The Innate Effector Programmes of Invariant Natural Killer T-cells.

by

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Started from the bottom: now we here!

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"What the caterpillar calls the end of the world, The Master calls a butterfly."- Richard Bach.

"BITTER: always a bit unanticipated. Coffee, chocolate, rosemary, citrus rinds, wine. Once, when we were wild, it told us about poison. The mouth still hesitates at each new encounter. We urge it forward, say, Adapt. A certain connoisseurship of taste, a mark for how you deal with the world, is the ability to relish the bitter, to crave it even, the way you do the sweet. Now, enjoy it."

- 'Sweetbitter,' Stephanie Danler.

Abstract

T-cell effector functions exemplify adaptive immunity in that they 'adapt' to the threat at hand via a programme of antigen recognition, clonal expansion and subsequent contraction, and the generation of long-lived memory cells as insurance against subsequent insult. Invariant Natural Killer T (iNKT) cells are a lipid-reactive lineage of T-lymphocyte which, via an invariant T-cell receptor and epigentically 'poised effector' status, combine both the specificity of adaptive immunity as well as the rapid 'trained' responses of innate immunity. While a lot has been written about adaptive, antigen-specific or TCR-driven mechanisms of iNKT functionality, less is known about non-TCR or innate cues that drive iNKT responses. This disquisition aims to explore the same via enquiring after the functional consequences of having unusually high cell-surface expression of the integrin and co-stimulatory molecule Leucocyte Function-associated Antigen-1 (LFA-1) as well as the means by which iNKT cells serve as cellular adjuvants in a humanised mouse model of EBV-driven lymphomagenesis.

For the former, we stimulated clonal or short-term polyclonal lines of human iNKT cells with plate-bound ICAM-1-Fc in the absence of antigen-presenting cells, but in the presence or absence of exogenous IL-12p70 to investigate the role that LFA-1:ICAM-1 interactions play in co-stimulating TCR-independent means of cell responsiveness. Surprisingly, we found that ICAM-1-fc alone is sufficient to drive IFNγ from iNKT cells, and does not require concurrent TCR or pro-inflammatory cytokine cues.

Immunotherapy work was performed within an *in vivo* model of EBV-driven lymphomagenesis using humanised mice. iNKT cells appear to promote tumour clearance via the activation of

endogenous MHC-restricted T-cells. Whether or not the iNKT-TCR is involved in potentiating this programme of adjuvanticity will be a topic for future experiments.

Ultimately, the work curated here builds a case for the versatility of iNKT cells via their ability to be sensitive to changes in their milieu that do not always involve direct antigen presentation. This ability to ‘stay current’ is what posits iNKT cells as a vital conduit, bridging the ‘communication gaps’ between classically innate and adaptive immune responses.

Introduction

Innate-like Immunity:

A central theme in immunology is the separation of immunocytes based on whether they mediate innate or adaptive immunity. Some of the criteria that have defined an innate immune cell include the presence of germline encoded receptors with a limited range of antigen recognition coupled to a near-immediate response time but no generation of immune memory[1,2]. The Natural Killer (NK) cell, for example, has been long regarded a classic innate immune cell. It is a rapid responder to virally infected or neoplastically transformed cells, and the activation of NK cells depends not on the recognition of a specific antigen, but ligands associated with stress[3].

On the other hand, adaptive cells recombine antigen receptors to generate means to respond to a diverse antigen range, and while the response time of adaptive cells is slow in comparison, the response is specific i.e. one that *adapts* to the insult at hand. MHC-restricted T-cells are prime examples of adaptive cells which recognise threatening stimuli, increase in number and generate a response 'customised' to the threat, followed by a phase of contraction. As an added bonus, there is also the generation of immune memory such that subsequent offences are dealt with swiftly[1].

It has been long appreciated that cooperation between the innate and adaptive arms is required for a robust, functional immune response. Facilitating this communication appears to be the forte of an emerging class of immunocyte: innate-like lymphocytes. Innate-like lymphocytes combine (perhaps the best) features of both innate and adaptive cells[4]. They express recombined antigen receptors which, like germline-encoded pattern recognition receptors, recognise a limited repertoire of non-peptide antigens[5,6]. They respond rapidly, within minutes to hours of stimulation, but do not generate immunological memory or show any phenotypic changes at the cell-surface upon subsequent antigen exposure. Innate-like lymphocytes are known to secrete a variety of

immunomodulatory cytokines[7,8], and are amongst the earliest responders to pathology or transformation. If we think of the immune response as a cascade, the initial early response of innate-like immunocytes is the 'seeding crystal' or catalyst to a cascade which builds as it travels through subsequent levels of the immune response. Thus, the purpose of innate-like immunity is to potentiate what may eventually become a specialized, specific response.

Innate-like T-cells include invariant Natural Killer T (iNKT) cells, Mucosa-associated Invariant T (MAIT) cells and $V\delta2^+$ $\gamma\delta$ T-cells[5,6]. This dissertation shall focus on the biology of human iNKT cells, their responsiveness to innate, T-cell receptor (TCR)-independent stimuli and their ability to mediate tumour rejection.

T-Cell Receptor-mediated Activation in Classical and Innate-like T-cells:

In the 1970s Doherty and Zinkernagel demonstrated that simultaneous recognition of foreign peptide and self-major histocompatibility (MHC) molecules was required for T-cell activation[9,10]. The subsequent discovery of the $\alpha\beta$ TCR[11] gave us what is often thought of as the first signal for T-cell activation as well as the idea that T-cells are MHC-restricted[12,13] i.e. will only recognise foreign peptides when they are presented on a particular MHC molecule. TCR:peptide-MHC interaction alone is not sufficient to activate T-cells[14], and may result in anergy[15,16]. MHC-restricted T-cells, upon antigen recognition, also require costimulation[17] as mediated by a variety of co-stimulatory molecules of which the best characterized include the B7 family[18] of proteins and the integrin Leucocyte Function-associated Antigen-1 (LFA-1)[19–21]. Finally, pro-inflammatory cytokines like IL-12, IL-18 and type I interferon[22–27] also enhance T-cell functional responses.

To compare and contrast, the TCRs of innate-like T-cells are also made up of α and β chains, but are nearly invariant with human iNKT cells almost exclusively expressing $V\alpha24\text{-}J\alpha18$ TCRs[28]. Innate-like T-cells are also restricted by an antigen-presenting molecule. In the case of iNKT cells,

this is the MHC-like molecule CD1d[28,29], of the family of evolutionarily conserved CD1 molecules. The antigens that CD1d presents can be both self[30–36] or non-self lipids or glycolipids instead of linearized peptides. Alpha-galactosylceramide (αGC) has been appreciated as the prototypical iNKT TCR agonist[37,38]. The α in the name refers to the orientation of the glycosidic linkage between the fatty acid tail and sugar head group[39,40]. αGC, however, is a synthetic glycolipid derived from a sea sponge[38], however its biochemistry gave rise to the idea that α-linked glycolipids may be candidate antigens for the iNKT TCR. Given that mammals do not link lipid tails to carbohydrate head groups in the α orientation, this biochemical motif represented a foreign pattern that iNKT cells could react to. Indeed, α-linked glucuronic and galacturonic acids from *Sphingomonas spp*[41,42], a-linked glucosyl and galactosyl diacylglycerols from *S. pneumoniae*[43] and *B. burgdorferi*[41] as well as cholesterol esters from *H. pylori*[44] have all been shown to be recognized by iNKT cells. However, it is not clear that TCR-mediated recognition is the principal mode by which iNKT cells are activated during bacterial infections. Neither is it clear that traditional co-stimulatory molecules like B7/CD28 are absolutely required for iNKT activation. Most of the evidence seems to suggest that indirect activation via APC-derived cytokines may be critical for the functionality of iNKT cells.

TCR-independent Pathways of T-cell Activation:

The primary non-TCR stimuli which T-cells respond are pro-inflammatory cytokines. Naïve, antigen-inexperienced MHC-restricted T-cells are poor responders to cytokine signals alone. Analyses by Tough and Sprent showed that T-cells could be driven to make IFNγ in antigen/TCR-independent way in response to pro-inflammatory cytokines such as IL-12, IL-18 and Type IFNs. The predominant responders to this 'bystander activation' included $CD44^{hi}$ memory $CD8^{+}$ T-

cells[45–49]. Memory CD4$^+$ T-cells have also been shown to undergo cytokine-mediated activation, but less efficiently than their CD8$^+$ counterparts[50–52].

iNKT cells are also capable of being directly activated by pro-inflammatory cytokines including IL-12 [53,54], IL-18[55,56], IL-23[57,58] , IL-25[59] and IL-33[60]. iNKTs are remarkable in that they have a high cell-surface expression of the IL-12R at the steady state[61], unlike classical MHC-restricted T-cells which, as stated above, need to become antigen-experienced in order to be able to undergo so-called bystander activation by pro-inflammatory cytokines. A variety of APCs make IL-12 in response to viral or bacterial ligands, and this IL-12 has been shown in both in vivo and in vitro systems to be able to activate iNKT cells[32,62–65]

One does not mean to suggest that TCR-dependent activation is dispensable for iNKT activation. Given how ubiquitously CD1d is expressed[66–68], it is very like that both TCR and cytokine signals work in synchrony to induce iNKT cell responses. Work from our lab has shown that sub-optimal TCR stimulation of iNKT cells epigenetically primes them to respond to subsequent cytokine signals[69,70].

Non-cytokine, TCR-independent Means of Activation:

The name 'natural killer T-cell' came to being because iNKT cells were initially characterized as CD3$^+$[24,71–74] cells which also expressed NK markers such NK1.1[75]. In time, it has been shown that this name is inaccurate: iNKT cells are not particularly cytolytic and not all iNKT cells express NK1.1[76]. NK1.1$^+$ T-cells are capable of making IFNγ when the ligand is cross-linked by anti-NK1.1 antibodies[77] but it is not known if iNKT cells may be able to do this. Human iNKT cells certainly express the NK-cell marker CD161[78,79] whose ligand CLEC2D is induced upon virally infected B-cells. It has been shown that CLEC2D-CD161 engagement induces IL-7 and IFNγ from T-cells, but depresses NK cell functions[80]. It would be interesting to see how CD161 modulates

iNKT functions in homeostasis and disease. Other activating NK receptors such as NKG2D also augment iNKT cell activation[81–83] while the presence of inhibitory NK cell markers on iNKTs act as a means of limiting iNKT responses[84,85].

Taken together, these means of iNKT activation show an integration of data from a variety of sources—TCR, cytokine receptors, and NK receptors and ligands, to lead to a response most appropriate for the context that the iNKT cells finds itself in. Further chapters of this dissertation will show the role of the costimulatory adhesion receptor LFA-1 in mediating iNKT activation as well as iNKT-classical-T interactions in anti-tumour responses.

PLZF: The Molecular Directive of Innate-like, Adapative-esque iNKT Features:

But what makes iNKT cells so distinct from classical MHC-restricted T-cells given that they both arise from the same pool of precursor cells? Here, too, there are both similarities and stark differences between iNKT and classical T-cell ontogeny. Pioneer thymocytes which may be, depending on signaling and context, coaxed into differentiating into B-cells, T-cells, NK cells or various cells of the myeloid lineage are c-kit^{+} and do not express CD4 or CD8. This stage—Double Negative-1[86,87] or DN1—proceeds to DN2 which is characterized by expression of CD44 and CD25 followed by DN3 wherein the cells are CD44^{-} CD25^{+}. The DN3 stage is where commitment to the T-cell lineage occurs. It is here that TCRs are rearranged, and cells undergo swift proliferation into double positive (DP) CD4^{+} CD8^{+} thymocytes[88–91].

This shows that not only does iNKT emergence occur quite late into T-cell development, but that a lot of the signal transduction that pushes thymocytes from one stage to the next is identical for both iNKT and classical T-cells. It is at the double-positive (DP) stage that iNKT cells part ways from their MHC-restricted counterparts.

Cells of the DP stage which have rearranged the canonical iNKT TCR[92] (Vα24-Jα18 in humans) have the ability to recognise self-lipid antigens presented on CD1d expressed by cortical thymocytes (rather than thymic epithelial cells)[93,94]. While the identity of these self-lipids is not known and the mechanism by which the self-lipids are loaded onto CD1d also remains elusive, it is clear that Cathepsin L is essential for lipid loading and subsequent iNKT development[95].

The CD1d recognition event represents a strong TCR signal to incipient iNKT cells which, when coupled to homotypic interactions of members of receptors of Signaling Lymphocytic Activation Molecule (SLAM) family, SLAMF1 and SLAMF6[96–99] and the expression of Early Growth Response proteins 1 and 2 (EGR1 and EGR2)[100] are definitive events in the commitment to the iNKT lineage. The expression of EGR1 and 2 is what, in fact, directs the upregulation of the transcription factor Promyelocytic Leukaemia Zinc Finger (PLZF)—the signature transcription factor of iNKT cells, as well as other innate-like T-cells[100–102].

Yet, the kinetics of PLZF differs amongst $PLZF^+$ T-cells. While PLZF levels are high during the development of iNKT cells, and eventually wane to a more moderate and stable expression as mature iNKTs emerge into the periphery[8], mucosa-associated invariant T-cells or MAIT cells do not express PLZF until quite late in their development i.e. when they are high expressors of CD44 and have rearranged their canonical invariant TCR (Vα7.2-Jα33). MAIT cells, like iNKT cells, originate in the thymus and separate from classical MHC-restricted T-cells at thd DP stage as described above. Like iNKT cells, the positive selection of MAIT cells also occurs via an unconventional antigen presenting molecule, in this case, MR-1 which is known to present products of microbial vitamin B metabolism[103–106]. MAIT cells, like, iNKT cells can be activated by pro-inflammatory cytokines in a TCR-independent manner[107], appear to home preferentially to the gut lamina propria[105], can co-secrete IFNγ and IL-17A[108], and while certainly high for LFA-1

expression (as our work shows), they are, amongst T-cells, the highest expressors of the integrin Very Late Antigen-4 (VLA-4)[109], though it remains unclear if this a PLZF-dependent characteristic.

