proline-rich intracellular tail[192, 193]. The difference in intra-cellular motifs translates to differential binding to intracellular signal transducers and consequently to different functions.

2B4 is expressed basally on NK cells[194], γδT cells[195], monocytes[196], eosinophils[197], basophils[198] as well as a subset of CD8 T cells[199] in mice. It was first discovered and has subsequently been extensively characterized in murine NK cells. IL2 expanded NK cells showed non-MHC restricted killing through the ligation of 2B4 using a monoclonal antibody[194]. This suggested that 2B4, like CD2, has an activating role in NK cell cytotoxicity in mice. Subsequent studies with *2b4*$^{-/-}$ mice showed an enhanced clearing of CD48+ melanoma cells in an *in vivo* mouse model of cancer, while 2B4 sufficient wild-type mice were unable to clear the CD48+ tumors[200]. Hence, in this model, the function of 2B4 was compatible with being an inhibitory receptor for NK cell cytotoxicity. Similar observations were made in *in vitro* studies wherein lymphokine-activated killer (LAK) cells from 2B4-sufficient mice were inefficient in killing CD48+ targets as compared to LAK cells from *2b4*$^{-/-}$ mice[201, 202]. Conversely, lack of CD48 on target cells enhanced the effect of killing by 2B4 sufficient NK cells[202]. Hence, 2B4 has now been proven to be an inhibitory receptor for murine NK cells.

The human 2B4 receptor was cloned and identified based on sequence homology to mouse 2B4 and human CD48[203]. Analysis of human peripheral blood mononuclear cells (PBMCs) showed that 2B4 is expressed on NK cells, γδT cells, CD8 T cells, a small percentage of CD4+ T cells[198], monocytes[198], basophils[198] and eosinophils[197], which is similar to the distribution of 2B4 in mice[199]. Functional studies showed that ligation of 2B4 with a monoclonal antibody caused an augmentation of NK cell killing of tumor targets[198, 203, 204, 205]. Additionally, blocking of the 2B4-CD48 interaction caused a reduction in CD48+ tumor cell lysis[198, 206]. Furthermore, disruption of the 2B4-CD48 binding by mutating key amino acids involved in the interaction resulted in drastically reduced cytotoxicity of NK cells[207]. Cytokine secretion primarily of IFNγ also increased upon engagement of 2B4 on human NK cells[204, 205]. Hence, it was concluded that 2B4 is an activating receptor for human NK cells[198, 203, 204] and the 2B4-CD48 interaction was necessary for NK cell functions[198, 204, 207].

The murine 2B4 receptor has been shown to exist in two isoforms, a long-form (2B4-L) and a short form (2B4-S) that are products of alternative splicing[208]. Both isoforms have similar extracellular regions (V and C Ig-like domains) and share the transmembrane domain but differ in their intracellular domains. The long form has 4 immunoreceptor tyrosine-based switch motifs (ITSM) in its cytoplasmic domain whereas the short form cytoplasmic tail contains only 1 ITSM[208]. A discrepancy was noted in the expression of both isoforms where in one study equal expression was observed in NK cells[208] while the other showed increased 2B4-L isoform in LAK cells[209]. In functional studies, when the 2B4-L isoform was stably transfected into a NK cell line, reduced target cell killing was observed. In stark contrast, transfection with the 2B4-S isoform conferred enhanced cytotoxicity[210]. Hence, it was concluded

that the 2B4-L isoform is inhibitory, while the 2B4-S isoform is activating. The human 2B4 receptor also has two isoforms, h2B4-A and h2B4-B. While the mouse 2B4 isoforms differ in their intracellular ITSM motifs, both isoforms of human 2B4 have the same 4 ITSM motifs[211]. Human 2B4-A and B differ only in their extracellular domain (C2 region) wherein h2B4-B has five extra amino acids as compared to h2B4-A[211]. Like the studies done in mice for isoform function evaluation, human NK cell lines transfected with h2B4-A mediated CD48+ target cell lysis whereas h2B4-B transfected lines could not lyse CD48+ cell lines. Hence, it was concluded that h2B4-A is an activating receptor and h2B4-B is an inhibitory receptor[212].

Upon further evaluation of the signaling mechanisms of the 2B4 receptor it was found that when the 2B4 receptor is ligated, the ITSMs are phosphorylated by the Src family of intracellular tyrosine kinases[206]. Src homology 2 (SH2) domain-containing adaptor proteins like SAP, SHP-1, and SHP-2 can then bind to the ITSMs to modulate the receptor function[206, 213, 214, 215]. Strikingly, it was found that the first, second and fourth ITSM can only bind SAP, which mediates a positive signal, whereas the third ITSM can bind SHIP, SHP-1, and SHP-2, which mediate negative signals[215]. The binding of SAP to only the first ITSM was found to be adequate for triggering cytotoxicity, which explains how the 2B4-S form that has only 1 ITSM motif mediates an activating signal. Fyn is usually recruited by SAP, which leads to a cascade of downstream events that cause positive NK cell activity[158, 159, 216]. Additionally, SAP competitively binds to the ITSMs to block the binding of the inhibitory tyrosine phosphatases SHP-1, SHP-2, and SHIP[215]. While these studies were done in mice, the findings can also be extended to human NK cells. In the human disease XLP, the lack of SAP causes NK cells to have defective cytotoxicity[213, 217, 218]. This is because, in the absence of SAP, inhibitory

adaptor proteins can bind to the ITSMs and abrogate NK cell activity[206, 213, 215]. Indeed, in line with this observation, it was observed that immature human NK cells had a high expression of 2B4 but no expression of SAP. When these immature NK cells were ligated through 2B4, they failed to kill CD48+ tumor targets, while completely mature NK cells had robust cytotoxicity against the same targets. Hence, the 2B4 receptors expressed in immature NK cells were inhibitory since they lacked SAP to transduce the activation signal. This hypothesis was further strengthened when SHP-1 transcripts were found that can mediate a negative or inhibitory signal of the 2B4 receptor[219]. The inhibitory form of 2B4 on immature NK cells was shown to be useful since these cells do not upregulate the MHC-specific inhibitory receptors until later in the maturation process. This can potentially render them autoreactive to the other developing hematopoietic cells. In this case, 2B4 acts as a fail-safe mechanism to inhibit the killing of neighboring cells [219]. While there are studies suggesting that SAP is necessary for the inhibitory functions of 2B4 in murine NK cells[220, 221], there is evidence that this may not be true. In a seminal study[201], it was found that 2B4 plays an inhibitory role in NK cell cytotoxicity in the presence or the absence of SAP. This finding suggested that SAP is dispensable for the role of 2B4 in murine NK cells[201].

Around the same time as 2B4 was first discovered on NK cells, it was also shown to be present on $\gamma\delta$ T cells in the skin[194, 195]. In a striking similarity to NK cells, 2B4 expression on $\gamma\delta$ T cells seemed dependent on IL2 since the surface expression of 2B4 decreased in the absence of IL2. Functionally blocking 2B4 using monoclonal antibodies increased the $\gamma\delta$ T cells' killing potential[195]. CD8+ T cells, on the other hand, were shown to have low 2B4 expression on naïve cells but upon activation with cytokines like IL2, IL-4 and IL-15, 2B4 was robustly upregulated[199]. Furthermore, both

2B4-S and 2B4-L isoforms were equally expressed in activated CD8 T cells[199]. In the initial studies, it was postulated that 2B4 was activating on CD8 T cells since the presence of 2B4 augmented T cell cytotoxicity[222] whereas blocking the 2B4 receptor led to a reduction in proliferation[199, 223] and activation[223]. Mechanistically, it was shown that 2B4 associates with LAT to carry out its lytic function[224]. A shift in thinking occurred when adoptive T cells were used to treat cancers[225, 226] and when chronic viral infections[227] were studied. Both CD4 and CD8 T cells were found to upregulate 2B4 as a marker of exhaustion in melanomas[225] and lung cancers[226]. The presence of 2B4 in a lung cancer model in mice also contributed to the reduced secretion of IFNγ and increased death of T cells[226]. In a recent study, the blocking of 2B4 in an *in vivo* mouse model of head and neck squamous cell carcinoma showed a decrease in total tumor volume and an increase in tumor-infiltrating CD8 T cells[228]. The inhibitory role of 2B4 in T cells was also observed in other conditions such as transplantation[229, 230] and sepsis[231]. An interesting phenomenon of the role of SAP and 2B4 in CD8 T cell function was studied by a few groups[232, 233]. One group found that SAP is necessary for CD8 T cell proliferation and cytokine production by associating with 2B4 but only when the antigen is presented by B cells and not by the other APCs[232]. Another group found that like NK cells[221], the amount of 2B4 expressed on the cell as well as the availability of intracellular adaptor proteins governs the activity of 2B4 on CD8 T cells[233, 234]. In a chronic viral infection model in mice, blocking of 2B4 on intermediate expressors reduced virus-specific responses due to an activating phenotype of 2B4. Conversely, 2B4 blockade on 2B4 high T cells augmented their anti-viral response owing to an inhibitory 2B4 phenotype in exhausted CD8 T cells[234]. Similar observations were made in virus-specific CD8 T cells wherein crosslinking of the

receptor under low levels increased the cytotoxicity and IFNγ production, which was not observed in higher levels of 2B4 expression[233].

Similar to what was initially observed in mice, earlier studies done in human T cells pointed to an activating role of 2B4. Various studies showed that upon activation human CD8 T cells upregulated 2B4, perforin, granzyme and secreted IFNγ. This indicated that during the expression of 2B4, CD8 T cells were displaying an effector phenotype[235, 236, 237, 238]. A subsequent study from T cells obtained from leukemia patients showed that 2B4 was significantly upregulated on CD8 T cells and while these cells retained the capacity to produce IFNγ, they did not have cytolytic capacity[239]. In addition, the upregulation of 2B4 was shown by various studies to occur simultaneously with other inhibitory receptors like PD-1[240, 241, 242, 243, 244] and CD160[238, 241, 242], pointing to an inhibitory role of 2B4 in human T cell function.

Role of SLAM receptors in iNKT cell cytotoxicity

Invariant NKT cells have demonstrated lineage-specific roles of various receptors. SAP and SLAM have been shown to be dispensable for NK and T cell ontogeny[202, 245, 246, 247] whereas SAP is critical for iNKT cell development. While the role of SLAM family receptors in iNKT cell development has been studied, its role in iNKT cell cytotoxic responses remains to be defined. As mentioned earlier, the SLAM family (SLAM*f*) of receptors is comprised of seven well-defined members. To address the role of SLAM*f* receptors in iNKT cell cytotoxicity, we performed an *in vitro* cytotoxicity assay where iNKT cells were co-cultured with radiolabeled EL4 tumor targets in the presence of a cocktail of SLAM*f*-Fc fusion proteins (fusion of the extra-cellular domain of the

individual SLAM*f* receptors to an IgG1 Fc domain). While iNKT cells from C57BL/6 (B6) mice robustly lysed the syngeneic EL4 tumor targets, this function was significantly reduced in the presence of the SLAM*f*-Fc fusion proteins. As the assay was done with a cocktail of various SLAM*f*-Fc fusion proteins, our next approach was to identify which SLAM*f* receptors could be contributing to the observed response. To that end, iNKT cells from mice that lacked specific SLAM*f* receptors were obtained by flow cytometry and analyzed in a similar cytotoxicity assay. We found that mice that lacked homotypic SLAM receptors (*Slamf1*$^{-/-}$, *Slamf3*$^{-/-}$, *Slamf5*$^{-/-}$, *Slamf6*$^{-/-}$) showed comparable iNKT cell cytolytic capacity as noted in B6 iNKTs. This suggested that either of these homotypic SLAM receptors were dispensable or redundant in function. This was further confirmed by killing assays with B6 iNKT cells using single homotypic SLAM*f*-Fc fusion proteins. Unlike the homotypic SLAM*f* receptors, 2B4 and CD48 mediate heterotypic interactions. Like the other homotypic receptors, the cytolytic activity of *Cd48*$^{-/-}$ iNKT cells was comparable to B6 iNKTs. However, the addition of only the CD48-Fc fusion protein significantly impaired the killing of tumor targets, suggesting that heterotypic interaction of CD48 with 2B4 regulates iNKT cell killing. However, prior studies have shown that 2B4 is not expressed on thymic iNKT cells beyond the double positive stage during ontogeny[174]. Contrary to these reports, we observed that a subset of thymic iNKT cells has surface expression of 2B4. Thus, the specific goals of the current study are 1) to examine the kinetics and tissue distribution of 2B4 expression in resting and antigen-stimulated iNKT cells and 2) to evaluate the role of 2B4 in iNKT cell cytotoxicity.