Also $PLZF^+$ is the most frequent γδ T-cell subset in humans: $V\gamma9V\delta2^+$ T-cells. It is understood that αβ and γδ T-cells emerge from a common thymic precursor[110], and rearrange TCRs via V(D)J recombination. The commitment to the γδ lineage is thought to occur at the DN3 stage (described above) with *TCRd, TCRg* and *TCRb* rearrangements occurring at the DN2 stage of T-cell development[111,112].Vγ9Vδ2 T-cells are PLZF expressors, though when the acquisition of PLZF occurs during development in humans remains unknown. It must be noted that an 'innate-like' subset of murine γδ T-cells—the Vγ1Vδ6.3s—shadow an iNKT-like ontogeny, including kinetics of PLZF expression[113]. Unfortunately, it isn't quite clear to me that murine Vγ1Vδ6.3 γδ T-cells are equivalent to human Vγ9Vδ2s, and thus I will stop here with regards to talking about the development of this subset. Abundant in peripheral blood in humans and activated by minute amounts of phosphoantigens, what the cognate antigen-presenting molecule for Vγ9Vδ2 T-cells is still remains enigmatic. Yet, like iNKT and MAIT cells, Vγ9Vδ2 T-cells also express high LFA-1, NK cell markers, and are capable of co-secreting T_H1, T_H2 and T_H17 cytokines[114]. What follows is a table summarizing some of the salient features of the $PLZF^+$ T-cell trinity outlined above after which I shall return to a discussion of PLZF-mediated gene regulation with a focus on iNKT cells.

Cell Type	*TCR Rearrangement*	*Prototypical ligands.*	*Antigen Presenting Molecule*	*Tissue Localisation*
iNKT	Vα24-Jα18	Glycosphingolipids, Lysophospholipids, α-linked glycolipids.	CD1d.	Omentum.
MAIT	Vα7.2-Jα33	Small molecule vitamin B metabolites of microbial origin.	MR1.	Intestinal mucosa.
Vγ9Vδ2$^+$ γδ T-cells	Vγ9Vδ2	Phosphoantigens of the isoprenoid metabolic pathway.	Unclear; perhaps butyrophilins?[115]	Peripheral blood.

Table 1. **Comparing and contrasting PLZF$^+$ T-cells.** A summarizing of the differences between PLZF$^+$ T-cells delineated at the level of TCR rearrangements, mode and nature of antigen presentation as well as tissue locales in humans where they are most commonly found.

PLZF regulates a variety of genes responsible for the homing patterns of iNKT cells e.g. high LFA-1 cell surface expression[116], lowered CD62L, upregulated CD44 and expression of CCR4 which binds CCL17—a chemokine produced by cross-presenting DCs[117–119]. Among immune functionality-related genes, PLZF binds the genes responsible for IL-12R and IL-18R expression, and in the absence of PLZF, iNKT cells display low levels of these pro-inflammatory cytokine receptors[120]. PLZF, in conjunction with c-maf, regulates IL-4 and IL-10 secretion from iNKT cells[117]. While the exact mechanism is not known, PLZF is also responsible for IFNγ production from iNKT cells[118].

More interestingly, ectopic induction of PLZF into classical T-cells enables them to acquire the 'poised effector state' that iNKT cells exist in at the steady state. Forced PLZF expression gives classical T-cells the ability to co-secrete IFNγ, IL-4, GM-CSF and IL-17 while also acquiring the CD62L^{lo} CD44^{hi} phenotype consistent with iNKT cells. Unlike iNKT T-cells, forced PLZF expression did not make classical T-cells Granzyme B competent or enable the expression of NK-related markers like NK1.1 or NKG2D. This suggests that PLZF is both required and sufficient to imbue cells expressing with iNKT-like features[121].

Upon maturation, iNKT cells emigrate from the thymus into the peripheral tissues bearing a memory phenotype characterized by cell-surface expression of low CD24, high CD44 and the presence of T-cell activation markers such as CD69 and CD122[122]. This is in contrast to recent MHC-restricted $CD3^+$ thymic emigres who express a naïve phenotype. In humans, iNKT cells make up anywhere from 0.01-0.1% of peripheral blood T-cells[78,123]. In mice, iNKT cells are highly enriched in the sinusoids of the liver where they are tethered via LFA-1:ICAM-1 interactions[116], though this is not the case in humans[124]. While widely distributed in human tissues, iNKT cells appear to be enriched in omental fat where they comprise of ~10% of T-cells present[125].

So, why study such a rarefied population of T-lymphocytes anyway? What can such a small population of lymphocytes matter after all?

iNKT Cells in Health and Disease:

My personal discovery of iNKT cells occurred while doing a literature review for my Master's thesis. My MS work was in a murine model of experimental allergic asthma, and around the time I was writing my MS thesis (~2012-13), there was considerable interest in the potential roles iNKT cells play in allergic asthma. And with good reason: here is a population of T-lymphocytes known to patrol the lungs and capable of co-producing T_H1 and T_H2 cytokines. Thus it is appropriate that I start this section with the importance of iNKT cells to allergy.

Mice do not spontaneously develop allergic asthma. In murine models where allergic asthma is induced, it has been unclear whether iNKT cells are required for allergic disease or not. In CD1d-KO mice which lack iNKT cells, allergic mice failed to develop airway hyperresponsiveness (AHR) upon challenge[126,127]. Consistent with these are analyses of human sputum and bronchoalveolar lavage fluid which show increase numbers of iNKT cells in allergic individuals versus healthy controls[128,129]. Yet, other analyses have shown that iNKT cells are dispensable for

AHR[130,131] and do not make up a significant proportion of lymphocytes found in bronchoalveolar lavage fluid or sputum of asthmatic patients[132,133].

The differences seen could relate to the aetiology of the asthma, whether or not the allergen is presented on CD1d, the chemokines and cytokines that are induced in the epithelium upon allergen challenge: regardless, it is apparent that iNKT are a dynamic, adaptable population of T-lymphocytes which can be protective or pathological depending on context.

The Janus-like nature of iNKT cells is especially apparent in models infectious disease. A prime example is *Ehrlichia chafeensis*-mediated disease. *E.chafeensis* is a Gram-negative bacterium lacking LPS. It does, however, produce glycolipid moieties that are presented on CD1d, and lead to iNKT cell activation. In models of this disease, iNKT cells promote bacterial clearance by potentiating DC maturation and CTL expansion, but they also promote sequelae associated with toxic-shock-like syndrome via prodigious production of TNFα, IL-10 and IFNγ[134]. The same theme of a 'well meaning' iNKT response which turns pathogenic has also been demonstrated in several models of polymicrobial sepsis[135–137] and multiple sclerosis[138,139].

Lipid perception of iNKT cells posits them as sentinels and, perhaps, players in the development and pathogenesis of metabolic disorders. Of particular relevance to this disquisition is atherosclerosis. Atherosclerosis is an obstruction of arterial intima by cholesterol-rich plaques, and thus is a harbinger of potentially fatal conditions such as coronary heart disease, stroke, myocardial infarction, peripheral artery disease and chronic kidney disease[140–142]. The aetiology of atherosclerotic plaques is being actively investigated, but it is clear that T-cell derived IFNγ is required to direct pro-atherogenic myelomonocytic populations[143], and that atherogenesis is associated with an upregulation of adhesion molecules (e.g. ICAM-1)[144–146]. Incidentally, iNKT cells—known for the lipid antigen proclivity, the ability to secrete IFNg and express high levels of

cell surface LFA-1 (cognate ICAM-1 receptor)—make up 3% of T-lypmhocytes infiltrating atherosclerotic plaques[147,148]. Moreover, murine models have shown that atherosclerosis is ameliorated in iNKT-deficient mice[149–151]. Given that one of my projects shows that ICAM-1:LFA-1 ligation is enough to drive IFNγ secretion from iNKT cells, it is tempting to herald this pathway as being singularly important for the initial burst of gamma interferon during incipient atherogenesis. Yet, the importance of the CD1d-iNKT-TCR axis in the promotion of atherosclerosis cannot be ignored[152], and it is very like that in a physiological context both ICAM-1 and CD1d cooperate to bring forth a cytokine response from iNKT cells. Thus, the finding in chapter I presents an avenue worth exploring when asking how atherosclerosis is initiated.

The ability of innate lymphocytes to respond to both TCR and cytokine-originating cues as well as other inflammatory or danger-associated prompts such as the upregulation of neoplasia or stress-associated ligands, it is no surprise that innate T-cells are seen as valuable agents of cellular immunotherapy against cancer. In humans, the frequency or functionality of iNKT cells is highly correlated with overall survival in cancers such as neuroblastoma, medulloblastoma, melanoma, prostate cancer, and head and neck cancers[153,154]. In the case of tumours that express CD1d, iNKT cells may be direct cytolytic effectors[56,155,156]. Alternatively, iNKT cells can also promote the adaptive anti-tumour response by 'training' dendritic cells to express activation markers such as CD70, CD80 and CD86 which position them as better activators of conventional CD4 or $CD8^+$ T-cells[157,158]. Yet, paradoxes remain. In studies of haematological malignancies, iNKT cells appear to inhibit anti-tumour responses via the production of T_H2 cytokines[159,160]. It remains unclear as to whether it is the tumour milieu or the antigenic lipids in the immediate environment that enable a suppressive iNKT phenotype. Thus, the 'humanised' NSG mouse model detailed in chapters II and III presents a valuable pre-clinical avenue to address similar mechanistic questions.

Ultimately, these findings from models of disparate disease show that iNKT cells are a potent force in the final outcome of an immune response. Going back to the early idea of immune-response-as-cascade, iNKT cells, though a minute population, represent that initial spark which eventually builds into a conflagration. Thus, iNKT cells are attractive therapeutic targets and potential agents of immunotherapy once their basic biology is more satisfactorily understood.

Chapter I: LFA-1 Ligation by High Density ICAM-1 is Sufficient to Activate IFNγ Release by Innate T lymphocytes

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Abstract

By binding to its ligand ICAM-1, LFA-1 is known to mediate both adhesion and co-stimulatory signaling for T cell activation. The constitutively high LFA-1 cell surface expression of invariant natural killer T (iNKT) cells has been shown to be responsible for their distinctive tissue homing and residency within ICAM-rich endothelial vessels. However, the functional impact of LFA-1 on the activation of iNKT cells and other innate T lymphocyte subsets has remained largely unexplored. In particular, it is not clear whether LFA-1 contributes to innate-like pathways of T cell activation, such as IFN-γ secretion in response to IL-12. Using recombinant ICAM-1-Fc to stimulate human iNKT cells in the absence of APCs, we show that LFA-1 engagement enhances their IL-12-driven IFN-γ production. Surprisingly, however, exposure to high densities of ICAM-1 was also sufficient to activate iNKT cell cytokine secretion independently of JAK/STAT signaling. LFA-1 engagement induced elevated cytoplasmic Ca^{++} and rapid ERK phosphorylation in iNKT cells, and the resulting IFN-γ secretion was dependent on both of these pathways. Analysis of freshly isolated human PBMC samples revealed that a fraction of lymphocytes that showed elevated LFA-1 cell surface expression produced IFN-γ in response to plate-bound ICAM-1-Fc. Responding T cells expressed PLZF, a transcription factor that is characteristically expressed by innate lymphocytes, and included iNKT cells, MAIT cells, and $V\delta2^+$ γδ T cells. These results

delineate a novel integrin-dependent pathway of IFN-γ secretion that is a shared feature of innate T lymphocytes.

Introduction

T cells are typically considered to epitomize adaptive immunity. However, it has recently become clear that a fraction of T lymphocytes share with innate lymphocytes the expression of a master transcription factor, Promyelocytic Leukemia Zinc Finger (PLZF)[161]. PLZF is expressed by innate lymphoid cells (ILCs) and human NK cells[101,162,163], but is suppressed in conventional adaptive T cells[102], suggesting it is exclusively associated with an innate functional status. The best known $PLZF^+$ T cells are invariant natural killer T (iNKT) cells[118,164]. iNKT cells utilize a canonical TCRα chain rearrangement that is paired with a limited set of TCRβ chains, recognize conserved lipid antigens presented by non-classical CD1d antigen presenting molecules, and have innate-like functional properties including mediating rapid effector cytokine responses upon primary challenge[28,37,67,165]. Additional subsets of T lymphocytes now known to express PLZF include mucosal-associated invariant T (MAIT) cells and certain γδ T cells[166,167]. These subsets resemble iNKT cells in that they utilize canonical TCR rearrangements, recognize conserved non-classical antigens, and have innate-like functional properties[168,169]. Thus, based on their constrained TCR structures, specificity for conserved ligands, and shared transcriptional program, these T cell subsets can be grouped into a distinct compartment called "innate T lymphocytes"[4]. The specific features of innate T cells that are conferred by their shared expression of PLZF, and that may thus set them apart as a group from adaptive T lymphocytes, remain largely unexplored.

One such distinctive characteristic conferred by PLZF is upregulated expression of the integrin Leucocyte Function-associated Antigen-1 (LFA-1)[116]. LFA-1 plays critical roles in T cell migration via binding to its adhesion ligand Intracellular Adhesion Molecule-1 (ICAM-1), which is

expressed on vascular endothelium and other cell types[170]. The elevated LFA-1 expression of murine iNKT cells has been shown to be responsible for their stable residency in the sinusoids of the liver, which are endothelial vessels that are high in ICAM-1[116,171]. Similarly, under steady state conditions iNKT cells have been observed by intravital microscopy to constitutively patrol other ICAM-rich areas of the vasculature, including pulmonary endothelial surfaces[172]. Additionally, both human and murine iNKT cells have been found to be recruited to atherosclerotic plaques, which are inflamed vascular endothelial areas where ICAM-1 levels may be elevated[147–149,173]. Thus, the elevated LFA-1 expression level of iNKT cells likely plays a key role in their distinctive tissue recruitment and residency patterns. However, what has been less clear is whether their high LFA-1 status impacts the functional responses of iNKT cells.

LFA-1 also plays a key role during TCR-mediated activation. TCR signaling from initial antigen recognition induces the unfolding of LFA-1 from its low-affinity state into higher affinity conformations that are able to bind to ICAM-1[174–176]. LFA-1 binding to ICAM-1 binding leads to the rapid activation of Src-family kinases (e.g. Lck, ZAP-70) and an ensuing signaling cascade resulting in the activation of PLCγ and triggering of downstream Ca^{++} signaling[177–180]. Ultimately, the signaling events contributed by LFA-1 are thought to lower the threshold of antigen required for productive T cell activation[181–183]. iNKT cells are known to recognize certain unusual glycolipids, such as α-galactosylceramide (α-GalCer), as highly potent TCR agonists. However, it is not clear that many pathogenic microbes produce α-GalCer or closely related lipids, and lipids of this type derived from other physiological sources appear to be present in only extremely small quantities[184,185]. In contrast, other highly abundant lipid species (e.g. lyso-phosphatidylcholine and β-linked glycosylsphingolipids) serve as weak TCR agonists for iNKT cells[33,34]. Thus, since iNKT cells probably experience only low levels of TCR stimulation in most physiological settings due to

a scarcity of high affinity and/or an abundance of low affinity antigens, LFA-1 mediated co-stimulation likely plays an important role during their TCR-mediated activation.

Less clear is whether LFA-1 also impacts other pathways of iNKT cell activation. For example, iNKT cells can also become activated directly by pro-inflammatory cytokines (e.g. IL-12), without requiring a concurrent TCR signal[53,69,70]. Indeed, prior studies have suggested that this TCR-independent pathway of activation is the dominant means by which iNKT cells become activated during many bacterial infections *in vivo*, and that the resulting IFN-γ secretion by iNKT cells plays an important protective role[186]. It has been established that the IFN-γ produced by adaptive T cells in response to TCR stimulation is secreted in a directional manner towards the immunological synapse, which is also where LFA-1 is aggregated at the cell surface[187], and we have observed a similar phenomenon for iNKT cells responding to DCs presenting the potent lipid antigen α-GalCer (supplementary Figure 1A). However, in the case of TCR-independent IFN-γ secretion by iNKT cells, it is not clear whether cell contact, and thus LFA-1 engagement, plays any role. This is an important question, since the physiological effects of IFN-γ secretion probably depend largely on the identity and functional characteristics of the recipient cells, and thus IFN-γ that is produced in the context of an immunological synapse with an APC may serve a different purpose than IFN-γ that is produced during contact with other cells, or that is produced in a manner that is independent of cell contact.

We previously observed that cytokine-stimulated human iNKT cells secreted substantially more IFN-γ when B-lymphoblastoid cells were also present, even if the B-lymphoblastoid cells lacked CD1d and thus were not able to deliver TCR stimulation to the iNKT cells[70]. Moreover, the IFN-γ produced by iNKT cells during exposure to IL-12 on an ICAM-1 coated surface appeared to co-localize intracellularly with the tubulin nexus representing the microtubule organizing center

(supplementary Figure 1B), which has been shown in migrating T cells to be positioned adjacent to the area of the plasma membrane where LFA-1 is aggregated[188]. Finally, it has also been observed through intravital microscopic analysis that intravenous injection of the cytokines IL-12 and IL-18 leads to rapid IFN-γ production by the iNKT cells patrolling hepatic sinusoids[189]. Intriguingly, the iNKT cell response in this situation was associated with migration arrest, suggesting that it was associated with conformational changes and signaling by LFA-1. Together, these observations suggested to us that LFA-ICAM interactions might facilitate or enhance TCR-independent IFN-γ secretion by iNKT cells. Therefore, in this analysis we set out to investigate the impact of LFA-1 engagement on TCR-independent IFN-γ secretion by iNKT cells.

Results

ICAM-1binding to LFA-1 co-stimulates cytokine-driven IFN-γ secretion by human iNKT cells

Since it was previously shown in a murine model system that PLZF confers elevated LFA-1 expression, and murine iNKT cells have constitutively high levels of this integrin, we first sought to confirm whether human iNKT cells also show high expression of LFA-1. PBMCs purified from healthy adult subjects were stained for CD3 to identify T cells, and co-stained for CD11a to detect LFA-1, and CD1d tetramer to distinguish iNKT cells. Flow cytometric analysis revealed that the iNKT cells were amongst the brightest CD11a-expressing T cells (Figure 1A, left plots). Analysis of samples from multiple unrelated donors revealed that this was consistently the case, with the iNKT cell subset showing on average ~1.7-fold brighter CD11a staining than tetramer-negative T cells (Figure 1A, right plot).

We next investigated the impact of ICAM-1 exposure on iNKT cell IFN-γ secretion in response to IL-12. Human iNKT cells were incubated in culture medium containing recombinant human IL-

12p70 in the presence or absence of plate-bound ICAM-1-Fc for 24h. Culture supernatants were harvested and tested for secreted IFN-γ by ELISA. While exposure to IL-12 alone was sufficient to induce iNKT cell IFN-γ secretion, the IFN-γ amounts were consistently elevated in the presence of plate-bound ICAM-1-Fc (Figure 1B), suggesting that ICAM-1 exposure co-stimulated the IL-12-driven IFN-γ production. Since LFA-1 is comprised of two subunits, CD11a (integrin α L) and CD18 (integrin β 2), we tested the involvement of each of the subunits. iNKT cells were incubated with IL-12 and plate-bound ICAM-1-Fc in the presence or absence of blocking antibodies against CD11a and CD18. Blockade of either CD11a or CD18 resulted in abrogation of the ICAM-dependent enhancement of iNKT cell IFN-γ secretion, and anti-CD11a blockade completely abrogated the response (Figure 1C). Together, these results demonstrate that ICAM-1 binding to LFA-1 co-stimulates iNKT cell IFN-γ secretion that is driven by IL-12.

LFA-1 stimulation alone is sufficient to induce iNKT cell IFN-γ secretion

To further investigate the requirements for ICAM-1 to promote IFN-γ secretion by iNKT cells, we titrated the density of the plate-bound ICAM-1-Fc. Low densities of ICAM-1-Fc showed no detectable co-stimulatory effect, but as the density increased we observed an abrupt enhancement of iNKT cell IFN-γ secretion in response to IL-12 (Figure 2A, grey circle symbols). Surprisingly, however, at densities of ICAM-1-Fc above this inflection point we observed that iNKT cell IFN-γ secretion was induced even in the absence of IL-12 (Figure 2A, black triangle symbols). To confirm that this effect was not due to IL-12 mediated stimulation, we tested the effect of inhibiting JAK signaling, which is required for IL-12-mediated activation of iNKT cells. Addition of an inhibitor of JAK1 and 2 did not affect iNKT cell IFN-γ secretion that was induced by exposure to

ICAM-1-Fc alone. (Figure 2B). These observations suggested that ICAM-1 exposure may drive IFN-γ secretion by iNKT cells directly via signaling through LFA-1.

Since LFA-1 stimulation is known to induce Ca^{++} signaling in T cells[178,190], we investigated whether iNKT cell IFN-γ secretion in response to ICAM-1 exposure was affected by a calcium signaling inhibitor. iNKT cells were exposed to plate-bound ICAM-1-Fc or anti-CD3 mAb as a positive control, in the presence of titrated concentrations of cyclosporine A (CsA), a drug that inhibits the Ca^{++}-dependent, NFAT-activating serine phosphatase calcineurin[191,192]. IFN-γ secretion in response to either anti-CD3 or ICAM-1 was completely abrogated in the presence of CsA (Figure 2C). We also assessed cytoplasmic calcium levels in iNKT cells exposed to an ICAM-1 coated surface. iNKT cells were cytoplasmically labeled with Fluo-4, a dye that increases its fluorescence in proportion to the surrounding Ca^{++} concentration, and placed onto glass slides coated with ICAM-1-Fc and blocked with poly-L-Lysine. Microscopic imaging every 20 seconds revealed that the iNKT cells on ICAM-1-coated slides appeared comparatively stationary, whereas most of those placed onto control slides coated with poly-L-Lysine were more migratory (data not shown). Analysis of the Fluo-4 signal intensity over time for a selection of cells from each condition revealed greater spiking of Fluo-4 signal intensity over time in the ICAM-1 exposed iNKT cells compared to isotype control (Figure 2D). Together, these results suggest that Ca^{++} signaling resulting from LFA-1 engagement by ICAM-1 is involved in this pathway of iNKT cell IFN-γ production.

We have previously documented that iNKT contact with APCs potentiates IFN-g secretion in response to subsequent IL-12 stimulation, and that this effect is highly dependent on ERK phosphorylation in iNKT cells[69]. To investigate whether MAPK signaling may play a role in LFA-1 mediated activation of iNKT cells, we first confirmed that anti-CD11a mAb stimulation is

sufficient to induce ERK phosphorylation in Jurkat T cells. Jurkat cells were exposed to anti-CD11a mAb stimulation for varying amounts of time, then Western blotting of lysates was performed to detect total or phosphorylated ERK. As shown in Figure 3A, anti-CD11a mAb treatment resulted in comparatively efficient ERK phosphorylation, with signals peaking after 6-9 minutes of stimulation, and then declining. The ERK phosphorylation signal was abrogated when anti-CD11a stimulation was performed in the presence of the MEK inhibitor U0126 (Figure 3A). Similar to Jurkat cells, when human iNKT cells were exposed to anti-CD11a mAb stimulation, we observed evidence of ERK phosphorylation by Western blotting, and this was inhibited in the presence of U0126 (Figure 3B). To evaluate whether ERK phosphorylation was required for iNKT cell IFN-γ secretion in response to ICAM-1, iNKT cells were incubated on ICAM-1-Fc coated plates or with anti-CD3 mAb, in the presence of titrated concentrations of U0126. While the iNKT cell IFN-γ response to anti-CD3 mAb stimulation was partially inhibited by U0126, the response to plate-bound ICAM-1-Fc was almost completely abrogated by the presence of U0126 (Figure 3C). Thus, compared to TCR-activation, iNKT cell IFN-γ secretion in response to LFA-1 stimulation appears to be more highly dependent on ERK signaling.

ICAM-1 exposure induces IFN-γ production by human innate lymphocytes directly ex vivo

We next investigated whether exposure to an ICAM-coated surface was sufficient to induce IFN-γ production by primary cells. Freshly isolated human PBMCs were incubated for 18 hours (the final 12 hours in the presence of monensin) in culture wells coated with 5 μg/ml ICAM-1-Fc or with an isotype-matched negative control mAb. The cells were resuspended, stained for cell surface markers, then fixed and permeabilized and stained to assess intracellular IFN-γ. Flow cytometric analysis revealed that a small fraction (typically about 1%) of the lymphocyte population produced IFN-γ after exposure to plate-bound ICAM-1-Fc, but not to the isotype-

matched control mAb (Figure 4A). The cells that produced IFN-γ after ICAM-exposure had higher expression of CD11a compared to those that did not respond (Figure 4B), although, notably, not all of the $CD11a^{hi}$ cells showed IFN-γ production (Figure 4A). Similar to what we had observed for cultured iNKT cells, this response was abrogated by the presence of either CsA or U0126 (Figure 4C and D).

Since the population that produced IFN-γ in response to plate-bound ICAM-1-Fc appeared substantially more abundant than iNKT cells, which usually make up less than 0.1% of the lymphocytes in peripheral blood, we investigated the phenotypic characteristics of the responding cells. Typically, the majority of the responding cells were T cells, while the remainder were NK cells (Figure 5A). However, the T cells that produced IFN-γ^+ contained a significantly higher proportion of $CD56^+$ cells than the non-responding T cells (mean 59% $\pm$ 14% $CD56^+$ for responding T cells vs. mean 16% $\pm$ 8% $CD56^+$ for non-responders, p=0.03). Moreover, the responding T cell population showed elevated staining for PLZF compared to the T cells that did not respond (Figure 5B), suggesting that the ICAM-responsive subset is largely comprised of innate T cells. Consistent with this, using TCR-specific reagents we detected not only iNKT cells, but also MAIT cells and $V\delta2^+$ T cells within the ICAM-1-responsive population (Figure 5C). These results suggest that the ability to produce IFN-γ directly in response to a high surface density of ICAM-1 is a shared feature of human innate T lymphocytes and NK cells.

Discussion:

The results presented here delineate a novel pathway of integrin-mediated T cell activation. While prior studies have emphasized the ability of LFA-1 to contribute to T cell activation by lowering the threshold required for productive TCR signaling, we show here that innate T lymphocytes can

directly utilize LFA-1 signaling to activate IFN-γ secretion. Since LFA-1 ligation typically occurs in the context of interactions with $ICAM^+$ APCs, and LFA-1 engagement can enhance IFN-γ secretion in response to IL-12 stimulation, this activation pathway may facilitate the targeting of IFN-γ secretion towards specific types of recipient cells during inflammatory responses. Moreover, since IL-12 is not required, LFA-1-mediated activation may allow innate T cells to promulgate inflammatory responses independently of myeloid APCs in situations where an initiating event has led to the up-regulation of ICAM on other cell types. For example, events such as the deposition of oxidized LDL or exposure to TNFα or IL-1β are known to upregulate expression of ICAM-1 on vascular endothelial cells[144,193]. Since, exposure to IFN-γ causes VCAM-1 upregulation by vascular endothelial cells, the integrin-mediated activation of iNKT cells in this context might lead to the arrest and subsequent extravasation of monocytes and other cell types integral to the formation of atherosclerotic plaques.