APPENDIX

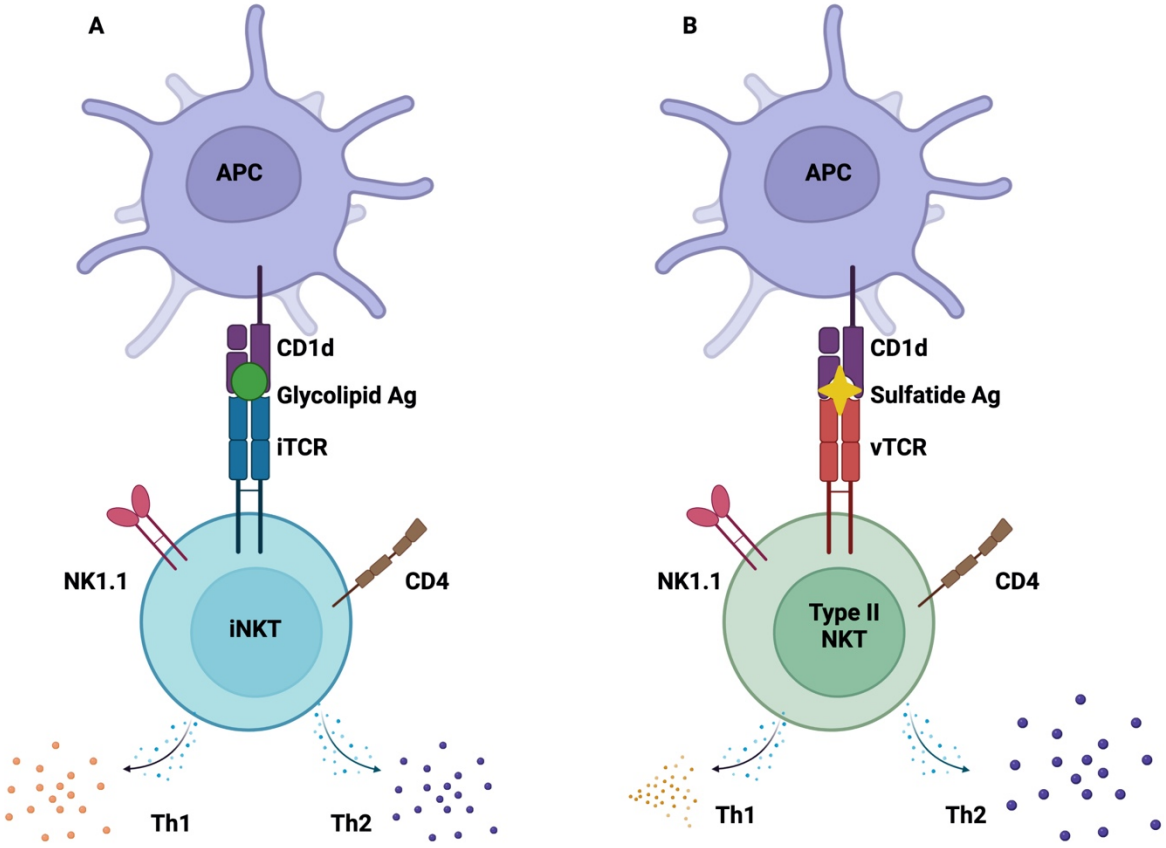


Figure 1. Invariant NKT and Type II NKT cells

A) Type I NKT cells have an invariant TCR (iTCR), are glycolipid reactive, and produce both Th1 and Th2 cytokines when stimulated. B) Type II NKT cells have a more variable TCR (vTCR), recognize many antigens including sulfatides and produce more Th2 cytokines than Th1 cytokines when stimulated. Both iNKTs and Type II NKTs share the expression of NK1.1, occur as either CD4 or DN cells and recognize the antigen in the context of CD1d.

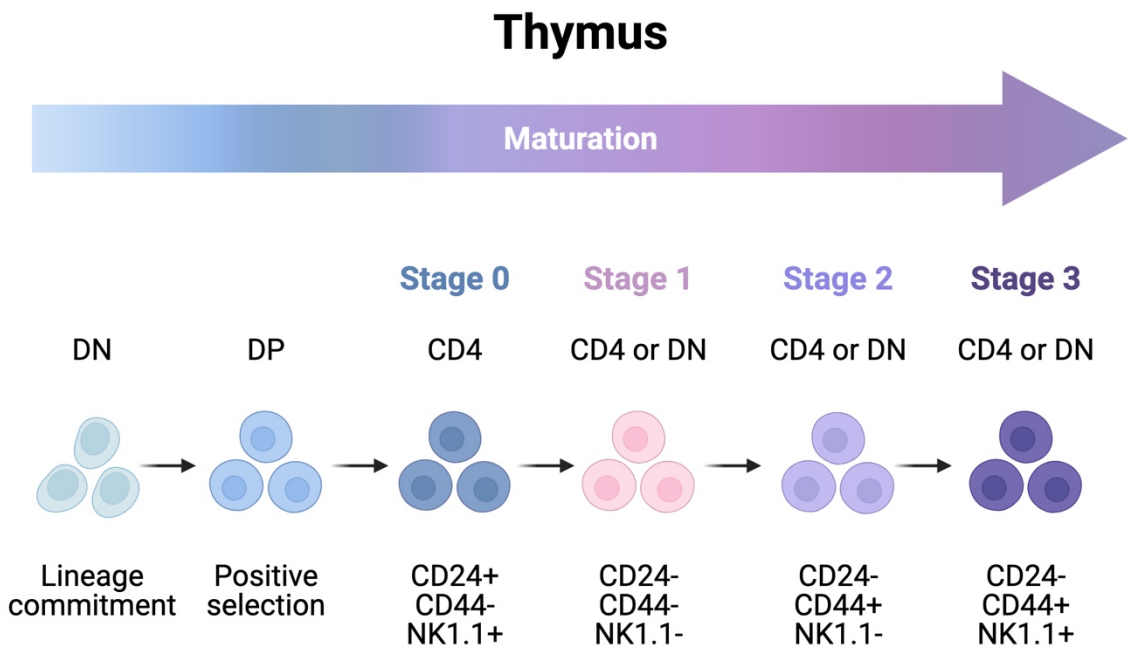


Figure 2. Sequential lineage development model of iNKT cells

Invariant NKT cells develop and mature in the thymus. Lineage commitment occurs at the double negative (DN) stage after which positive selection is undertaken in the double positive (DP) stage. Immediately post positive selection developing iNKTs are CD24+ (Stage 0) after which they sequentially downregulate their CD24 (Stage 1), upregulate their CD44 (Stage 2) and complete maturation with expression of NK1.1 (Stage 3). Once fully developed and mature, iNKT cells egress to the peripheral organs like the spleen and the liver.

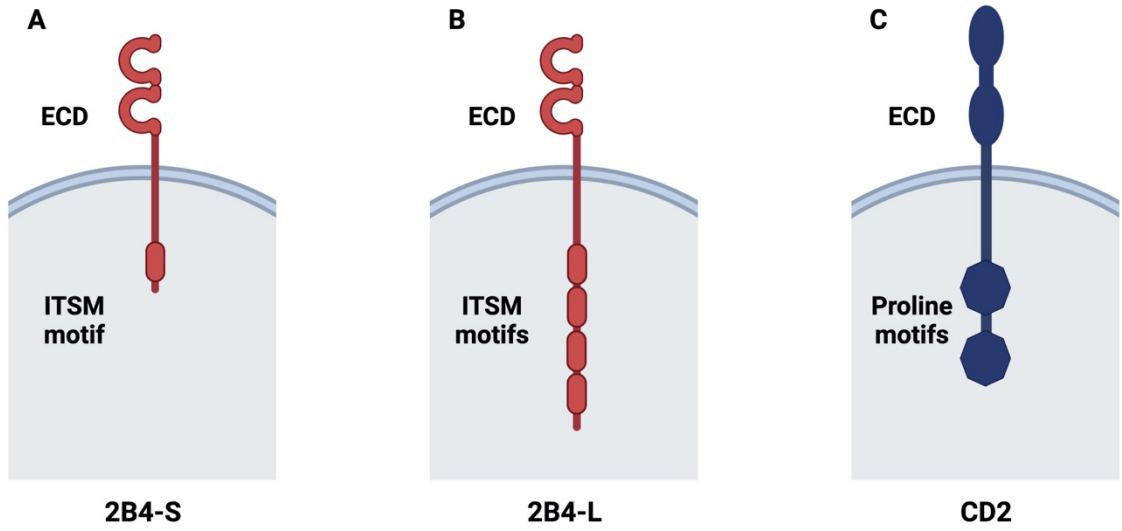


Figure 3. 2B4 and CD2 receptor structures

The 2B4 receptor occurs in two isoforms. A) The 2B4-S isoform consists of an extracellular domain (ECD) with a single intracellular ITSM motif whereas B) the 2B4-L isoform contains 4 ITSM motifs. C) The CD2 receptor also consists of an extracellular domain, a transmembrane domain, and an intracellular domain with proline motifs as compared to the tyrosine rich domains of the 2B4 ITSM motifs.

CHAPTER 2- METHODS AND MATERIALS

Mice

C57BL/6 (B6) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA), bred, and housed under specific pathogen-free conditions at Michigan State University. Male and female, age-matched mice (8-12 weeks) were used for experiments. All animal studies were approved by the Institutional Animal Care and Use Committee at Michigan State University (protocol number: PROTO202100207)

Cell lines and reagents

DN3A41.2 (1.2) cells, a mCD1d-autoreactive NK T cell hybridoma, were a kind gift from Dr. Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA). EL4 cells derived from a murine T lymphoma were obtained from American Type Culture Collection (Manassas, VA, Cat. No. TIB-39) and firefly luciferase-expressing EL4 cells (EL4-Luc) were from Caliper Life Sciences (Hopkinton, MA). 1.2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100U/mL Penicillin, 100U/mL Streptomycin, 2mM L-glutamine and 50μM 2-mercaptoethanol (2-ME). EL4 and EL4-Luc cells were cultured in DMEM supplemented with 10% fetal calf serum, 100U/mL Penicillin, 100U/mL Streptomycin and 2mM L-glutamine. PBS44 and OCH, which are α-galactosylceramide analogues, were purchased from S&D Lipopharma (Provo, UT) and Enzo Life Sciences (Farmingdale, NY), respectively.

Generation of cell lines expressing chimeric 2B4 receptors

1.2 cell clones expressing 1) full length 2B4 (2B4-WT), 2) full length CD2 (CD2WT), 3) a chimeric fusion protein consisting of the 2B4 extracellular domain fused to the

CD2 intracellular domain (2B4-CD2), and 4) the inverse chimera containing the CD2 extracellular region fused to the 2B4 intracellular domain (CD2-2B4) were previously generated in the lab (unpublished data). Briefly, 1.2 cells were transduced using second generation lentiviral vectors[248, 249] encoding either the full length 2B4, full length CD2 or the chimeric receptors 2B4-CD2 and CD2-2B4. After lentiviral transduction the 1.2 cells were selected in RPMI media supplemented with 10% fetal calf serum, 100U/mL Penicillin, 100U/mL Streptomycin, 2mM L-glutamine and 2μg/mL Puromycin. Stable expression of the surface expression of the 2B4 or CD2 receptor was confirmed by flow cytometry prior to use in each experiment.