One of the key features of the LFA-1-mediated activation pathway we observed is that it requires a threshold density of ICAM-1 in order to activate iNKT cell IFN-γ secretion. This may not be surprising, since it is known that LFA-1 binding to ICAM-1 involves a complex interplay between the conformational state of the LFA-1 molecule, (which can adopt low, intermediate, or high affinity states), and the avidity status induced by activation-induced clustering of LFA-1 at the cell surface. Thus, it is possible that the presence of a comparatively high density of ICAM-1 is sufficient to induce conformational changes in the LFA-1 molecules expressed by innate T lymphocytes that are associated with *de novo* signal transduction. It is intriguing to speculate that the high cell surface expression level of LFA-1 on innate T cells is thus a critical aspect of their ability to undergo independent LFA-mediated activation, since their elevated level of LFA-1 may enhance avidity-based interactions with ICAM.

We show here that activation of iNKT cells by LFA-1 involves both Ca^{++} and ERK signaling. We have previously demonstrated that human iNKT cells are highly responsive to weak TCR stimulation that generates ERK phosphorylation but only a minimal level of Ca^{++} signaling. Moreover, we found that weak TCR engagement (even when it is not sufficient to directly induce a productive response) leads to acetylation of the *IFNG* locus of iNKT cells, and this epigenetic status renders them receptive to TCR-independent stimulation by IL-12 and IL-18[70]. It thus seems likely that the epigenetic status of the *IFNG* locus of innate T lymphocytes may also play a critical role in their ability to produce IFN-γ in response to LFA-1-mediated signals. Consistent with this, we observed that exposure to plate bound ICAM-1-Fc selectively induced IFN-γ secretion, with little evidence of other cytokines typically produced by human iNKT cells, such as GM-CSF, IL-13, or IL-4 (data not shown). In this regard, however, the functional response induced by LFA-1 contrasts with TCR-mediated activation of iNKT cells, which induces both T_H1 and T_H2 cytokines, and instead appears more similar to that induced by pro-inflammatory cytokines such as IL-12 . Thus, the selective induction of iNKT cell IFN-γ secretion mediated by the LFA-1 pathway is consistent with prior reports suggesting that LFA-1 engagement promotes T_H1 functions in iNKT cells[194], as well as studies suggesting that LFA-1 ligation promotes T_H1 responses more globally[195–198].

This analysis outlines a previously unrecognized pathway of innate T cell activation that relies only on signals resulting from LFA-1 binding to ICAM-1, and does not require concurrent TCR or pro-inflammatory cytokine signaling. It will be of significant interest in the future to determine whether this pathway allows innate T cells to influence other cell types in situations where cognate antigen presenting molecules or relevant antigens are lacking. For example, naive T cells undergoing TCR-mediated activation have been shown to up-regulate their cell surface ICAM-1 and form clusters with other T cells[199]. LFA-1 induced IFN-γ secreted by innate T cells in this

context could thus promote T_H1 polarization of the naive T lymphocytes. Alternatively, this pathway may contribute to the ability of innate T cells to mediate TCR-independent IFN-γ secretion during viral infections[200–203].

Materials and Methods

Peripheral blood mononuclear cell (PBMC) isolation. Venous blood was obtained from healthy adult male or female subjects in accordance with a UW-IRB approved protocol. Freshly drawn blood samples were mixed with heparin sodium (10U per ml of blood) (Sagent Pharmaceuticals), and mononuclear cells were purified by density-gradient centrifugation (Ficoll-Paque Premium; GE Healthcare).

iNKT cells. Human iNKT cells used in these analyses included previously established clonal lines[204,205], and polyclonal cultures resulting from short-term in vitro expansion of cells sorted using α-GalCer loaded CD1d tetramer provided by the NIH tetramer facility at Emory University[206,207]. Clonal and polyclonal iNKT cell cultures were maintained in culture medium comprised of RPMI 1640 diluted with glucose-free RPMI (Biolgical Industries) to yield a final glucose concentration of 7.5mM, 3% human AB serum (Atlanta Biologicals), 10% heat-inactivated bovine calf serum (HyClone), 1% Penicillin-Streptomycin, 1% L-glutamine and 200 U/ml IL-2 (Peprotech). iNKT cultures used for experiments were of 98-100% purity as assessed by flow cytometric analysis with α-GalCer loaded CD1d tetramers.

ICAM-1 stimulation of iNKT cells. High protein-binding 96-well plates (Corning) were coated with recombinant human ICAM-1-Fc (Acro Biosystems) or an isotype-matched negative control mAb (clone MOPC21, Sigma Aldrich), then washed with PBS to remove unbound ICAM-1. iNKT cells (50 $x10^5$ per well) were added in culture medium lacking IL-2. Where indicated, the

following compounds were included in the culture medium: 20 U/ml recombinant human IL-12p70 (Peprotech); the indicated concentrations of U0126 (Invivogen) or Cyclosporin A (Sigma Aldrich); 1 μg/ml anti-human CD11a blocking mAb (Invitrogen, Clone HI111); 1 μg/ml anti-human CD18 blocking mAb (Biolegend, Clone TS1/18); or the indicated concentrations of JAK Inhibitor I (Calbiochem). Each stimulation condition was performed in four replicate wells. iNKT cells were stimulated for 18-24h at 37 ^{o}C and 5% $C0_2$, then culture supernatants were harvested and assayed for IFN-γ using a sandwich ELISA (capture antibody clone MD-1 from Biolegend; biotinylated detection antibody clone 4SB.3 from BD Pharmingen). IFN-γ concentrations were determined by comparison to a standard curve of recombinant human IFN-γ (Peprotech) assayed in parallel.

Microscopic analysis of iNKT cell cytoplasmic Ca^{++}. iNKT cells were labeled with Fluo-4 (Calbiochem) according to the manufacturer's instructions, then seeded 7.5x 10^4 cells onto glass chamber slides (iBidi) coated with ICAM-1 (5 μg/ml) then blocked with 2.5% BSA or coated with poly-L-Lysine alone. The slides were placed into a 37 ^{o}C and 5% CO_2 chamber, and images were taken every 20 seconds for 30 minutes using a Nikon Ti-Eclipse inverted wide-field microscope's GFP channel. For Fluo-4 analysis, cells determined to be <50% maximum intensity at the start of image acquisition were manually tracked over 30 minutes (90 frames) with changes to per cell 2-D mean fluorescence intensity (MFI) gauged using FIJI/ImageJ2 software (imagej.net/Fiji).

Detection of ERK phosphorylation. Jurkat T cells (2 x 10^6) or iNKT cells (1.5 x 10^6) were stimulated with anti-human CD11a mAb (Biolegend, Clone TS2/4) for the indicated times in culture medium lacking IL-2. Cells were lysed using CellLytic-M^{TM} (Sigma Aldrich) buffer infused with one tablet of Phosphostop^{TM} (Roche) phosphatase inhibitor. Lysates were run on a

12% SDS-PAGE gel, then transferred onto a polyvinylidene difluoride (PVDF) membrane using a Transblot Turbo™ semi-dry transfer system (Biorad). Membranes were blocked overnight at 4 °C in a solution of Tris-buffered saline, 0.05% Tween-20, 5% non-fat dry milk (Sanalac), 5% bovine serum albumin (Fisher). Phosphorylated or total ERK bands were detected using a polyclonal rabbit anti-human phospho-ERK antibody (Cell Signaling Technology; Cat# 9101S) or polyclonal rabbit anti-human MAPK p44/42 ERK1/2 antibody (Cell Signaling Technology, Cat# 9102S), followed by detection using a goat anti-rabbit HRP-linked secondary antibody (Cell Signaling Technology; Cat# 7074S). Membranes were developed with Clarity Western ECL Substrate™ (Biorad) and analyzed using a VersaDoc Imaging System (Biorad). Total and phospho-ERK band intensities were quantitated using ImageJ software, and normalized by the corresponding signal from an adjacent empty lane to account for assay to assay variation in background.

ICAM-exposure and analysis of fresh PBMCs. PBMCs (1×10^5) were incubated for 18-24 hrs with plate-bound ICAM-1-Fc (coated at 5 μg/ml) or with an isotype-matched negative control mAb (5 μg/ml MOPC-21, Sigma Aldrich). PBMCs were resuspended and Fc receptors were blocked by incubation in a solution of PBS containing 25% human AB serum (Atlanta Biologicals) for 20 minutes at 4°C, then cell-surface staining was performed for 30 minutes at 4 °C using the following human-specific antibodies or tetramers: anti-CD3 (OKT3, Biolegend), anti-CD4 (OKT4, Biolegend), anti-CD8α (HIT8a, Biolegend), anti-CD8β (SIDI8BEE, eBioscience), anti-CD11a (HI111, Biolegend), anti-CD56 (5.1H11, Biolegend), CD1d tetramer (PBS-57 loaded, NIH Tetramer Core Facility), MR1 tetramer (5-OP-RU loaded, NIH Tetramer Core Facility), anti-Vδ2 TCR (123R3, Miltenyi Biotec). Cells were fixed and permeabilized according to the manufacturer's instructions using the BD Cytofix/Cytoperm kit (BD Biosciences), then stained with anti-IFN-γ (4S.B3, Biolegend), anti-PLZF (Mags.21F7, eBioscience), or the respective

negative control mAbs suggested by the vendor. Cells were then washed, resuspended in PBS and analysed on an LSR II flow cytometer (BD Biosciences). Staining data were analyzed using FlowJo analysis software (Tree Star Inc).

Statistical Analyses. An unpaired two-sided Student's T-test was used to assess replicates from different experimental conditions. A Mann-Whitney test was used to assess sample groups comprised of data aggregated from multiple independent analyses. Sets of paired samples were analyzed using a Wilcoxon-matched pairs analysis.

Figures:

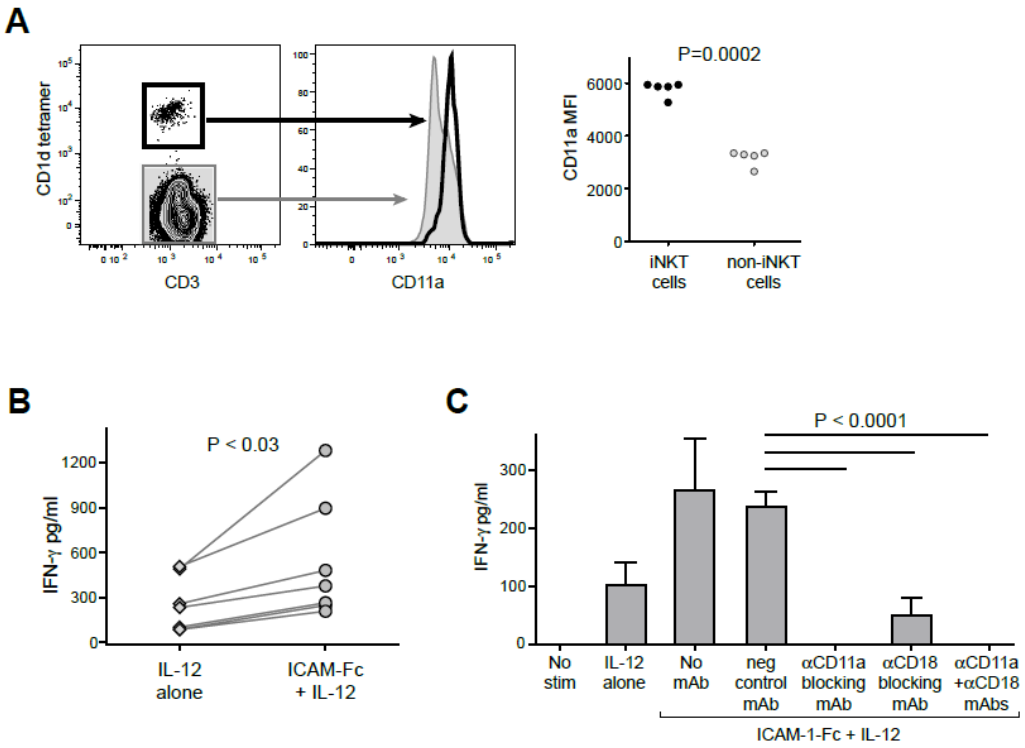


Figure 1. **Elevated LFA-1 expression on human iNKT cells co-stimulates IFN-γ secretion in response to IL-12p70. A)** Freshly isolated human PBMCs were stained with antibodies against CD3 and CD11a, and with αGalCer loaded CD1d tetramer and analyzed by flow cytometry.

Plots on left show CD11a expression by CD1d-tetramer positive (heavy black line) compared to tetramer-negative (grey shaded) for one representative experiment. Plot on right shows results from analysis of PBMC samples from five unrelated healthy adults. Mean fluorescence intensity (MFI) of CD11a staining is plotted for CD1d-tetramer positive (iNKT cells) and tetramer-negative (non-iNKT cells). **B)** iNKT cells were incubated for 24h in medium containing 20 U/ml recombinant human IL-12p70, in the presence of plate-bound ICAM-1-Fc (coated at 5 μg/ml) or negative control mAb ("IL-12 alone"). Secreted IFN-γ was quantitated using a standardized ELISA. The plot shows aggregated results from 7 independent experiments, using

different iNKT cell lines. **C)** iNKT cells were incubated for 24h in culture medium alone ("no stim"), or in medium containing IL-12p70 in wells coated with a negative control mAb ("IL-12 alone"), or in medium containing IL-12p70 in wells coated with ICAM-1-Fc in the presence or absence of the indicated blocking antibodies, and secreted IFN-γ was quantitated by ELISA. The plot shows results from one representative experiment out of three; bars indicate means and standard deviations from 4 replicates per treatment.