Flow cytometry and cell sorting

Antibodies used for immunofluorescence staining had specificity towards TCRβ, 2B4, CD4, PLZF (BioLegend, San Diego, CA) and NK1.1, CD69 (BD Biosciences, San Hose, CA). Fluorochrome conjugated isotype antibodies were also purchased from either BioLegend or BD Biosciences. Fluorochrome conjugated CD1d-tetramer loaded with PBS-57 (CD1d-Tet) and unloaded controls were obtained from the NIH Tetramer Core Facility (Emory University, Atlanta, GA). For staining cell surface molecules, 0.4 - 2 X 10^6 cells were resuspended in 50μL FACS buffer (consisting of 1XPBS with 1% bovine calf serum), containing optimized concentrations of fluorochrome-conjugated monoclonal antibodies for 30 minutes at 4°C. Post-incubation, cells were washed once with ice-cold FACS buffer and resuspended at 200μL of FACS buffer. For intracellular staining, post surface staining and washing with FACS buffer, cells were resuspended in 200μL of Cytofix/Cytoperm™ solution (BD Biosciences) as per manufacturer's protocol. Cells were washed twice in 1X Perm/Wash™ solution (BD Biosciences) and

incubated with an optimal concentration of fluorochrome-conjugated monoclonal antibodies (prepared in in 1X Perm/Wash™ solution) for 1 hour at 4°C. Cells were washed twice with 1X Perm/Wash™ solution and then resuspended in 200μL of FACS buffer for flow cytometric analysis. Data was collected on a BD™ LSR II flow cytometer and analyzed using FlowJo software (FlowJo LLC; Ashland, OR, USA). Isolation of hepatic lymphocytes was carried out via density gradient using Percoll (GE Healthcare). The mononuclear cells thus obtained were counted and used for *in-vitro* activation assays.

In vitro iNKT cell activation

Hepatic lymphocytes from B6 mice or DN3A4-1.2 cells (1.2) were cultured on plate-bound Ultra-LEAF™ purified anti-mouse CD3ε and LEAF/Ultra-LEAF™ purified anti-mouse CD28 antibodies (Bio Legend) at 10μg/mL and 5μg/mL, respectively, or left unstimulated for 6-66 hours. At the end of incubation, cells were harvested, stained, and analyzed through flow cytometry for activation (CD69) and 2B4 surface expression.

In vitro cytotoxicity studies

Untransfected 1.2 cells or cell lines overexpressing the full length 2B4 receptor (2B4WT), the full length CD2 receptor (CD2WT), the chimeric 2B4 receptor (2B4-CD2), or the chimeric CD2 receptors (CD2-2B4) were cultured in triplicate wells with EL4-Luc target cells loaded with 200ng/mL PBS44 for 18 hours. D-firefly luciferin potassium salt (Perkin Elmer) was added at 75 μg/mL and plates read for bioluminescence (flux = photons/second) in a micro-beta plate reader (Perkin

Elmer)[250]. Percentage specific lysis was calculated as (Spontaneous BLI signal – experimental BLI signal)/ (Spontaneous BLI signal – minimum BLI signal). Minimum BLI signal was measured by complete lysis of target cells by using 0.1% Igepal (Sigma-Aldrich) in water.

Quantitative Real-Time PCR

Cells were lysed in TRIzol solution (Thermo Fisher Scientific, Waltham, MA) and total RNA was extracted as per the manufacturer's protocol. RNA (200ng-1000ng) was reverse transcribed into cDNA using the Superscript III cDNA synthesis kit according to the manufacturer's instructions (ThermoFisher, Cat. No. 18080051). Real-time quantitative PCR was performed using SYBR green primers and PowerUp™ SYBR™ Green Master Mix using Quant Studio™ 3 system (Applied Biosystems) according to the manufacturer's instructions. The following isoform specific primers were used: 2B4L forward 5'- AGC AGA ATT CCC CTG GAG AT, 2B4L Reverse 5'- TTC CTG GAA GCC TGG ACT AC, 2B4S Forward 5'- TGT TCA GCT CCC TTC TAG CTT T, 2B4S Reverse 5'- TCT ATT TCC CAT TTT TCT CTG CTC. Relative gene expression data (fold change) was calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal reference control.

Statistics

To determine statistical significance, either Student's unpaired t-test with Welch's correction, or a two-way ANOVA was used, as indicated in the figure legends. Significance is shown as *($p<0.05$), **($p<0.01$). Statistical analysis was performed using PRISM software (GraphPad, San Diego, CA, USA).

CHAPTER 3- RESULTS

Type I and Type II NKTs have differential distribution of 2B4

Various immune cells like NK[194] and γδ T cells[195] express 2B4 under resting conditions. To analyze the pattern of 2B4 expression on iNKT cells we analyzed the organs where they are the most abundant, i.e., the thymus, spleen, and liver. By using PBS57-loaded-CD1d tetramers and anti-TCRβ antibodies we identified the iNKT cell population in each organ (Fig. 4A, top panel). Flow cytometry for 2B4 (Fig. 4A, lower panel) revealed that iNKTs in the spleen and the liver did not express 2B4. On the contrary, a small fraction of iNKT cells in the thymus showed the presence of the 2B4 receptor. An alternative method of identification of total NKTs is through the expression of surface NK1.1 and TCR (Fig. 4B, upper panel). Analysis of 2B4 expression on this population revealed basal expression of 2B4 in the thymus, spleen, and liver (Figure 4B, lower panel and 4C). Total NKT cells is comprised of iNKT and Type II NKT subsets based on their reactivity to PBS57-loaded CD1d tetramer, and they have different tissue distributions. In line with earlier studies, we found that Type II NKTs were more prevalent in the spleen (50-60%) while iNKTs had abundance in the thymus and liver (80-90%) (Figure 4D). Since we found that the total NKTs have a high surface expression of 2B4, we next analyzed which fraction of the two NKT subsets were contributing to the 2B4 expression. Hence, we stained for 2B4 and found that consistent with our finding in Figure 4A, iNKTs showed minimal 2B4 expression basally in the spleen and the liver (<5%) and have a small population of cells that express 2B4 in the thymus (20-25%) (Figure 4E, top panel and Figure 4F). In sharp contrast, Type II NKTs had significantly higher basal levels of 2B4 with the most dramatic expression in the spleen (60-80%) (Figure 4E, lower panel and Figure 4F).

2B4 is upregulated on iNKT cells upon activation

Since we found that 2B4 is basally absent on iNKT cells, we next analyzed 2B4 expression following activation. To that end, we isolated total mononuclear cells from B6 livers and polyclonally activated them using αCD3 and αCD28 for various time periods (6, 24, 48 and 66 hours). Upon activation, iNKT cells are known to rapidly downregulate their TCR[251] which posed a hurdle in distinguishing them from other lymphocytic subpopulations in the total liver mononuclear cells. To address this, we used a combination of surface and intracellular markers to specifically gate on iNKT cells while excluding conventional T and NK cells from the mixed lymphocyte population (Figure 5A). Upon analysis, we found that the 2B4 receptor is upregulated on the surface of iNKT cells as early as 6 hours post-activation and continues to rise until 48 hours, after which it is stably expressed at least until 66 hours post-activation (Figure 5B, top panel, and Figure 5C). To confirm that the iNKTs were undergoing adequate stimulation we stained them for the surface expression of the activation marker CD69. We observed that CD69 was adequately upregulated at various time points (6-66hours) as compared to the unstimulated cells (Figure 5B, bottom panel, and Figure 5D). At each of the time points where CD69 was upregulated we saw the expression of 2B4 suggesting that the presence of 2B4 was indeed a function of iNKT cell activation.

One of the limitations of using NK1.1+ PLZF+ CD4+ to identify iNKT cells is that we could only analyze 2B4 expression on CD4+ iNKTs. Moreover, recent literature shows that Type II NKT cells are also CD4+[252]. To ensure that our findings are specific to iNKT cells, we used an iNKT cell hybridoma cell line DN3A4-1.2, henceforth referred to as 1.2 cells, in activation studies. When 1.2 cells were stimulated like the primary

hepatic mononuclear cells, we found that 2B4 is upregulated at 6 hours and shows steady expression up to 66 hours (Figure 6A, C), which is in line with our previous observation in Figure 5B, C. Similar to what we observed in primary cells, 1.2 cell activation led to significant upregulation of CD69 (Figure 6B, D) at the same time points where we observed the presence of 2B4.

2B4 negatively regulates iNKT cell cytotoxic responses

The 2B4 receptor is known to exist in two isoforms which are derived as splice variants of from the same precursor messenger RNA[208]. In mice, the long-form (2B4-L) has 4 intracellular ITSM motifs while the short-form (2B4-S) has only 1 ITSM motif[208]. To analyze the relative expression of the two isoforms, we polyclonally activated 1.2 cells for 24 hours and performed qPCR analysis. Relative to the unstimulated cells the 2B4-S form was minimally expressed (Figure 6E, F). In contrast, the 2B4-L form was significantly upregulated as compared to both the unstimulated cells as well as the 2B4-S form following activation (Figure 6E, F).

To define the function of 2B4 on iNKT cells post activation, we performed an *in vitro* cytotoxicity assay using 1.2 cells that have expression of 2B4 (2B4+) or do not express it (2B4-) (Figure 7A). The target cells used were luciferase expressing-EL4 cells (EL4-Luc) that exhibit surface CD48 (Figure 7B). Similar to the chromium release cytotoxicity assay in which we measure the difference in initial and final levels of radioactive chromium to gauge iNKT cytolysis, the luciferase assay allows the analysis of tumor target killing by examining the change in the bioluminescence signal. Upon performing the luciferase assay, we observed that unlike 2B4- 1.2 cells, 2B4+ cells

showed a significant reduction in killing (Figure 7C, D, E), indicating an inhibitory role for 2B4 in iNKT cell cytolytic activity. This was further confirmed when we observed that 1.2 cells which overexpress the 2B4 full-length receptor (2B4WT) completely failed to kill EL4 tumor cells (Figure 8A-D). Unlike the contradictory roles of 2B4 in NK and T cell function, our study provides the most direct evidence of an inhibitory role of 2B4 in iNKT cell killing even in the presence of a strong antigen.

2B4 intracellular domain contributes to the negative regulation of iNKTs

We next analyzed if the inhibitory effect of the 2B4 receptor was due to intracellular motifs or its extracellular domain. To this end, we used 1.2 cells that were previously generated in the lab and overexpressed chimeric receptors. Specifically, 1.2 cells either expressed 1) full-length CD2 (CD2WT) (Figure 8A, B), 2) chimeric 2B4 containing its extracellular domain while containing the intracellular CD2 motifs (2B4-CD2) (Figure 9A, B), 3) chimeric CD2 with the extracellular domain of CD2 and intracellular ITSM motifs of the 2B4 receptor (CD2-2B4) (Figure 9A, B). Interestingly, in cytotoxicity assays, we observed that the CD2-2B4 cells had reduced killing (Figure 9C, D) when compared to wild-type cells, which is in stark contrast to the increased killing seen by CD2WT cells (Figure 8C, E). These findings suggest that the inhibitory role of 2B4 is in part due to the intracellular motifs responsible for signal transmission. Furthermore, CD2WT cells exhibited increased killing even in the presence of the weak antigen OCH (Figure 8F, H), which further strengthens the previous findings of the important activating role of the CD2 receptor in iNKT cell functions.

2B4-CD2 chimeric receptor mediates robust lysis of T cell tumor targets

Excitingly, we saw the highest cytolytic activity carried out by the 2B4-CD2 cells compared to wildtype 1.2 cells in the presence of the strong antigen PBS44 (Figure 9C, E). The switch from no killing seen in the 2B4WT cells (Figure 8C, D) to the drastically elevated cytolysis mediated by 2B4-CD2 (Figure 9C, E) was striking and suggested that the 2B4 receptor response can be transformed by altering its intracellular domains. Surprisingly, 2B4-CD2 had significantly elevated cytotoxicity even in the presence of a weak antigen like OCH (Figure 9F, H), which bolsters the observation of superior functional capacity of the 2B4-CD2 receptor when compared to wild-type iNKT cells and is consistent with the stronger binding of 2B4 with CD48.

APPENDIX

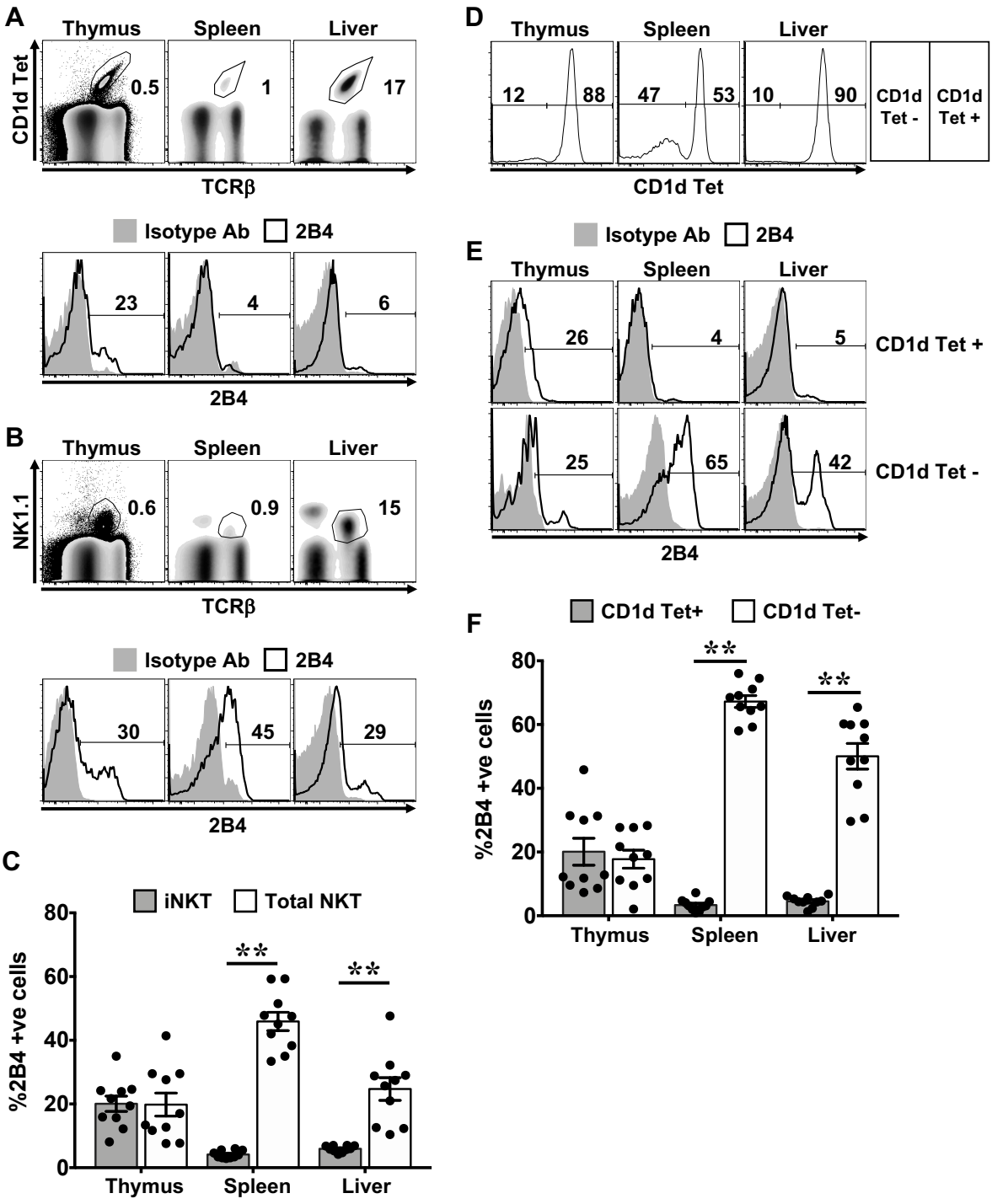


Figure 4. 2B4 is not expressed on resting iNKT cells

PBS57-CD1d tetramer (Tet)+TCR-β+ iNKT cells (A) or NK1.1+ TCR-β+ NKT cells (B) from the thymus, spleen, and liver of wild type (B6) mice were stained with fluorescently labelled anti-2B4 (bold histogram, A and B lower panel) or isotype

Figure 4. (cont'd)

control (grey solid histogram, A and B lower panel) and analyzed by flow cytometry. (C) Average percentages of 2B4+ cells from the different organs are shown. Data represents the mean ± standard error of the mean (SEM) obtained from 10 mice. (D) CD1d-Tetramer reactivity of total NKT cells in (B, upper panel) was analyzed in the thymus, spleen, and liver of B6 mice. (E) Representative histograms of 2B4 expression on CD1d Tet+ (upper panel) and CD1d Tet- fractions of NK1.1+ TCR-β + NKT cells (lower panel) as compared to their isotype controls. (F) Average percentage of 2B4 expression on CD1d Tet+ and CD1d Tet- cells from the organs of B6 mice. Data represents the mean ± SEM of 10 age-matched mice. Statistical significance in C, F was determined by Student's unpaired t-test with Welch's correction. (*$p<0.05$, **$p<0.01$).

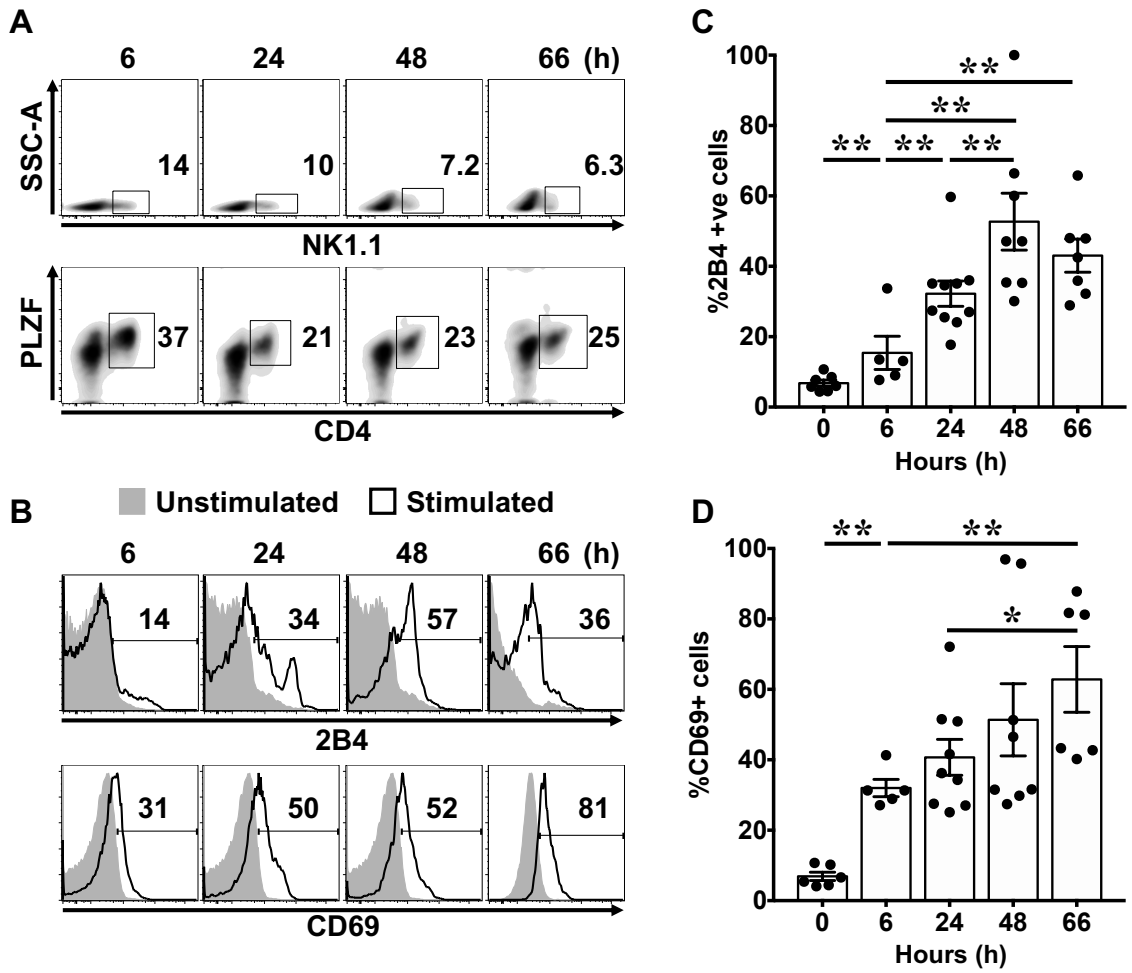


Figure 5. 2B4 is upregulated on primary intra-hepatic iNKTs post-activation

(A) Liver lymphocytes from B6 mice were incubated in the presence of plate-bound αCD3 and αCD28 antibodies for the time points as indicated. iNKTs were identified by flow cytometry as NK1.1+ (upper panel) PLZF+ CD4+ cells (lower panel). (B) 2B4 (upper panel) and CD69 (lower panel) expression on activated iNKTs (bold histogram) compared to unstimulated iNKTs (grey solid histogram) is shown at the respective time points. Pooled data showing 2B4 expression (C) and CD69 expression (D) at different time points. Data in (C) is from 5 experiments with 5-10 livers and data in (D) is from 5 independent experiments with 5-9 livers and is represented as mean ± SEM. Statistical significance in C, D was determined by Student's unpaired t-test with

Figure 5. (cont'd)

Welch's correction. (*p<0.05, **p<0.01).

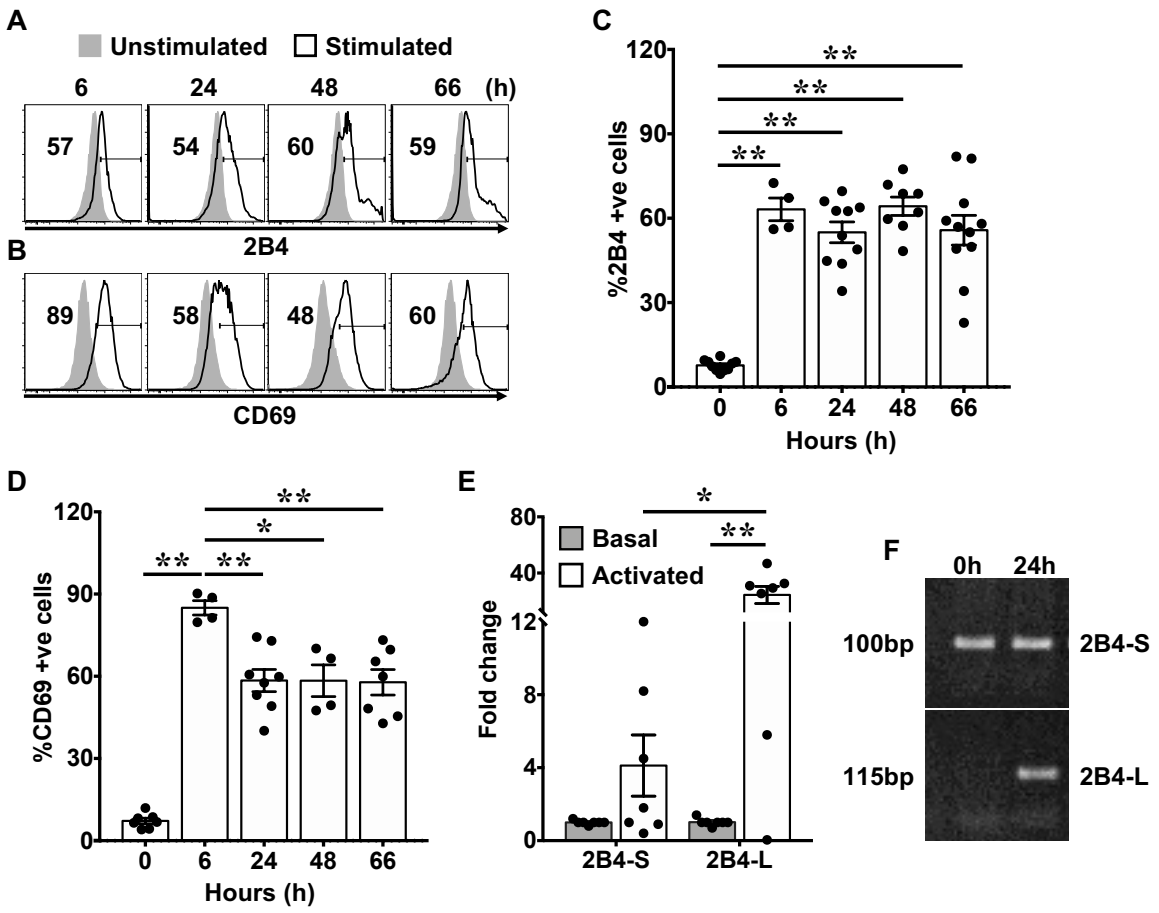


Figure 6. Activated 1.2 cells predominately express the long-form of 2B4

Mouse iNKT cell line DN3A4-1.2 (1.2) cells were activated in the presence of plate-bound αCD3 and αCD28 antibodies for the time points as indicated. Surface expression of (A) 2B4 and (B) CD69 was evaluated through flow cytometry in activated cells (bold histogram) compared to unstimulated cells (grey solid histogram). (C) Pooled data of 2B4 expression on 1.2 cells from 8 experiments is shown and represented as mean ± SEM. (D) Pooled data of CD69 expression on 1.2 cells from 5 independent experiments are shown and represented as mean ± SEM. (E) Fold change obtained by qPCR of 2B4 short isoform (2B4-S) and 2B4 long isoform (2B4-L) at 24 hours post-activation (white bars) compared to unstimulated cells (grey bars) is shown. Data is from 7 independent experiments and is expressed as mean ± SEM. (F) Representative gel image of amplicon obtained from qPCR of 2B4-S (upper panel)

Figure 6. (cont'd)

and 2B4-L (lower panel) is depicted. Statistical significance in C, D, E was determined by Student's unpaired t-test with Welch's correction. (*$p<0.05$, **$p<0.01$).