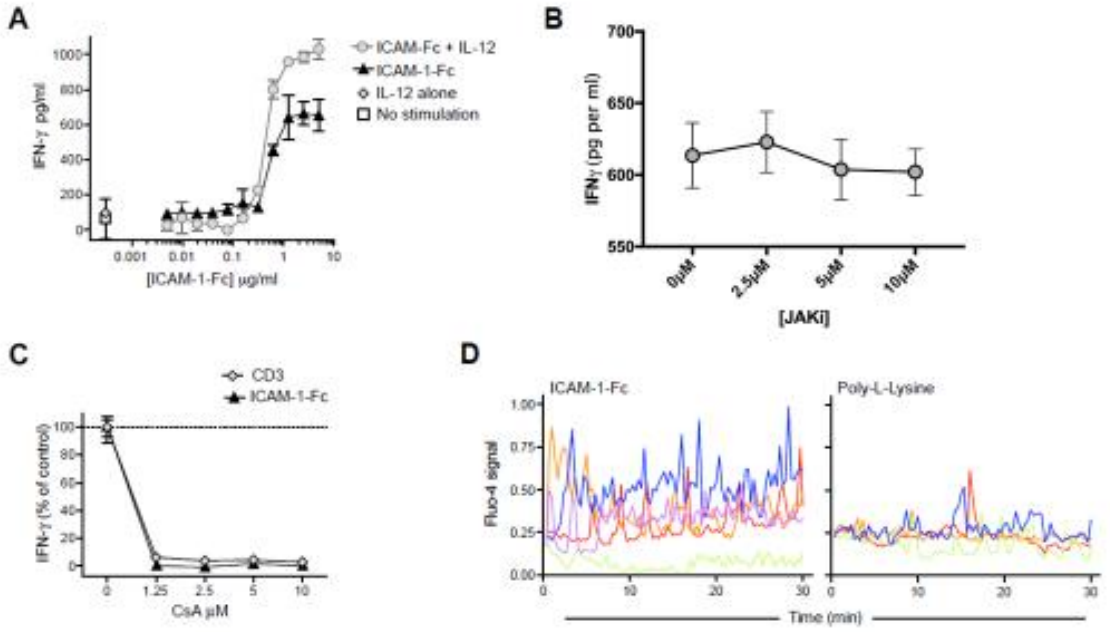


Figure 2. Exposure to a high density of ICAM-1 promotes iNKT cell IFN-g secretion in a manner that is independent of IL-12, but that involves Ca^{++} signaling. A) iNKT cells were incubated for 24h in wells coated with titrated doses of ICAM-1-Fc, in the presence or absence of 20 U/ml IL-12p70. Secreted IFN-γ was quantitated by ELISA. The plot shows results from

one representative experiment out of three; symbols indicate means and standard deviations four replicates per treatment. **B)** iNKT cells were incubated in wells coated with ICAM-1-Fc, in the presence of the given concentrations of a JAK2 inhibitor, and secreted IFN-□□was quantitated by ELISA. Similar results were obtained in two independent analyses. **C)** iNKT cells were incubated for 24h with plate-bound ICAM-1-Fc (coated at 5 mg/ml), or with 0.5 □g/ml soluble anti-CD3 mAb, in the presence of the indicated concentrations of cyclosporin A (CsA), and secreted IFN-γ was quantitated by ELISA. The plot shows the amount of IFN-γ as a percentage of the amount produced in each condition in the absence of CsA; symbols represent the means and standard deviations (not always visible on the scale shown) of four replicates per condition. Similar results were obtained in four independent analyses. **D)** iNKT cells labeled with the calcium indicator dye Fluo-4 were placed on slides coated with (what concentration?) ICAM-1-Fc or poly-L-Lysine, and images were taken at 20 second intervals using a fluorescence microscope. The plots show the Fluo-4 signal intensities over time for individual iNKT cells (indicated by different color lines) in each condition.

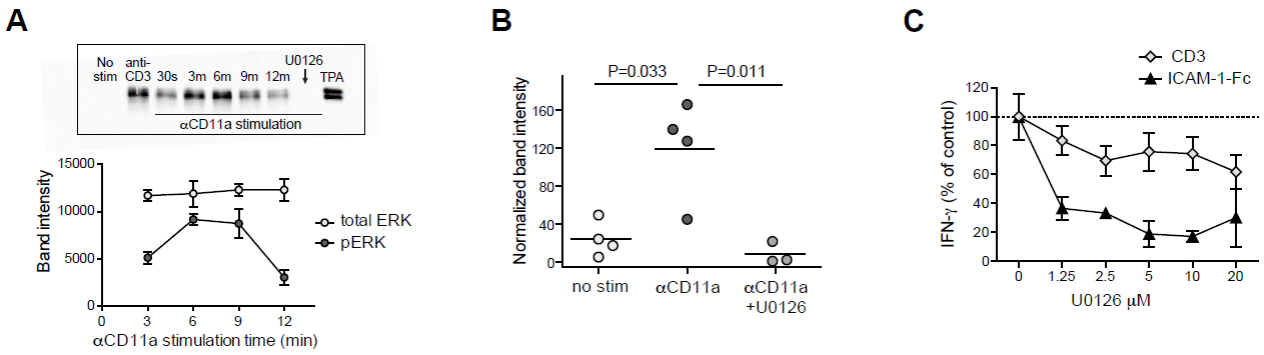


Figure 3. iNKT cell IFN–γ secretion in response to LFA-1 stimulation is dependent on ERK phosphorylation. A) Jurkat T cells were stimulated with anti-CD3 mAb for 5 minutes or

with anti-CD11a mAb for the indicated times, then lysed and subjected to Western blotting using detection antibodies against either total ERK or phospho-ERK. The blot at the top shows the phospho-ERK results from a representative experiment; "U0126" indicates Jurkat cells that were stimulated five minutes with anti-CD11a in the presence of 5 ∝M of the MEK inhibitor U0126; "TPA" indicates Jurkat cells that were stimulated with 20nM 12-O-Tetradecanoyl-phorbol-13- acetate for 10 minutes. The plot at the bottom shows the means and standard deviations from three replicate analyses of total ERK and phospho-ERK Western blots. **B)** iNKT cells were stimulated with anti-CD11a mAb 5 minutes in the presence or absence of 5 μM U0126, then the cell lysates were subject to Western blotting to detect phospho-ERK. The plot shows the aggregated results from 3-4 independent analyses, with band intensities normalized by the signal from an adjacent empty lane to account for assay to assay variability. **C)** iNKT cells were incubated for 24h with plate-bound ICAM-1-Fc or with soluble anti-CD3 mAb, in the presence of the indicated concentrations of U0126, and secreted IFN-γ was quantitated by ELISA. The plot shows the amount of IFN-γ as a percentage of the amount produced in each condition in the absence of U0126; symbols represent the means and standard deviations (not always visible on the scale shown) of four replicates. Similar results were obtained in three independent analyses.

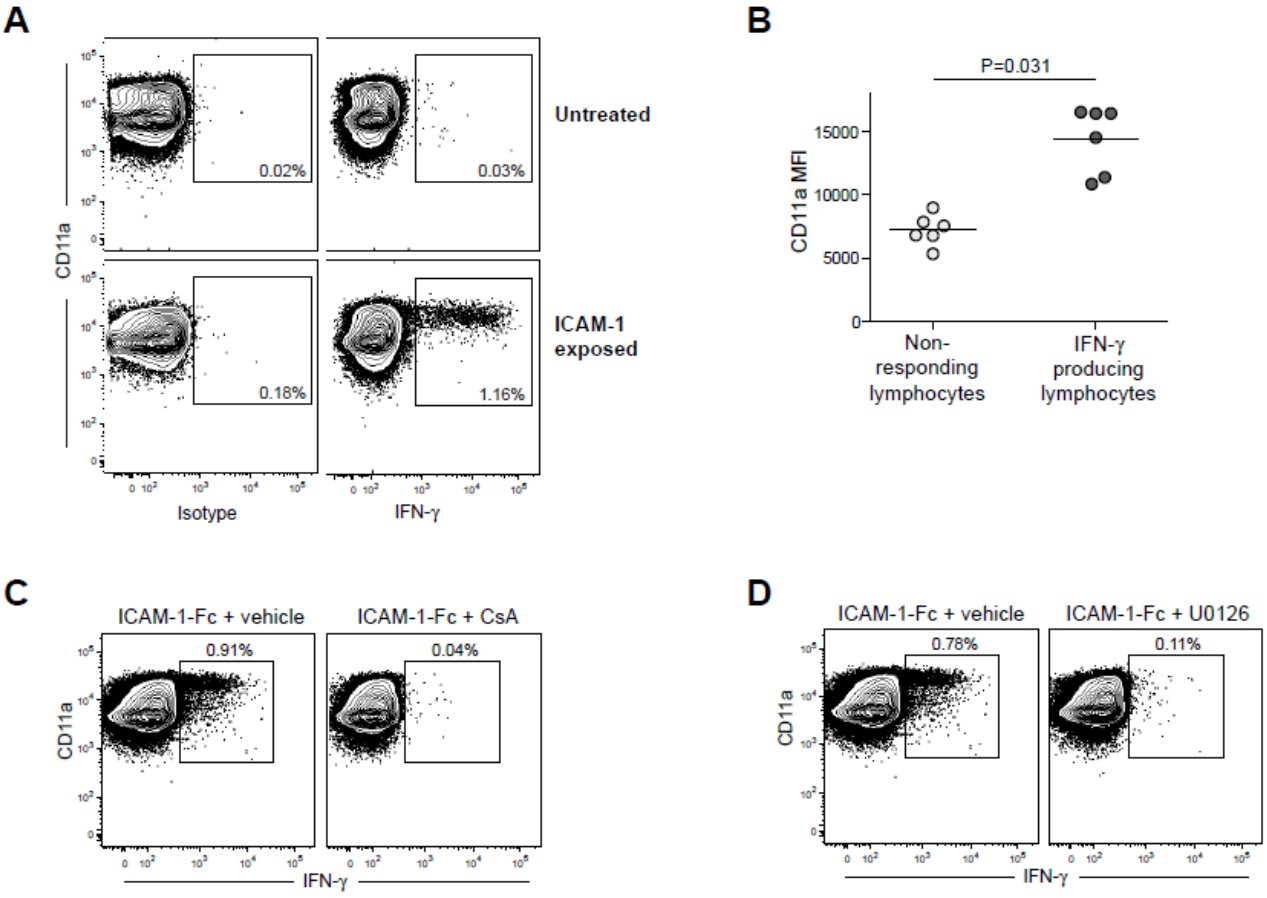


Figure 4. Exposure to high density ICAM-1 activates IFN-g production by a small subset of human lymphocytes directly ex vivo. A) Freshly isolated human PBMCs were incubated in culture wells coated with 5 μg/ml ICAM-1-Fc or with an isotype-matched negative control mAb, then subjected to flow cytometric analysis to assess CD11a expression levels and intracellular IFN-γ. Plots show results from analysis of the total lymphocyte gate as assessed by forward and side scatter. **B)** Aggregated results from analyses of PBMCs from six unrelated healthy adults, showing CD11a mean fluorescence intensity (MFI) for the lymphocyte population that stained positively for IFN-γ compared to those that did not. **C and D)** Freshly isolated human PBMCs were incubated in culture wells coated with 5 μg/ml ICAM-1-Fc in the presence of 5□M CsA (panel **C**) or 5μM U0126 (panel **D**), or vehicle alone, and then subjected to flow cytometric

analysis to assess CD11a expression levels and intracellular IFN-γ.

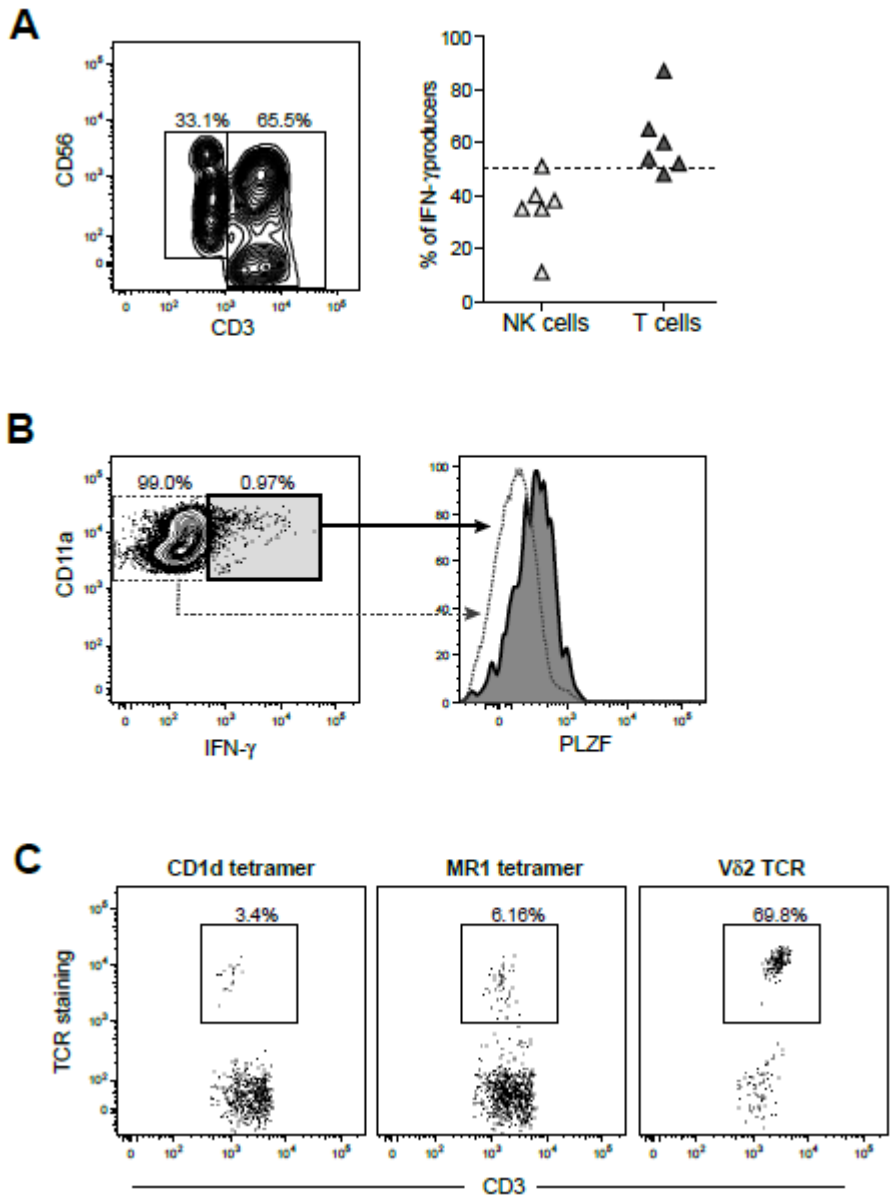
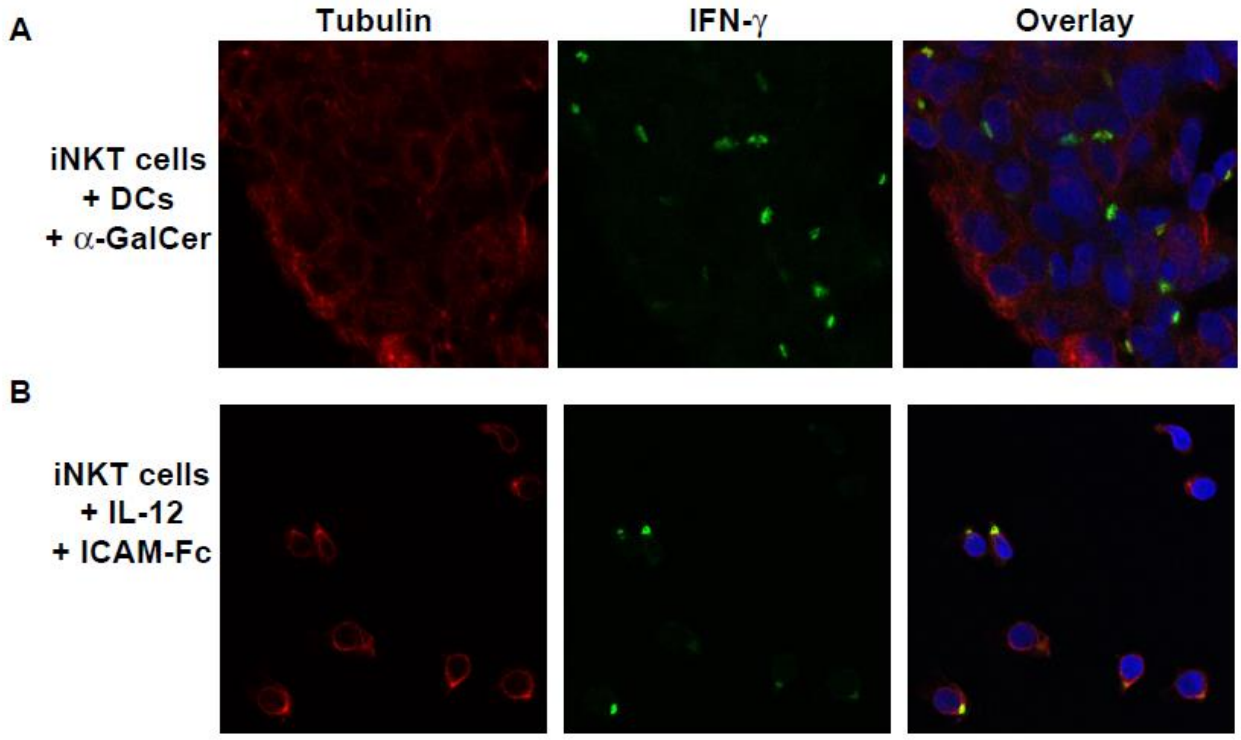


Figure 5. The subset that responds to ICAM-1 is comprised of NK cells and innate T cells.

A) Freshly isolated human PBMCs were exposed to plate-bound ICAM-1-Fc, and analyzed by

flow cytometry to characterize the IFN-γ producing cells. Contour plot on left plot is gated on the IFN-γ producing subset from one PBMC sample, and shows CD3 and CD56 staining; scatter plot on right shows the fraction of the IFN-γ producing subset identified as NK cells ($CD56^+CD3^-$) vs. T cells ($CD3^+$) for PBMC samples from six unrelated healthy adults. **B)** Analysis of the IFN-γ producing cells (grey shaded) compared to the non-responding population (dotted line) for expression of the PLZF transcription factor. **C)** Fraction of the IFN-γ producing subset identified as iNKT cells (left), MAIT cells (center), or Vγ9Vδ2 T cells (right).



Supplementary Figure 1. Fluorescence microscopy showing IFN–γ production by human iNKT cells. A) Human iNKT cells were co-incubated with a 1:1 ratio of α-GalCer pulsed monocyte-derived dendritic cells, then stained with DAPI (blue) to identify cell nuclei, anti-tubulin (red), and anti-IFN-γ (green). Similar to what has been previously established for conventional T cells, the IFN-γ staining appears tightly localized at the interface between iNKT cells and DCs. **B)** Analysis of iNKT cells incubated on slides coated with ICAM-1-Fc in medium containing IL- 12p70. IFN-γ staining appears to co-localize with a nexus of tubulin staining that corresponds to the microtubule organizing center (MTOC).

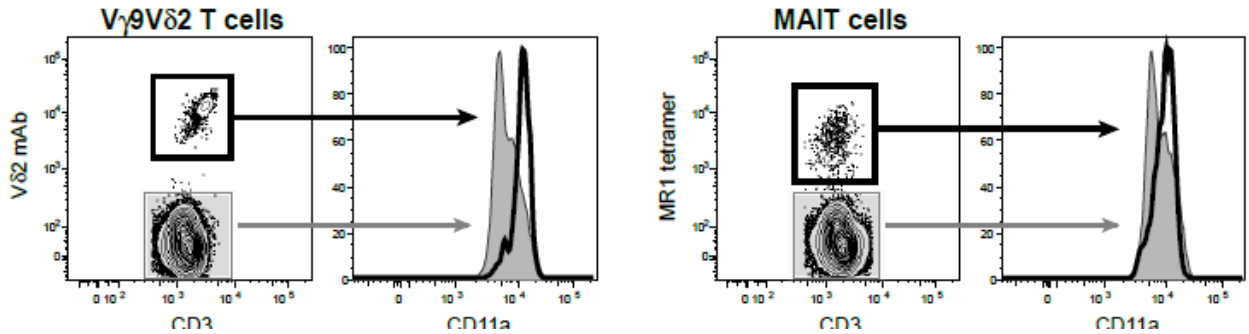


Figure 2. Flow cytometric analysis showing CD11a expression levels of human peripheral blood MAIT and Vδ2^{+} T cells. Freshly isolated human PBMCs were stained with antibodies against CD3 and CD11a, and with MR1 tetramer or anti-Vδ2 mAb and analyzed by flowcytometry. Histogram plots show overlayed CD11a expression for the gated populations shownin the neighboring contour plots: CD11a of the Vδ2^{+} T cell or MAIT population (heavy black line) is overlayed on that of the remainder of the T cells (grey shaded)

Chapter II: Expansion and Adoptive Transfer of Human Vδ2$^+$ T cells to Assess Antitumor Effects *in Vivo.*

This chapter has been co-written with Drs. Nicholas Zumwalde, PhD and Jenny Gumperz, PhD. My contributions to this are descriptions of preparing CBMC for EBV infection and engraftment, identifying and harvesting tumours, and preparing tumour tissue for histological and immunohistochemical analyses. This chapter provides an excellent framework for the methods used to investigate iNKT-mediated immunotherapy in this exact model. Text from this chapter will be part of the book series Methods in Molecular Biology: Cancer Immunosurveillance, published by Springer Nature.

Anti-tumor functions of γδ T cells. Since they were first identified about 30 years ago, it has been clear that human γδ T cells display potent cytotoxicity towards tumor target cells[208]. Human gd T cells lyse target cells in an MHC-unrestricted manner, and have been shown to efficiently kill a variety of neoplastic cell types, particularly those of hematological and epithelial origin[209–211]. Based on their ability to eradicate primary human tumor cells *in vitro*[212–216], and on studies showing that human γδ T cells can control xenografted human tumors in immune-deficient mice *in vivo*[217–222], pilot clinical trials have been undertaken to investigate γδ T cell-based immunotherapies in cancer patients[223,224]. While the results of these studies have overall been promising (a recent meta-analysis of 13 clinical trials that used γδ T cell-based immunotherapies and involved patients with advanced or metastatic cancer found a total Effective Rate of 0.407 with a p value <0.014[225]), the mechanistic pathways used by human γδ T cells to mediate anti-tumor effects *in vivo* remain poorly understood. For example, it is not clear whether their anti-tumor effects are necessarily due to their cytotoxic functions, since a number of studies have suggested that γδ T cells may also

promote antigen-specific anti-tumor responses by acting as highly stimulatory antigen presenting cells (APCs) for HLA-restricted T cells[226–229] Hence, methodologies that allow for investigation of mechanisms underlying the anti-tumor effects of human γδ T cells *in vivo* are of considerable interest.

EBV model system. To generate a an experimental model for investigating the anti-tumor effects of human γδ T cell adoptive therapy, we have used Epstein-Barr virus (EBV) to drive the *de novo* formation of human B-lymphomas *in vivo*. EBV is a completely human-specific γ–herpesvirus that infects B lymphocytes, causing dysregulated proliferation[230]. Primary EBV infection is typically controlled by cytolytic lymphocyte responses[231]. However, in situations where the initial viral infection is overwhelming or the cytolytic response is sub-optimal, failure to contain the virus leads to potentially fatal B cell proliferation. This is particularly common when pediatric patients who are naive to EBV are transplanted with organs or tissue from an EBV-infected individual; the ensuing pathology is thus termed post-transplant lymphoproliferative disease (PTLD)[232]. PTLD is an increasingly common complication after allogeneic stem cell transplantation[232,233].

We have taken advantage of the fact that human umbilical cord blood lymphocytes are naive to EBV to establish an experimental system resembling post-transplant lymphoproliferative disease. By transferring freshly isolated human umbilical cord blood mononuclear cells (CBMCs) and EBV into immunodeficient mice, the initially healthy human B cells become infected by EBV and undergo neoplastic transformation *in vivo* during the ensuing 2-3 weeks. Typically, about 80-90% of the mice will ultimately develop invasive lymphomas within the peritoneal cavity [28]. The lymphomas are heavily infiltrated by autologous human $CD4^+$ and $CD8^+$ T cells derived from the umbilical cord blood sample[234]. However, the B cells in the lymphomas express

immunosuppressive ligands (e.g. PD-L1, PD-L2) that hold the anti-tumor functions of the T cells in check[235]. Thus, this model provides the opportunity to evaluate both the immunosurveillance and tumor rejection functions of human γδ T cells. By adoptively transferring human γδ T cells within the first 1-2 weeks after the injection of CBMCs and EBV their impact on virally infected cells that are only nascently neoplastic can be evaluated (i.e. immunosurveillance). Alternatively, by waiting to administer the γδ T cells until 3-4 weeks, their effects can be evaluated in the context of established tumors containing an immunosuppressive environment[236].

The following sections provide detailed descriptions of methods for setting up the EBV-driven lymphoma model in immunodeficient mice, expanding $V\delta2^+$ T cells from human PBMCs, and harvesting and analyzing lymphomas and other tissues from the mice.

2 Materials

2.1 Engraftment of NSG mice with EBV-exposed human umbilical cord blood cells

2.1.1 Preparation of EBV

1. A solution of the lytic M81 strain of EBV, stored in frozen aliquots at -80 ^oC or in LN_2 (*see* Note 1). Virus is stored in 200 μl aliquots,at a concentration of 200 IU/μl. Thus, each vial contains a total of 40,000 IU of virus, which is sufficient for 20 mice (the number of mice that can usually be generated from one cord blood sample).

2.1.2. Preparation of human umbilical cord blood cells and exposure to EBV

1. Human umbilical cord blood mononuclear cells (*see* Note 2).

2. Ficoll-Paque Premium.

3. 50 ml conical tubes (sterile).

4. RPMI 1640 medium (keep cold; 2-8°C).

5. Culture Medium: RPMI 1640, 10% bovine calf serum (heat inactivated), 3% human AB serum, 1% L-Glutamine, 1% Penicillin-Streptomycin.

6. Ammonium-Chloride-Potassium (ACK) lysis buffer (keep cold; 2-8°C).

7. Sterile PBS, tissue culture grade.

2.1.3 Injection of EBV-treated CBMCs into immunodeficient mice

1. 6-8 week old NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice (Jackson Labs).

2. Tuberculin syringes and 28.5 gauge needles.

2.2 Expansion of Vδ2$^+$ T cells from adult peripheral blood and adoptive transfer into EBV-infected mice

2.2.1 Expansion of human Vδ2$^+$ T cells

1. Anticoagulant treated peripheral blood obtained from healthy adult donors after informed consent, or purified peripheral blood mononuclear cells (PBMCs).

2. Ficoll-Paque PLUS.

3. T cell culture medium: RPMI-1640, 15% bovine calf serum (heat inactivated), 3% human AB serum, 1% L-Glu, 1% Penicillin-Streptomycin, 200 U/mL interleukin-2 (IL-2).

4. Zometa (zoledronic acid; Novartis).

5. Antibodies to assess expansion and purity of γδ T cells, such as: anti-Vδ2 (clone B6), anti-Vγ9 (clone B3), anti-CD3 (clones OKT3 or HIT3a), all available from BioLegend.

6. Flow cytometer for analysis of expanded γδ T cells.

7. Freezing solution (15% DMSO in bovine calf serum) and cryovials.

2.2.2 Adoptive transfer of human Vδ2$^+$ T cells

1. Sterile PBS, tissue culture grade.

2. Tuberculin syringes and 28.5 gauge needles.

3. Isoflurane and isoflurane chamber for mouse anesthesia

2.3 Collection and analysis of tumors and spleen tissue

2.3.1 Harvesting tumor and spleen tissue

1. Forceps.

2. Surgical scissors.

3. Surgical foam board.

4. Pins.

2.3.2 Determining tumor burden and preparing tissues for further analysis

1. 50 ml conical tubes, pre-weighed.
2. Scale.
3. 10% Formalin solution, neutral buffered.
4. Filter mesh and plungers for manual dissociation of tissues, or gentleMACS dissociator (Miltenyi Biotec).
5. PBS

2.3.3 Histological analysis of tissues

1. Biopsy/embedding cassettes.
2. Absolute ethanol and solutions of 70% and 90% ethanol.
3. Xylene.
4. Forceps.
5. Scissors.
6. Base molds.
7. Heating block.
8. Paraffin wax.