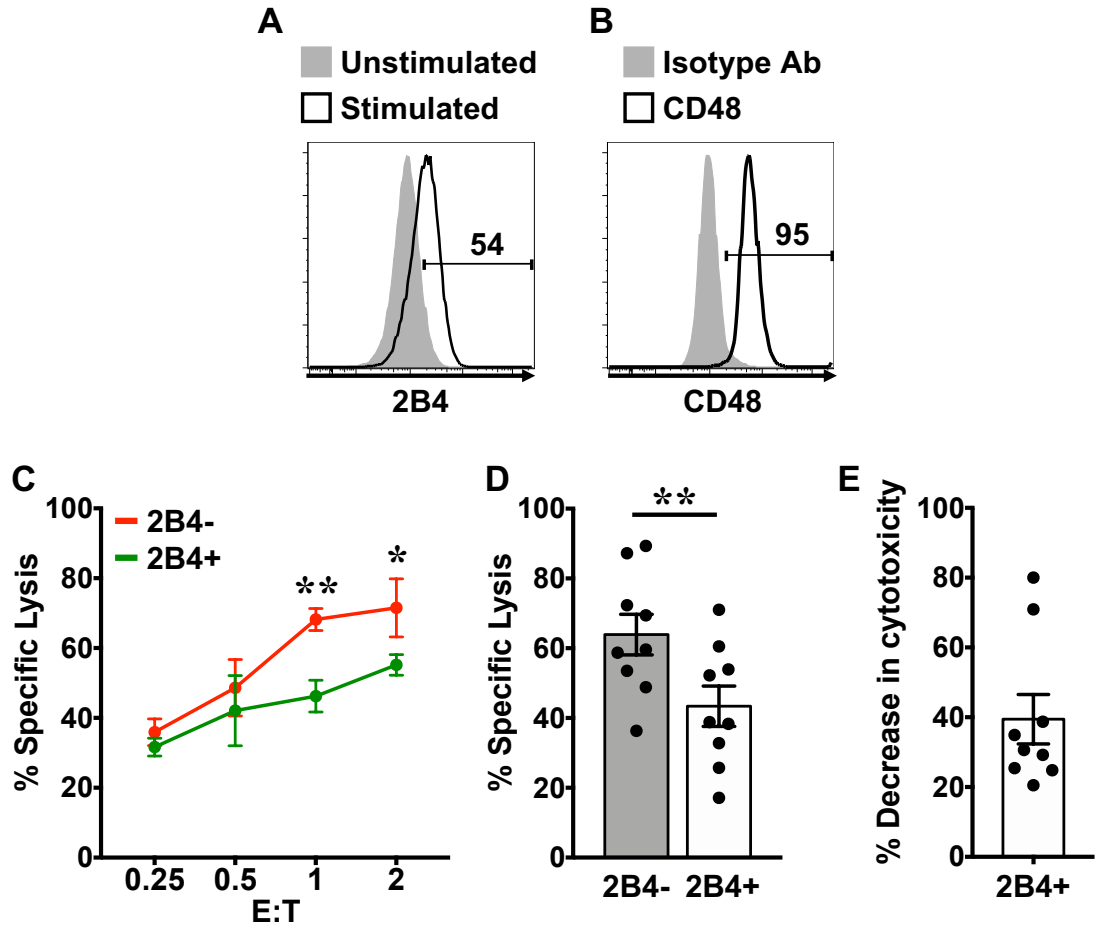


Figure 7. iNKTs expressing 2B4 have reduced cytotoxicity

(A) 2B4 expression on 1.2 cells stimulated with plate bound aCD3 and aCD28 antibodies for 24 hours (bold histogram) compared to unstimulated 1.2 cells (grey solid histogram). (B) Surface expression of CD48 (bold histogram) compared to its own isotype (grey solid histogram) on EL4-Luc target cells, assessed by flow cytometry. (C) *In vitro* cytolytic activity of 1.2 cells that express 2B4 (2B4+) and do not express 2B4 (2B4-) against PBS44-loaded luciferase-expressing EL4 (EL4-Luc) tumor targets at increasing E:T ratios. Data points are from triplicates of 1 of 2 experiments and the error bars represent standard deviation (SD). Statistical significance was determined by two-way ANOVA (*$p<0.05$, **$p<0.01$). (D) Percent specific lysis in 2B4- (grey bars) and 2B4+ 1.2 cells (white bars) against EL4-Luc cells at effector: target ratio 1:1.

Figure 7. (cont'd)

(E) Mean percent reduction in cytolysis of EL4-Luc by 2B4+ iNKTs compared with 2B4- iNKTs. Data shown in D, E is represented as mean ± SEM from 9 experiments and statistical significance in (D) was determined by Student's unpaired t-test with Welch's correction. (**p<0.01).

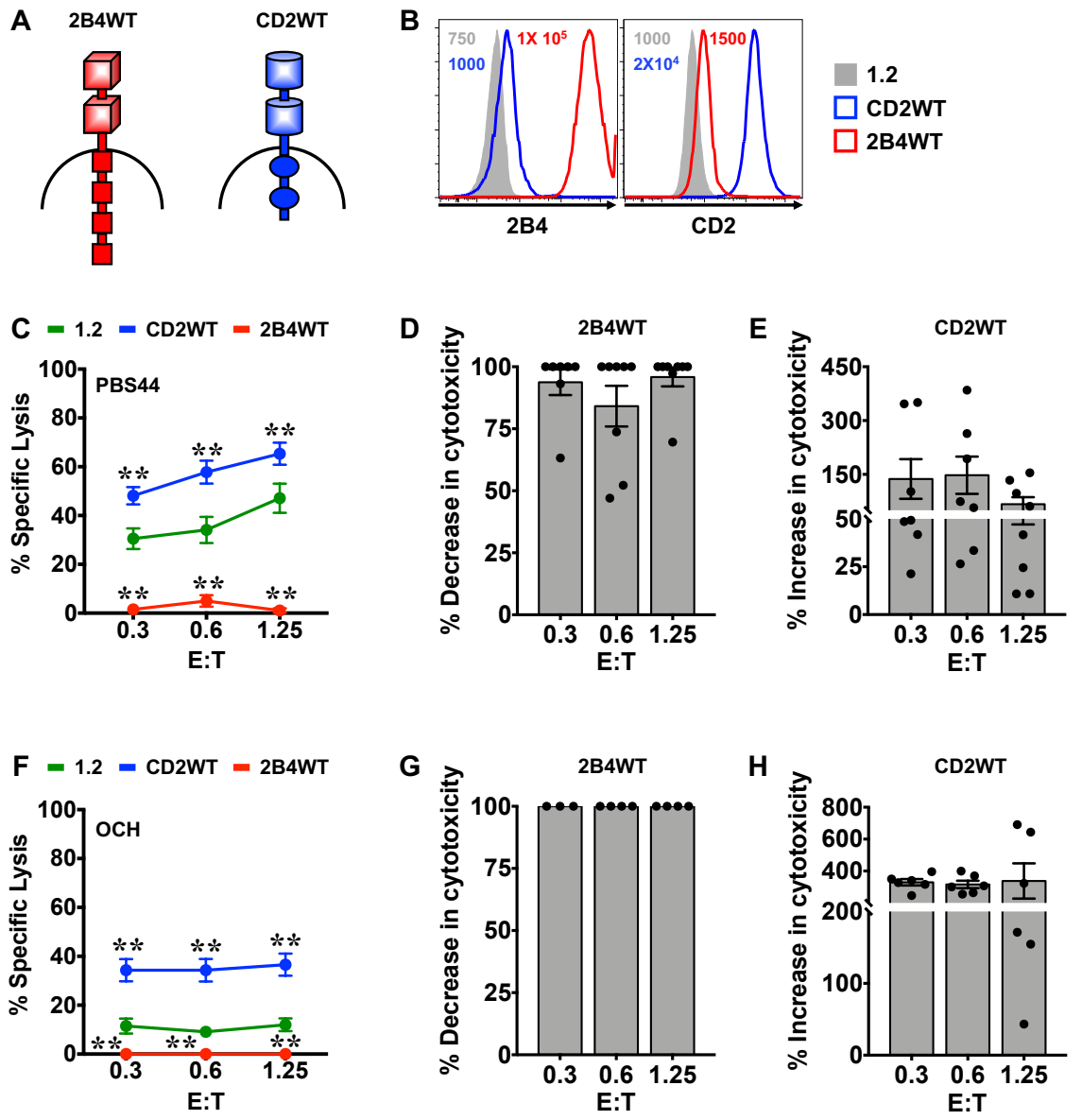


Figure 8. 2B4 is a negative regulator of iNKT cell cytotoxicity

(A) Schematic showing the full-length 2B4 (2B4WT) and CD2 (CD2WT) clones which were expressed using lentiviral transduction and selected with media containing 2μg/mL puromycin. (B) Expression of 2B4 and CD2 by CD2WT (blue open histogram) and 2B4WT (red open histogram) was confirmed by flow cytometry and is compared to untransduced 1.2 cells (solid grey histogram). Numbers in the histogram indicate mean fluorescence intensity (MFI). Lysis of PBS44-loaded (C) or OCH-loaded (F) EL4-Luc cells by 1.2 cells, CD2WT- and 2B4WT- transduced 1.2 cells at increasing

Figure 8. (cont'd)

effector to target ratios (E:T) is shown. Mean percent decrease in killing by 2B4WT-transduced 1.2 cells (D) or increase in killing by CD2WT-transduced 1.2 cells (E) as compared to 1.2 cells using PBS44 as antigen and percent decrease in cytolysis by 2B4WT-transduced 1.2 cells (G) and increase in cytolysis by CD2WT-transduced 1.2 cells (H) as compared to control 1.2 cells, using weak antigen OCH is depicted for the indicated clones. In C-H, the data is represented as mean ± SEM from 3-8 experiments. Statistical significance in percent specific lysis of EL4-Luc cells by CD2WT- and 2B4WT--transduced 1.2 cells as compared to untransduced 1.2 cells in C, F was determined by 2-way ANOVA. (*$p<0.05$, **$p<0.01$).

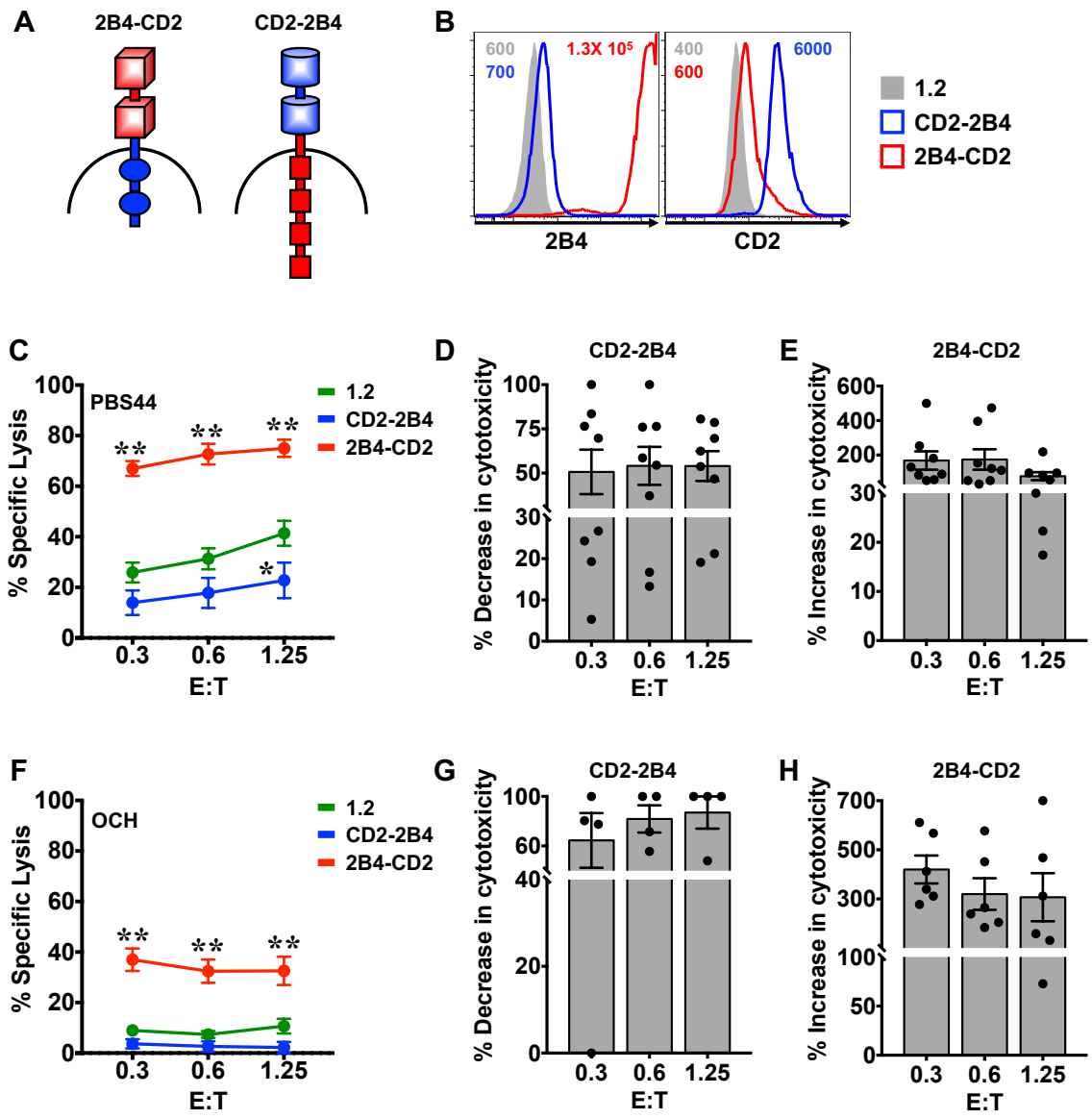


Figure 9. 2B4-CD2 chimeric receptor mediates robust lysis of T cell tumor cells

(A) Schematic showing the 2B4-CD2 construct which contains the extracellular domain of 2B4 with intracellular motifs of CD2 and the CD2-2B4 construct that contains the extracellular domain of CD2 and the intracellular motifs of 2B4, both expressed in 1.2 cells. (B) Over-expression of 2B4 and CD2 in the CD2-2B4-transduced 1.2 cells (blue open histogram) and 2B4-CD2-transduced 1.2 cells (red open histogram) was confirmed by flow cytometry and is compared to untransduced 1.2 cells (solid grey histogram). Numbers in the histogram indicate mean fluorescence

Figure 9. (cont'd)

intensity (MFI). Lysis of PBS44-loaded (C) or OCH- loaded (F) EL4-Luc cells by 1.2 cells, CD2-2B4- and 2B4-CD2-transduced 1.2 cells at increasing effector to target ratios (E:T) is shown. Mean percent decrease in killing by CD2-2B4 cells (D) and increase in killing by 2B4-CD2 cells (E) using PBS44 as antigen as compared to 1.2 cells and mean percent decrease in killing by CD2-2B4 cells (G) and increase in killing by 2B4-CD2 cells (H) as compared to 1.2 cells, using weak antigen OCH is depicted. In C-H, the data is represented as mean ± SEM from 4-12 experiments. Statistical significance of percent specific lysis of EL4-Luc cells by CD2-2B4 and 2B4-CD2 cell, respectively, as compared to 1.2 cells in C, F was determined by 2-way ANOVA. (*$p<0.05$, **$p<0.01$).

CHAPTER 4- DISCUSSION

Immune reactions are controlled by a delicate balance between activating and inhibitory signals transmitted by different cell surface receptors. This balance ensures that the immune response to stimuli is neither weak, nor is it exaggerated. In the case of iNKT cells, several immune receptors have been shown to regulate their responses. In this study, we specifically focused on the role of SLAM*f* receptors in iNKT cell anti-tumor functions. We found that, unlike other SLAM*f* receptors that are basally expressed on iNKTs, 2B4 is largely absent from iNKT cells at rest, except for a small thymic subset. Type II NKT cells, on the other hand, have a robust expression of 2B4 basally. Upon activation, 2B4 is upregulated on iNKT cells and functions as a checkpoint molecule by inhibiting cytotoxic function. Excitingly, by swapping the intracellular domain of 2B4 with an activating receptor we saw an unprecedented augmentation (200-400% increase) in tumor target killing. Collectively, our study brings into focus the untapped potential of modulating 2B4 activity for iNKT cell immunotherapy.

Our studies revealed a few striking observations. One of the major findings was that 2B4 is not expressed in iNKT cells basally except for a small subset of thymic iNKTs. This is surprising since the other members of the SLAM-family receptors have a constitutive expression on iNKT cells[164, 174]. Recent literature suggests that total NKTs differ functionally depending on the organ from which they are isolated. This can be explained in part by our findings that 2B4 is expressed differentially on specific subsets (iNKTs vs Type II NKTs). Type II NKTs express abundant surface 2B4 in all organs even though they make up a small portion of the liver and thymic total NKT cells. We have also observed that 2B4 is dispensable for the development of iNKTs (data not

shown) while the lack of its downstream binding partners like SAP and Fyn are critical for iNKT cell ontogeny[160, 166].

NK, T and iNKT cells also rely on SAP signaling for their cytotoxic functions[166, 237, 253]. Similarly, iNKTs share various other characteristics with NK cells and T cells[20, 24] Like T cells, iNKTs have a TCR, express CD4, and share multiple co-stimulatory molecules, like CD28, ICOS, 41BB, etc. Invariant NKTs also express NK1.1, which is a canonical NK cell marker in B6 mice, as well as other NK cell-specific receptors, like NKG2D, and cytotoxic molecules, like perforin and granzyme[20, 24, 254]. The absence of 2B4 expression in peripheral iNKTs is similar to the expression of 2B4 on T cells[199]. In contrast, NK cells have a very high constitutive expression of 2B4[194]. Unlike in iNKTs, studies have shown that the presence of 2B4 on developing NK cells acts as a failsafe mechanism that restricts their killing of neighboring cells before other regulatory receptors are expressed[219]. Even though iNKTs have multiple similarities with both NK and T cells, the differential 2B4 expression in iNKTs points to a lineage-specific role. Analyzing our data further we see that iNKT cells upregulate 2B4 within 6 hours of activation. In stark contrast, 2B4 on NK cells was observed to be upregulated on Day 7 of LAK cell generation[208] while 2B4 on T cells was observed on Day 5 post activation[199]. One of the reasons for the quick upregulation of 2B4 on iNKTs may be that they are innate T cells and have a short reaction time to the glycolipid antigens[20, 24, 254]. Upon activation, iNKT cells express the long form of the 2B4 receptor, which plays an inhibitory role in cytotoxicity. In other cell types like NK and T cells, the function of 2B4 was not defined as easily since these cells upregulate both the activating and inhibitory isoforms of 2B4 post-stimulation. When the 2B4 receptor was first discovered, it was thought to be a positive regulator of function based on the

phenotype noted in human X-linked lymphoproliferative disease (XLP) patients. In XLP, various mutations in the *SH2D1A* gene result in the absence or functional deficiency of the SAP protein, which causes functional defects in NK and T cells. Initial studies showed that in SAP deficient NK cells 2B4 acted as an inhibitory receptor[253]. On the contrary, in SAP sufficient NK cells 2B4 functions as an activating receptor[253], wherein ligation of 2B4 caused an augmentation of tumor target killing[198, 203, 204, 205] and cytokine secretion[204, 205]. Similarly, in IL2 expanded murine NK cells an increased killing through the ligation of 2B4 using a monoclonal antibody was observed[194]. This suggested that murine 2B4 also had an activating role in NK cell cytotoxicity. However, in line with our findings, more recent studies point to an inhibitory role for the murine 2B4 receptor. Specifically, studies done with *2b4*$^{-/-}$ mice[200, 202] showed an enhanced clearing of CD48+ melanoma cells while 2B4 sufficient wild-type mice were unable to clear the CD48+ tumors[200].

The binding of 2B4 to CD48 causes the first ITSM motif in the 2B4 cytoplasmic domain to be phosphorylated on the tyrosine residue (do you know by what tyrosine kinase?), which in turn recruits SAP and leads to transduction of the downstream signals[215]. Since in XLP patients 2B4 on NK cells behaves as a negative receptor, it was suggested that SAP is the sole decider for the function of 2B4. An elegant study showed that the physiological role of the 2B4 receptor is based on a combination of factors[221]: the amount of 2B4 expression, the degree of 2B4 ligation by CD48, and the availability of SAP. The researchers used cell lines that were transfected such that 2B4 and SAP were expressed at different levels as well as dose titrations of monoclonal antibodies to ligate the 2B4 receptor to different magnitudes. They found that at a low level of 2B4 receptor surface expression, 2B4 behaves as an activating

receptor whereas higher expression levels cause the receptor to be inhibitory. This may be because when there is upregulation in the 2B4 receptor with a limited SAP supply, not enough SAP is available to engage the ITSMs and keep up the activation signal. When SAP is abundantly available, even with high surface expression of 2B4, it behaved as an activating receptor. Physiologically, it can be speculated that when more CD48 is available on the APC, 2B4 is further upregulated. Indeed, this study found that as crosslinking of the 2B4 receptors increases due to more availability of CD48 on the APCs, the function of the 2B4 receptor switches from activating to the inhibitory form. This finding is of significance since CD48 is widely present on hematopoietic cells and its expression is modulated in disease settings[213, 255], which can in turn alter the function of the 2B4 receptor.

Fundamental differences in the function of 2B4 in T cells, when compared to iNKTs, could be explained in a few different ways. The initial studies were done on either freshly isolated T cells or those which were expanded for 5-10 days, either from the spleen or the lymph nodes of mice[199, 222, 224]. During the early phase of T cell expansion, it was identified that both the activating and the inhibitory form of 2B4 were expressed, and it behaved as an activating receptor. In stark contrast, subsequent studies analyzed the role of 2B4 in tumor-bearing mice between 21-30 days, at which point the 2B4 receptor appeared to be a negative regulator of T cell function[225, 226, 228]. These studies show that the kinetics of 2B4 is important for its function wherein 2B4 in the early stages of upregulation acts as an activating receptor, which switches to an inhibitory receptor in the later stages of upregulation. Similarly, the type of antigenic stimulation could also be affecting the function of 2B4. Stimulating freshly isolated human T cells from a healthy donor with strong activating signals through αCD3 and

αCD28 or PMA/i has been shown to elicit a positive functional response in the presence of the 2B4 receptor[236]. On the other hand, stimulating human T cells from donors suffering from cancers with strong ligands in the presence of 2B4 elicited an inhibitory response[239, 256]. A possible explanation could be that T cells that encounter the tumor microenvironment could have chronically encountered weak tumor antigens which caused the upregulation of the inhibitory form of 2B4. Subsequent stimulation with strong activators like αCD3 αCD28 *ex vivo* will continue to elicit an inhibitory response. Hence, here we see that the function of the 2B4 receptor is based not only on the kinetics of the upregulation of the receptor but also on the antigenic stimulation it is provided.