2.3.4 Flow cytometric analysis of spleen or tumor tissues

1. Fc blocking solution consisting of 20% human AB serum in PBS.

2. Tubes appropriate for reading samples on flow cytometer.

3. Flow cytometry sample buffer consisting of a filtered solution of 1 mg/ml BSA in PBS.

4. Flow cytometer capable of detecting at least 4 channels (preferably 8 or 9).

5. Antibodies for human cell markers (all available from BioLegend): anti-CD45 (clone HI30), anti-pan HLA-A,B,C (clone W6/32), anti-CD3 (clone OKT3 or HIT3a), anti-CD19 (clone SJ25C1) or anti-CD20 (clone 2H7), anti-Vδ2 (clone B6) or anti-Vγ9 (clone B3). Antibodies against CD19 and CD20 can be used in the same color improve detection of EBV-infected B-cells.

3 Methods

3.1 Engraftment of NSG mice with EBV-exposed human umbilical cord blood cells

3.1.1 Preparation of EBV

1. Thaw sufficient virus for the experiment by briefly incubating one or more frozen aliquots at 37 ^{o}C. (Typically, 200 IU of virus is used per 10^6 CBMCs and each mouse is injected with 10^7 CBMCs, although these numbers may vary depending on the activity of the virus and the availability of CBMCs.)

3.1.2 Preparation of human umbilical cord blood cells and exposure to EBV

1. If frozen purified CBMCs are used, thaw the cells and wash them according to the supplier's instructions, then resuspend at 10^8 cells/ml in culture medium. If whole cord blood is used, the

CBMCs must first be isolated by density gradient centrifugation.

2. Dilute the whole cord blood with 3 times the volume of cold RPMI, and place 35 ml each into 50 ml conical tubes. Slowly pipet 15ml of the Ficoll-Paque under 35 ml of the diluted cord blood.

3. Centrifuge at 400xg for 40-45 min in a centrifuge with a swinging bucket rotor without using brakes or acceleration.

4. After centrifugation, the blood will have separated into layers: plasma (clear top layer), CBMCs (cloudy or opaque layer at top of interface with Ficoll), dark red bottom layer containing granulocytes and erythrocytes. Aspirate the plasma layer without disturbing the CBMCs, and discard.

5. Gently aspirate the CBMCs and transfer into a fresh 50 ml conical tube.

6. Fill the CBMC-containing conical tube with RPMI, and centrifuge at 400xg for 15 min at 18°C with full acceleration and brake.

7. Discard the supernatant, resuspend the CBMC pellet in RPMI and spin at 300xg for 15 min at 18°C with full acceleration and brake.

8. Repeat step 7, but at 200xg. The spins described in steps 6-8 help remove platelets from CMBC. Check the pellet for red color (*see* Note 3).

9. Resuspend the CBMC pellet in 1.5-2ml culture medium, and count the cells. Dilute to $1x10^8$ cells/ml in culture medium.

10. Add 2000 IU of M81 per $1x10^7$ CBMC and incubate at 37°C, 5% CO2, for 2-4 h, to promote viral attachment to the B lymphocytes in the CBMC sample.

11. Wash the CMBC with culture medium to remove non-attached virions. Resuspend in sterile PBS (room temperature) at a concentration of $5x10^7$ cells per ml.

3.1.3 Injection of EBV-treated CBMCs into immunodeficient mice

1. Using sterile tuberculin syringes and 28.5 gauge needles, intraperitoneally inject 200 μl of the cell suspension (1×10^7 CBMC) per mouse.

2. Mice should be maintained in a specific pathogen-free facility using sterilized cages, bedding, food, and water. Tumors will form in the peritoneal cavity by about 21 days post-injection of EBV-exposed CBMCs; γδ T cell treatment can be performed at any time point depending on whether the goal is to investigate immunosurveillance functions (i.e. prevention of neoplastic cell outgrowth or tumor deposition), or effects that occur in the presence of established tumors (e.g. infiltration or eradication).

3.2 Expansion of $V\delta2^+$ T cells from adult peripheral blood and adoptive transfer into EBV-infected mice

3.2.1 Expansion of human $V\delta2^+$ T cells

1. If frozen purified PBMCs are used, thaw the cells and wash them according to the supplier's instructions, then resuspend at $2x10^6$ cells/ml in T cell culture medium. If whole peripheral blood is used, the PBMCs must first be isolated by density gradient centrifugation using Ficoll-PLUS, as described for CBMCs in section 3.1.2.

1. Dilute Zometa to a concentration of 5 μM in T cell culture medium.

2. Add equal volumes of PBMCs (0.5 ml) and 5 μM Zometa (0.5 ml) to wells of a 24-well plate, resulting in a density of $1x10^6$ cells/well and a final Zometa concentration of 2.5 μM (*see* Note 4).

3. Incubate the cells at 37 °C in a humidified incubator with 5% CO_2.

4. Monitor the cultures to assess cell growth and acidification of the culture medium. Add 1 ml fresh T cell culture medium after 4-6 days, or sooner if the wells appear to be turning orange or yellow. As the cells expand (typically in the second week of culture), split each well into a second well and supplement with fresh T cell culture media.

5. Flow cytometric analyses should be performed to assess purity of γδ T cells and expression of ligands of interest (e.g. PD-1). (*see* Note 5).

6. Cultures containing expanded $V\delta2^+$ T cells are typically harvested after 8-14 days, with the precise timing chosen according to the level of $V\delta2^+$ T cell enrichment and total cell number. If necessary, contaminating cell types can be removed from the expanded $V\delta2^+$ T cell culture by magnetic or flow cytometric sorting. (*see* Note 6).

7. The expanded $V\delta2^+$ T cells can also be stored frozen for up to 3 months prior to use. Chill a suspension of culture medium containing 2-10 x 10^7 cells/ml and mix with 2x volume of cold freezing solution (15% DMSO in bovine calf serum). Pipet into pre-chilled cryovials and immediately transfer to -80 ^oC.

3.2.2 Adoptive transfer of human Vδ2$^+$ T cells

1. If the expanded γδ T cells have been stored frozen, thaw and wash cells several times with sterile PBS to remove DMSO.

2. Count cells and resuspend at 2.5x10^7 cells/ml in sterile PBS.

3. Anesthetize mice that were injected with EBV-exposed CBMCs.

4. Intravenously administer 2.5-5x10^6 cells in a volume of 100-200 μl by retro-orbital injection. Control mice should be injected with an equivalent volume of sterile PBS.

3.3 Collection and analysis of tumors and spleen tissue

3.3.1 Harvesting tumor and spleen tissue

1. Tumors are typically visible in the peritoneal cavity by 21 days post-injection of EBV-exposed CBMCs; mice typically become moribund by about 32-34 days post-injection. Thus, analyses can be done at any time during this window.

2. Euthanize the mice as specified by your institutional Animal Care and Use Committee.

3. Place murine carcasses with the ventral side up on a surgical foam board, and pin the limbs to the board.

4. Using a pair of forceps, pull the skin near the urethral opening upwards, and make an incision with a pair of surgical scissors. The incision should cut along the midline, and go from the urethral opening up to the chin.

5. Using the forceps carefully pinch the peritoneal sac pulling upwards and making an incision identical to the one in step 4 to expose the viscera.

6. Tumors are most commonly found initially in peri-pancreatic areas, and at later stages often invade pancreas, bile duct, liver, intestines, and (less commonly) other tissues. Locate the stomach on the left side of the peritoneal cavity, and with a pair of forceps, begin to separate the pancreas from the stomach and the intestines, delicately severing connections between the pancreas and the thorax using either another pair of forceps or a pair of surgical scissors.

7. Pull out the spleen and attached pancreas, and separate the spleen. Spleens should be weighed and then placed into cold culture medium or fixative solution for later analysis (e.g. flow cytometry or histology).

8. Place the pancreas and any associated tissue on the surgical foam board to examine for the presence of tumors. (*see* Note 7). Carefully dissect the tumor tissue from the pancreas.

9. Using a pair of forceps, also examine the mesentery, stomach, intestine and liver lobes for tumors. These will appear as white/pinkish engorged masses resting on the tissue, and can be harvested using scissors.

3.3.2 Determining tumor burden and preparing tissues for further analysis

1. Decide whether tumors will be analyzed by histology or flow cytometry, and prepare 50 ml conical tubes with 5-10 ml 10% neutral buffered formalin (histology) or cold culture medium (flow cytometry). Weigh the tubes containing the appropriate buffer, and record the weight on the side of the tube. To determine the tumor burden, place all of the excised tumor tissue from

an individual mouse into a tube, and re-weigh using the same scale. Subtract the tube weight to obtain the weight of the tumor tissue.

2. Tissues collected for histology may be stored at room temperature in 10% neutral buffered formalin for 24-48h, before further processing.

3. Tissues that are to be analyzed by flow cytometry should be kept cold and processed immediately.

3.3.3 Histological analysis of fixed tissues

1. To prepare them for paraffin sectioning, cut the tissue into slices ~3mm thick. Tissue preparation is often performed by a core facility or a commercial service using an automated tissue processor. Alternatively, processing steps can be completed manually as described briefly in the following steps[237,238].

2. Place the tissues into appropriately labeled embedding cassettes. Put the cassettes in a beaker containing 70% ethanol, and incubate for 60 min at room temperature.

3. Transfer the cassettes to a beaker of 95% ethanol, and incubate for 60 min.

4. Transfer the cassettes to a beaker of absolute ethanol, and incubate for 60 min. Repeat twice.

5. Transfer the cassettes to a beaker containing xylene, and incubate for 20 min. Repeat twice, with the final incubation lasting 45 min to 24 h.

6. Place cassettes in a container of molten paraffin wax and agitate gently for 30 min. Repeat twice.

7. Fill block molds with molten paraffin wax and position tissue within the wax as desired for sectioning; place cassette on top as backing.

8. Tissue can now be sectioned using a microtome and placed onto slides. For each tissue, it is helpful to perform hematoxylin and eosin staining on the first slide of a series of serial sections to identify areas of interest (e.g. tissues containing lymphocytes that likely correspond to tumor or lymphoid tissue). Further serial sections can then be chosen and immunohistochemistry performed using specific antibodies, as described[234–236,239,240].

3.3.4 Flow cytometric analysis of spleen or tumor tissues

1. Prepare a single cell suspension from the tissue sample. Tissues can be dissociated manually by using the backside of a plunger from a plastic syringe to mash the tissue over filter mesh (40-100 μm), or automatically using a tissue dissociator. If desired, the single cell suspension can be stored frozen using the freezing procedure described in step 7 of section 3.2.1..

2. Centrifuge samples at 400xg for 5 min to pellet cells, and discard supernatant.

3. Resuspend in PBS and pass through a 40-100 μm filter mesh (*see* Note 7).

4. Suspend single cell suspension in 1 ml Fc-blocking buffer and incubate for 15 min at 4 ^{o}C.

5. Pellet cells by centrifugation and pour off supernatant.

6. Add cocktail of staining antibodies to the void volume in the tubes and incubate for 30 min at 4 °C.

7. Wash the samples with flow cytometry sample buffer, and resuspend in a volume of 0.3-0.5 ml.

8. Analyze samples on flow cytometer (*see* Notes 8-10).

4 Notes

1. We recommend using a lytic strain of EBV (e.g. M81, Akata) rather than the more commonly used B95-8 strain. B95-8 lacks EBV-encoded microRNAs and has an almost completely latent pattern of gene expression, whereas strains such as M81 contain the whole viral genome and show both lytic and latent gene expression. Since most of the EBV peptide epitopes recognized by $CD8^+$ T cells derive from lytic genes, the use of a lytic strain produces better virally-driven $CD8^+$ T cell expansion and thus also provides for better modeling of the immunosuppressive environment that can thwart anti-tumor T cell responses.

   The titer of the virus particles must established prior to use. To do this, we make use of an EBV construct that expresses the green fluorescent protein (GFP)[241]. Infectious viral particles are produced from 293 cell lines that were stably infected with the M81 strain of EBV following transfection with EBV BZLF1 expression vector as described[242]. The titer of infectious EBV is determined by assessing the number of fluorescent Raji cells derived from serial dilutions of the concentrated virus stock and calculating the green Raji unit (GRU) titer, as described[242].

2. Cord blood may be obtained as an anticoagulant-treated whole blood sample, or as purified cord blood mononuclear cells (CBMCs) that are supplied either fresh or frozen. The $CD34^+$ cells are not needed for this protocol, and so if desired these may be removed from the CBMCs by magnetic sorting prior to EBV-exposure.

3. If the CBMC appears red it likely has significant erythrocyte contamination. In this case,

resuspend in 10-15 ml of cold ACK lysis buffer and incubate for 5 min at room temperature. Lysis of erythrocytes should be evident by this time. Fill the tube with culture medium to dilute out the ACK lysis buffer, and spin the cells down at 300xg for 15 min.

4. In Zometa expanded PBMC cultures, Vδ2$^+$ T cells typically comprise 70-80% of the total CD3 population between days 7 and 11 post stimulation. However, the percentage of Vδ2$^+$ T cells can vary depending on the initial frequency of □□ T cells in the starting PBMC sample and the potency of the Zometa preparation, among other variables. It is therefore advisable to monitor the Vδ2$^+$ T cell expansion using flow cytometry, by testing the starting PBMCs at day 0, and on the day of harvest.