Another striking finding of our study is that 2B4 is a checkpoint molecule for iNKT cell functions, analogous to what has been described for CTLA-4 in T cells[257]. While CTLA-4 has been studied at length in T cells[258, 259] and successfully turned into a target for cancer immunotherapy, the studies on the 2B4 receptor in iNKTs are limited. Two prior studies have shown that iNKTs are detrimental to tumor control in a murine breast cancer model[260, 261]. Mice lacking iNKTs (*Ja18*$^{-/-}$) when administered with the anti-CTLA-4 monoclonal antibody had better survival and slower tumor growth than iNKT sufficient mice. This is suggestive of a tumor-supportive role of iNKT cells, which is at odds with our findings. One possibility for this observation is that since the tumor model in their study is in a Balb/c background, NKT2 cells are predominant[73], which can support tumor growth. On the other hand, the iNKT cell line used in our study is derived from C57BL/ B6, which have a Th1 bias. To the best of our knowledge, there are no studies done to discuss the kinetics of CTLA-4 expression or the effect of the CTLA-4 receptor on iNKT cell functions, which can shed light on the results observed by the

prior studies[260, 261]. While CTLA-4 is known to be expressed on a subset of T cells called regulatory T cells[262], we still do not know if 2B4 exists in a regulatory iNKT subset. Functionally, both 2B4 and CTLA-4 compete with costimulatory molecules, in this case, CD2[189], and CD28[263] respectively, for binding with their ligands. 2B4 binds with higher affinity to CD48 as compared to CD2[189, 264], like CTLA-4 to CD80/86 when compared to CD28[264]. CTLA-4 is indispensable for T cell survival, wherein the lack of CTLA-4 is lethal to mice due to loss of regulatory T cells and unchecked lymphoproliferation[265, 266, 267]. The lack of 2B4 has not shown similar lethal effects on survival in our lab's studies using *2b4*$^{-/-}$ mice (data not shown). This is probably because unlike CTLA-4 which has known roles in central[268] and peripheral[262] tolerance 2B4 does not have any described role in central tolerance. Like 2B4 and CTLA-4, Lag-3 is also not expressed basally on iNKTs but is upregulated post-TCR-mediated activation[269]. In human iNKT cells, the presence of Lag-3 caused a reduction of IFNγ secretion in both CD4+ and CD4- iNKT populations[270]. Invariant NKT cells, as we know, have a dual function of cytotoxicity as well as cytokine production. In our study, we have analyzed the role of 2B4 in murine iNKT cell cytolysis but have not looked into the role of 2B4 in cytokine production. This is because, unfortunately, the iNKT hybridoma 1.2 cells do not produce IFNγ and IL-4 upon stimulation[271].

Unlike 2B4, PD-1 is a well-characterized inhibitory receptor that has been described on iNKT cells but is basally expressed and can further increase upon activation[139]. Once upregulated, PD-1 persisted until at least 30 days from antigenic stimulation. PD-1 expressing iNKTs showed reduced killing, which is similar to what we see in 2B4 expressing iNKTs in our study. PD-1 on iNKT and T cells binds to PDL1 and PDL2 on APCs to mediate the inhibitory signals. Strikingly, blocking the PD-1-PDL1 axis in

iNKTs augmented their Th1 cytokine production whereas the absence of PD-1-PDL2 binding increased Th2 cytokine secretion[272, 273, 274]. Multiple studies showed augmentation of cytokine production and cytotoxicity by blocking the PD-1 pathway in iNKT cells *in vitro* after anergy has been established[139, 274, 275, 276, 277]. Surprisingly, though, in the *in vivo* models, the efficacy of blocking PD-1 was only observed when the antibodies were administered before or concurrently with the αGC treatment[139, 275]. Once anergy has been established, blocking PD-1 was shown to have no effect in the augmentation of iNKT cell responses. Indeed, we have shown that 2B4 is co-expressed with PD-1 on activated iNKT cells, which together could be driving the anergy response.

While our study was underway, other groups have identified various iNKT cell inhibitory receptors like B- and T- lymphocyte attenuator (BTLA4), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), and T cell immunoglobulin and mucin domain containing protein (Tim) family of receptors. Unlike 2B4, BTLA4 has a constitutive expression on resting iNKTs from the thymus, spleen, and liver[278, 279]. Recent studies have shown that blocking BTLA-4 reduced tumor progression and metastasis in a polyoma middle T oncogene-driven mammary tumor[280] and had an inhibitory effect on iNKT cell cytokine production[278, 279]. TIGIT, on the other hand, is an understudied inhibitory receptor that has a similar expression profile on resting thymic[281] and hepatic iNKT cells[282] like 2B4. The lack of the TIGIT receptor caused a skewing of the developing iNKT profile to NKT1 by reduction of NKT2 cells[281] showing that it has a role in iNKT cell development, which is not a function shared by 2B4. Among the Tim family of receptors, Tim-1 was found to be expressed basally in iNKTs. Tim1 when engaged through monoclonal antibodies in

iNKT cells both *in vitro* and *in vivo* reduced their Th1 cytokine production and increased Th2 cytokine production through modulation of T-bet and GATA-3[283].

The 2B4 receptor has marked similarity with the immunoglobulin superfamily member CD2. Unlike 2B4 which is an inhibitory receptor for iNKTs, our lab has demonstrated that CD2 is an activating molecule (data not shown). Interestingly, contrary to compensation of the CD2 receptor function by CD28 in T cells [284, 285, 286], CD2 is indispensable for iNKT functions. Consistent with our findings where CD2 deficient iNKTs failed to kill CD48+ tumor targets (data not shown), overexpressing CD2 in 1.2 cells (CD2WT) resulted in significantly elevated killing (increased by 150%) compared to untransduced iNKTs. The opposing functions of CD2 and 2B4 were more pronounced when we observed that iNKTs that overexpressed 2B4 (2B4WT) had complete abrogation of tumor target killing. In line with these findings, iNKTs with CD2 extracellular domain with the 2B4 intracellular motifs (CD2-2B4) showed a reduction in the killing of tumor targets suggesting the intracellular tail of 2B4 is inhibitory. Excitingly, when we swapped the intracellular domain of 2B4 and replaced it with that of CD2 (2B4-CD2) we saw the highest increase in killing (increased by 200%). This can be explained by the higher affinity of the extracellular portion of 2B4 to the CD48 receptor and the presence of activating CD2 motifs intracellularly driving a strong positive killing response.

The use of the 2B4-CD2 receptor to augment iNKT cell functions has tremendous translational value since we have appreciated unprecedented augmentation in killing (increased by 200-600%) which occurs even in the presence of a weak antigen like

OCH. OCH is a structurally modified form of αGC but has very nominal stimulation of iNKT cells as compared to robust stimulation observed by αGC[117]. In the presence of OCH, iNKT cells show drastically reduced killing of tumor targets[117] and have a Th2 skewing of their cytokines[287]. The reason for the reduced activation of iNKT cells by OCH was found to be a lower avidity for the TCR[288]. Since we know that 2B4 and CD48 have a high-affinity bond, it is possible that the presence of the extracellular 2B4 in the chimeric 2B4-CD2 receptor construct stabilizes the iNKT-target cell conjugation and allows for robust activation even in the presence of a weak antigen. This is particularly of great interest since the tumor microenvironment is rich in lipids and their metabolites, which can function as weak antigens and can be recognized by iNKT cells through CD1d[289, 290]. Additionally, the superior augmentation in killing by the 2B4-CD2 mutant cells was greater than that recorded in pre-clinical models of various CAR-iNKTs[144, 291].

Interestingly, spontaneous CTLA-4-CD28 fusion proteins have been found in pediatric and adult hematopoietic tumors[292, 293, 294, 295]. Experimental analysis with the CTLA4-CD28 fusion also showed increased proliferation and cytokine production by the T cells[294, 296, 297]. Along the same lines, the PD-1-CD28 fusion receptor was shown to enhance T cell cytotoxicity against tumor targets, elevated cytokine production, and proliferation[298, 299, 300]. Excitingly, the effects of using PD-1-CD28 in CAR-T cells have extended to solid tumors[299, 301] that have been largely refractory to immunotherapy.

The role of 2B4 in immune regulation extends beyond cancer and has been shown to play an important role in viral infections and other immune disorders such as

autoimmunity. In T cells, 2B4 has been established as a marker of exhaustion in viral infections[227]. Studies in CD8 T cells showed that 2B4 levels are higher in exhausted T cells in chronic viral infections along with PD-1 as compared to acute viral infections[233, 302]. Similarly, 2B4 is expressed on exhausted T cells in the context of various cancers[303, 304]. In autoimmune diseases like Systemic Lupus Erythematosus (SLE) varying effects of 2B4 expression on T cells were observed. One study showed that when 2B4 was expressed on effector memory T cells, there was an increase in cytotoxicity exacerbating the autoimmunity[305] while another study showed that 2B4 was reduced in cases of SLE with an overall reduction in CD8 T cell cytotoxicity[306]. Interestingly, studies have shown that polymorphisms in the 2B4 gene lead to a predisposition to acquiring SLE[307] and alternative splicing of 2B4 can govern SLE progression[308].

Future perspectives

2B4 has been a receptor of much intrigue in the recent past. While 2B4 has been previously studied in iNKTs in the context of human HIV[309], the kinetics of expression or the isoforms expressed were not delineated and hence the role of 2B4 in iNKT cell functions remained unclear. To the best of our knowledge, our study is the first to show that 2B4 is upregulated post iNKT cell activation and is predominantly expressed in its inhibitory isoform as a checkpoint molecule. Moreover, we have also shown that 2B4 plays an inhibitory role in iNKT cell cytotoxicity. A striking finding of our study is that, unlike other SLAM*f* receptors that are inhibitory in the absence of SAP, 2B4 is a unique SLAM*f* receptor that is inhibitory even in the presence of SAP.

Most of the SLAM*f* receptors studied in iNKT cells are basally expressed and are positive regulators of iNKT cell function. Like 2B4, Ly9 has been shown to have an inhibitory function as well[310]. Ly9 also has ITSM motifs in its cytoplasmic domain which can recruit SAP or SHP-2[311]. Interestingly, unlike 2B4, Ly9 is a homotypic receptor that is constitutively and highly expressed on iNKTs[312] and has been observed to negatively affect iNKT cell development[179]. SAP, which is a critical protein required for iNKT cell development and functions, has also been shown to be important for the development and function of Type II NKT cells[51]. Type II NKTs have been observed to be counterregulatory to iNKT cell functions in anti-tumor responses[313]. It is enigmatic that we found constitutive and abundant expression of 2B4 on Type II NKTs from various organs. The relevance and understanding of how the 2B4 receptor modulates Type II NKT response needs to be investigated.

While our studies have not shown *in vivo* persistence of the 2B4-CD2 cells, we provide a framework for these studies to be carried out in the future since our results show a remarkable increase in killing by these cells even in the presence of a weak antigen. Even though in this study we have only looked at the role of 2B4 in iNKTs and manipulated the 2B4 receptor to augment iNKT cell responses, there is immense scope in a cancer setting to further regulate 2B4 activity using blocking antibodies. Recent studies in a mouse model of head and neck cancer have shown that blocking 2B4 in T cells using antibodies is beneficial in controlling cancer progression[228]. How the blocking of 2B4 on iNKT cells, either by itself or in conjunction with other inhibitory receptors, affects cancer control is not known yet. Based on the success we have noted in augmenting iNKT cell cytotoxicity using the 2B4-CD2 receptor, we can also create a recombinant engineered CAR-iNKT cells that contain this chimeric receptor.

This is relevant since all hematopoietic cells express CD48[314, 315], which persists in various hematological cancers as well as in virally infected cells. Our finding that 2B4-CD2 augments iNKT cell responses in the presence of a weak antigen is also promising since CAR-iNKTs with the modified receptor can be used in solid tumors, which have been known to be largely refractory to other augmented T cell treatments.

Although our study has been focused on understanding iNKT cell functions in the context of cancer, we can also extend the usefulness of these findings to other immunological conditions, in which both iNKT cells and the 2B4 receptor are involved. The use of the CD2-2B4 or recruiting the inhibitory function of 2B4 using monoclonal antibodies can be theoretically used in autoimmune disorders, like SLE or asthma, in which iNKT cell functions are undesirable. Our study has been primarily performed to delineate the role of 2B4 in murine iNKTs and we know that there are fundamental differences between the mouse and the human 2B4 receptor. An important question to answer in subsequent studies is to identify the role of 2B4 in human iNKT cell functions. The future directions and studies that can be carried out based on this body of work are diverse, clinically relevant, and will lead to better modulation of various disease outcomes, most importantly in cancer.

APPENDIX

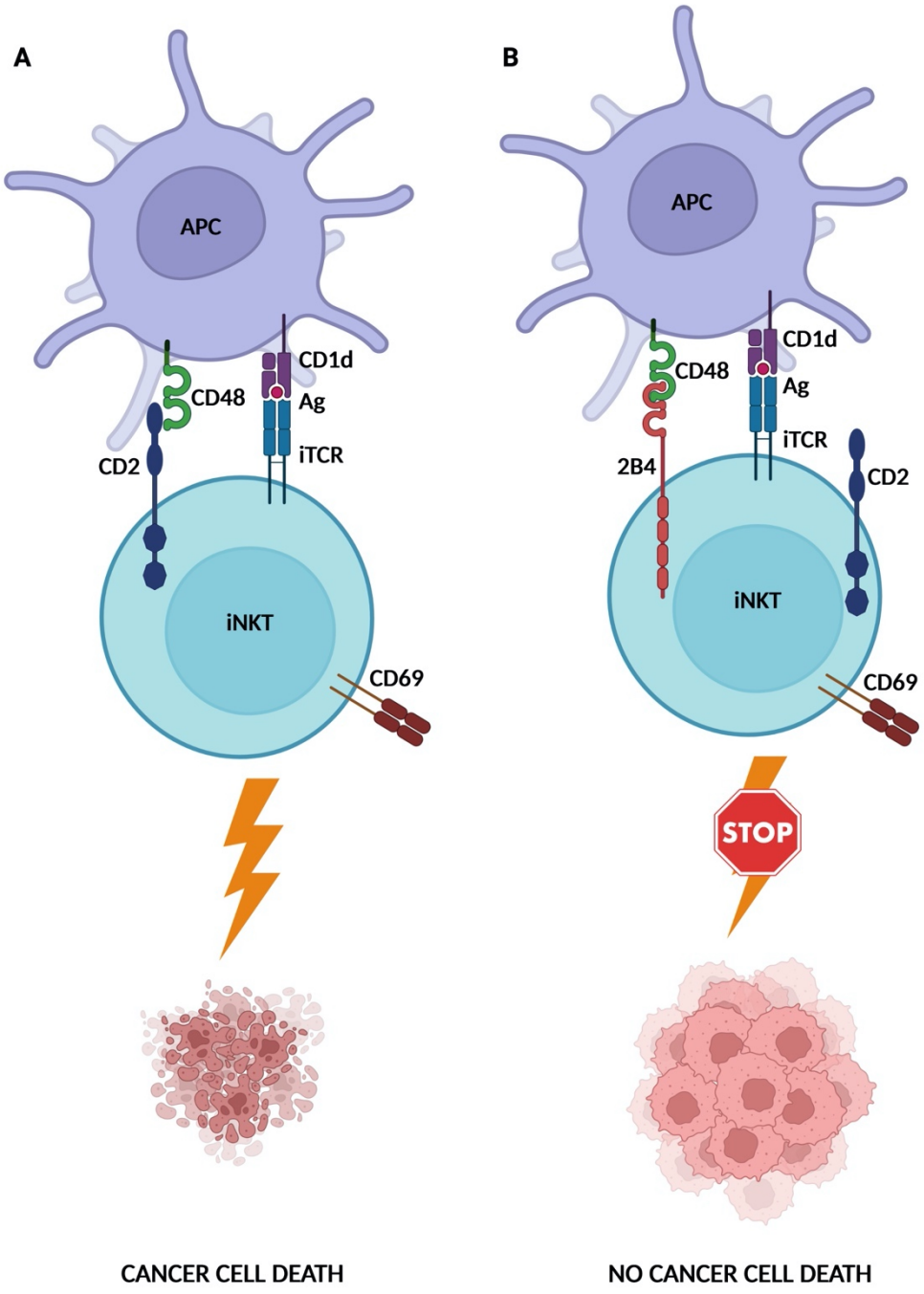


Figure 10. 2B4 is a checkpoint molecule for iNKT cell cytotoxicity

(A) Recognition of the glycolipid antigen by the TCR causes activation of the iNKT cell. CD2, which is basally expressed, binds to CD48, and mediates tumor cell killing. (B) Subsequently, 2B4 becomes upregulated and competes for CD48 binding with CD2, to mediate an inhibition of iNKT cell cytotoxicity.

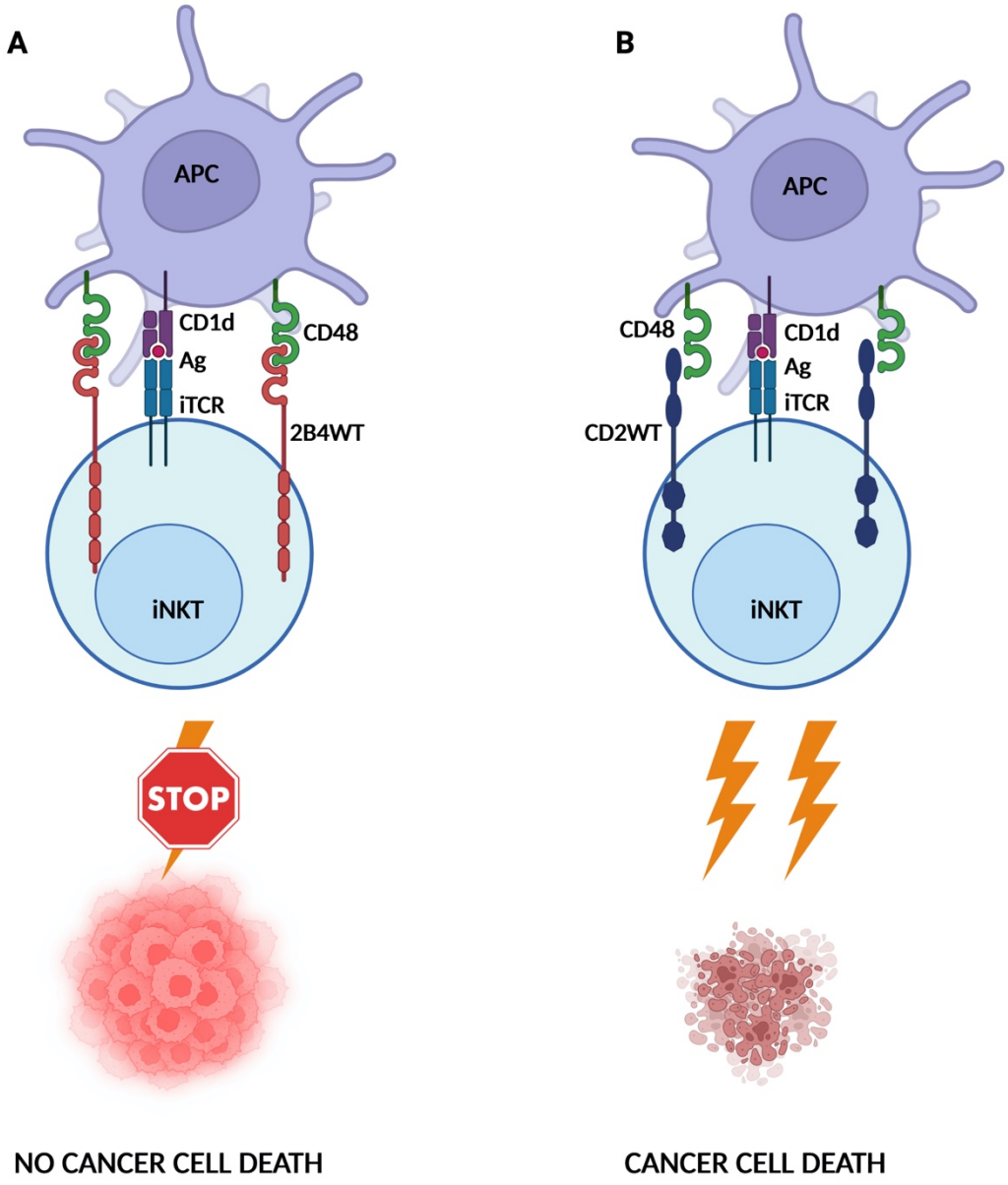
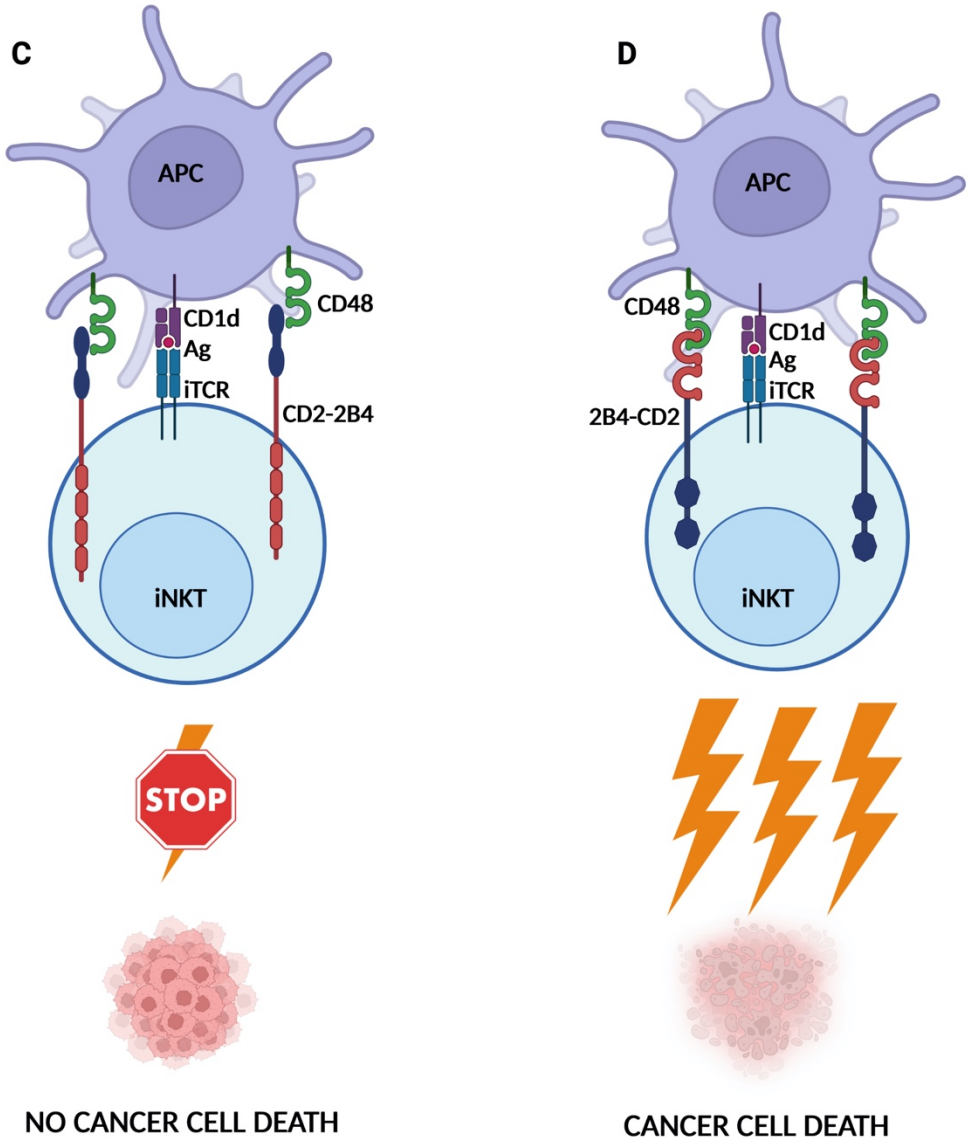


Figure 11. Effect of different iNKT receptor constructs on cancer cell killing

(A) Overexpression of the 2B4 receptor (2B4WT) leads to a complete abrogation of iNKT cell killing. (B) CD2WT iNKT clones have superior cytolytic capacity.

Figure 11. (cont'd)



(C) Swapping the CD2 intracellular domain with 2B4 ITSM motifs (CD2-2B4) shows a reduction in cancer cell cytolysis. (D) Unprecedented augmentation of killing by iNKT cells expressing 2B4 extracellular domain with CD2 intracellular motifs (2B4-CD2).

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