5. In the Zometa expanded Vδ2$^+$ T cell cultures, we have observed that PD-1 becomes transiently upregulated, typically peaking at about day 4, then returns to baseline by day 6-8 and remains low for the subsequent days of culture. Remarkably, the Vδ2$^+$ T cells maintain their low PD-1 expression after adoptive transfer [30]. Since low cell surface expression of PD-1 may be important for the anti-tumor functions of the adoptively transferred Vδ2$^+$ T cells, we recommend that flow analyses be performed to confirm that the transient elevation of PD-1 expression has subsided, prior to adoptive transfer.

6. We have not seen evidence that contaminating cell types in cultures of Zometa-expanded Vδ2$^+$ T cells have an impact on anti-tumor effects [30]. However, if desired, further sorting can be performed to increase Vδ2$^+$ T cell purity. Since most of the contaminating cells are usually αβ T cells, negative selection can be performed using pan-αβ TCR antibodies. However, since other populations (e.g. NK cells) are sometimes also present at low frequencies in the culture, if

an essentially 100% pure population of $V\delta2^+$ T cells is desired, it may be necessary to positively select the $V\delta2^+$ T cells using an antibody against Vδ2 or Vγ9. Importantly, we have not validated whether such positive selection using reagents that bind the TCR impacts the functioning of the $V\delta2^+$ T cells after adoptive transfer.

6. The viability of cells from tumor samples is often much lower than that of spleen samples. Tumor samples typically also require additional filtering compared to the spleen.

7. The mesenteric tissue wrapped around the pancreas can often be infiltrated with tumors or lymphoid aggregates giving it a bulbous, engorged appearance. Thus, while the pancreas itself is shaped like a flat pear with pink coloration (sometimes grey-ish when infiltrated by tumor cells), the tumors are usually lighter-colored masses with a firm texture.

8. Detection and analysis of human cells requires careful flow cytometric gating. It is helpful to set a singlets gate using side-scatter parameters, then gate on forward vs. side scatter to focus the analysis on live cells. It is best to use at least two different markers to identify the total human cell population, such as pan-HLA-A,B,C staining and anti-human CD45. Murine cells can also be excluded using antibodies against H-2, CD45, or other widely distributed murine antigens. Human cells identified by pan-HLA-A,B,C and anti-CD45 typically distribute into two nodes, with the HLA-A,B,C^{bright}/$CD45^{intermediate}$ population corresponding to B lymphocytes and the HLA-A,B,$C^{intermediate}$/$CD45^{bright}$ subset corresponding to T lymphocytes. The EBV-infected B lymphocytes often down-regulate CD20, and to a lesser extent CD19, from the cell surface at later stages in the infection. Thus, it can be helpful to stain for both of these markers in the same channel to amplify the signal. Nevertheless, even though histological analyses of the same tissue sample typically reveal abundant $CD20^+$ cells, flow

cytometric staining often reveals a distribution of CD3-negative human cells that range from clearly CD19/CD20 positive to essentially negative To accurately gauge expression of critical markers or to identify potentially rare cell types (e.g. adoptively transferred γδ T cells), it is helpful to set up parallel "fluorescence-minus-one" negative control samples, that are stained with all of the antibodies except the one of interest.

9. When staining for the adoptively transferred Vγ9Vδ2 T cells, it is best to use just a single TCR-specific reagent, since staining is often sub-optimal if multiple TCR-binding reagents (e.g. anti-Vγ9, anti-Vδ2, and anti-pan γδ mAbs) are all used in the same staining panel.

10. If delineating the adoptively transferred γδ T cells from those of the cord blood sample is important, HLA-A2 mismatching is helpful[236]. This is accomplished by staining CBMC and PBMC samples in order to identify samples that are mis-matched in regards to expression of HLA-A2 using an HLA-A2 specific antibody (clone BB7.2; BioLegend). After the HLA-A2 mis-matched γδ T cells are adoptively transferred, they can be readily distinguished from human cells that are derived from the cord blood sample by including the HLA-A2-specific antibody in the analysis panel.

Chapter III: CD4$^+$ iNKT Cells Promote Tumour Rejection in a Humanised Mouse Model of EBV-driven Lymphomagenesis.

This chapter is a compilation and commentary relating to data collected so far for a work very much in progress. The tentative author list for this manuscript is as follows:

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Abstract

Murine studies have highlighted the ability of invariant Natural Killer T (iNKT) cells as potent anti-cancer agents. Data from human trials which indirectly target iNKT cells also show that activating extant iNKT cells in various diverse cancers is an interventionist strategy of great promise. However, the utility of using iNKT cells as adoptive cell immunotherapy remains underexplored. To address this, we have used a pre-clinical model of de novo EBV-driven lymphomagenesis in the presence of an autologous T-cell compartment. Introduction of iNKT cells at timepoints when tumour masses are already established shows a significant reduction of tumour burden compared to early timepoints. This suggests that iNKT cells promote tumour rejection over immunosurveillance. That iNKT-instructed endogenous T-cells are capable of mounting EBV-specific responses when challenged ex vivo with viral motifs suggest that iNKT cells may be adjuvantising tumour-specific T-cells. Introducing iNKT-instructed T-cells into tumour-bearing mice set up with autologous cord blood shows a protective effect against subsequent neoplasia. Taken together, the results herein show that iNKT cells oversee a powerful and dramatic reduction in tumour burden by way of influencing the activation and memory of endogenous T-cells.

Introduction:

The principle behind adoptive cell immunotherapy for cancer involves isolating and *ex vivo* expanding tumour-specific T-lymphocytes, and infusing them back into the patient to promote tumour shrinkage or clearance[243]. Other variations have involved combining adoptive T-cell therapy with checkpoint blockade inhibitors[244], genetically manipulating the TCR to enable recognition of tumour-related antigens[245] or combining adoptive T-cell therapy with oncolytic viruses[246]. Limitations of these approaches include the potential for toxicity and neoplastic transformation, the need for autologous lymphocytes for adoptive transfer, and the generally cumbersome nature of genetic manipulations of primary cells and oncolytic virion productions.

Invariant Natural Killer T-cells offer an alternative to classical MHC-restricted adoptive T-cell therapy. Given the germline-encoded-like specificity of the iNKT TCR, there is no need to generate chimaeric antigen iNKT receptors. Moreover, iNKT cells are rapidly and potently activated not just by CD1d-borne lipids and glycolipids but by pro-inflammatory cytokines and other inflammatory stimuli (e.g. LFA-1). Finally, because of how highly conserved the CD1d/iNKT axis is in humans, iNKT cells are a 'donor-unrestricted' population of cells[247–249].

Human trials so far have indirectly targeted iNKT cells. Direct injection of αGC to solid-tumour bearing patients lead to increases in serum levels of IFNγ and GM-CSF and a tumourostatic effect, however these responses were dependent on basal, pre-treatment iNKT cell frequencies which were diminished compared to healthy controls[250]. While this iNKT depletion has been noted in prostate[251] and skin[252] cancers, this particular study suggests that iNKT loss may be more common across diverse cancer types. Increases in iNKT-mediated cytotoxicity, serum IFNγ levels and stabilization of progressive disease has been observed in patients treated with autologous

monocyte-derived dendritic cells pulsed with aGC in the contexts of non-small cell lung cancers[253,254], metastatic malignancies[255] and solid tumours[256]. Finally, direct administration of *ex vivo* expanded autologous iNKT cells has also been attempted for non-small cell lung cancer[257] and melanoma[258]. In both cases, there was stabilization of progressive disease but no tumour regression and increased serum IFNγ.

While it is apparent that iNKT cells are promoting a T_H1 immune profile leading to favourable patient outcomes, the mechanism behind how they do this remains elusive. Furthermore, whether or not iNKT cells can promote tumour clearance at all remains an open question. In the clinical trials referenced above, iNKT or iNKT-targetting immunotherapy was given at advanced stages of disease. The ability to model interactions between nascent and established neoplasms, and adoptively transferred iNKT cells *in vivo* would enable a molecular understanding of how iNKT cells are promoting an anti-tumour environment. Thus, immunodeficient mice engrafted with human cells are a powerful pre-clinical model where such interactions can be monitored and interrogated.

We have already shown that 'humanised' NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ (NSG) mice are capable of supporting tumours engendered by Epstein-Barr virus[234,235,239] (EBV), a human B-cell-tropic oncogenic virus, and that adoptive transfer of human γδ T-cells promotes tumour clearance[236]. NSG mice can successfully support human cells since they lack B and T-cells owing to a *Prkdc^{scid}* mutation leading to defects in V(D)J recombination[259]. The absence of common γ chain of the IL-2 cytokine receptor also prevents the development of murine adaptive immunocyte lineages as well as NK cells which could potentially kill the engrafted human cells[259,260].

In our EBV lymphomagenesis model, infected B-cells become lymphomas over the course of several weeks with macroscopic lymphoid aggregates or tumours visible ~4 weeks post-engraftment. The tumours themselves are infiltrated with endogenous $CD4^+$ and $CD8^+$ T-cells whose anti-tumour abilities are enervated by immune checkpoint receptors such as PD-1 and CTLA-4[235]. This model system, therefore, enables one to introduce immunotherapy at different timepoints during the course of tumourigenesis so as to see if iNKT cells are exerting anti-tumour effects early, at the level of immunosurveillance, by nipping in the bud transformed cells before they become tumours or via rejection of established tumour masses.

Results

De novo lymphomagenesis requires 1000-1500 IU of virus.

Human umbilical cord mononuclear cells (CBMC) were incubated with varying doses of M81 for 2 hours to enable viral attachment to B-cells. Infected cells were then injected into NSG mice intraperitoneally. Within ~4 weeks of injection, macroscopic lymphoid aggregates are visible in the peritoneum, normally growing upon the kidneys, in the hepato-biliary region, and sometimes invading the stomach and pancreas. As we have shown before, the lymphoid aggregates express EBV nuclear antigen-1 (EBNA1) and are also highly necrotised[236]. Titrating the viral dose revealed a dose-dependent decrease in the tumour masses recovered from the mice (Figure 6A). The 5000 IU dose gave more inconsistent tumour masses compared to the 1000 IU dose which gave a more uniform and subsequently reproducible tumour burden without killing the mice at the 4 week time-point. The viral titration experiments established an appropriate dose of virus at which to investigate the interplay between cellular immunotherapy and virally-mediated tumourigenesis,

iNKT cells mediate tumour rejection at 'late' timepoints when tumour masses are established.

We have shown that adoptive transfer of *in vitro* expanded γδ T-cells work to reduce tumour burden at early timepoints consistent with immunosurveillance, and are an effective immunotherapy against established tumour masses as well[236]. In order to investigate if iNKT cells the mode of immunotherapy that iNKT cells were facilitating—indeed, if they were facilitating tumour clearance at all since iNKT cells have been shown to suppress anti-tumour responses in humans and in diverse murine models[261–265], we administered *in vitro* expanded human iNKT cells to mice at early timepoints or late into the model. Early timepoints correspond with to days 0-3 post-infection while late timepoints refer to 23-25 days after injection with EBV-infected CBMC. We found that at early timepoints iNKT immunotherapy is ineffective at controlling tumour burden (Figure 6B), but there was near-complete eradication of macroscopic tumour masses when iNKT cells are introduced when neoplasia is apparent and visible.

iNKT cells mediate tumour-rejection within 36-48 hours of administration.

To investigate how rapidly iNKT cells were mediating tumour control, we administered immunotherapy on day 25 post-infection, and evaluated mice for tumours on days +1, +2, +3 and +4 post-immunotherapy. Tumour masses appeared reduced within 36-48 hours of iNKT administration (Figure 7A), and on day +3 of immunotherapy administration, there were no visible tumour masses. Correspondingly, T-cells were sorted from iNKT-treated or mock-treated mice from the specified timepoints, and co-incubated with EBV-infected, T-cell-depleted splenocytes. iNKT treatment was found to be associated with an increase in IFNγ production which positively correlated with the timing of tumour regression (Figure 7B).

The system is 'ready' for iNKT help 21 days post-engraftment of human cells into NSG mice.

In a variation of the above experiment, we decided to stagger iNKT delivery rather than timepoints of euthanasia to test the hypothesis that iNKT-mediated tumour control will occur at timepoints when macroscopic tumours first become visible. Our experience with the model has shown that day 18 post infection/engraftment represents one of the earliest timepoints wherein macroscopic tumours can be seen, so iNKT cells were administered at days 18, 21, and 25 post-infection, and mice were evaluated for tumour burden at day 29. While tumours were recoverable at days 18 and 21, day 21 showed a significant decrease in tumour burden in the iNKT-treated mice (Figure 8A). There was also a corresponding and sustained increase in IFNγ production from splenic T-cells produced by mice given iNKT cells on day 21 which positively correlated with the tumour regression (Figure 8B). The timing of this finding led us to hypothesise that there are endogenous factors or cells in the model which need to be 'ready' to accept iNKT-driven adjuvanticity, and the readiness does not occur until at least 21 days after engraftment of human cells in NSG mice. Given the increase in IFNγ levels detected, we decided to investigate the behaviour of endogenous cord-derived T-cells in the presence or absence of iNKT cells.

'iNKT-instructed' T-cells respond specifically to EBV peptide challenge, and may mediate protection from subsequent EBV-driven tumourigenesis.

T-cells from iNKT- or mock-treated animals were magnetically purified, and co-incubated with autologous T-depleted CBMCs serving as APCs with titrated doses of EBV peptides. iNKT-instructed T-cells showed a dose-dependent IFNγ response when challenged *ex vivo* (Figure 9A). Moreover, when these same T-cells were used as agents of immunotherapy in mice engrafted with autologous infected CBMC, they show potential for mediating a protective effect (Figure 9B).

iNKT immunotherapy drives tumour rejection, but does not confer a survival advantage upon treated mice.

Mice were infected with ~1500 IU of virus, and either mock-treated or given iNKT immunotherapy on day 25 post-infection. They were then euthanized when they became clinically ill. iNKT immunotherapy does not appear to extend the survival of treated mice (Figure 10). However, examination of the mice post-euthanasia revealed that iNKT-treated mice have a lower tumour burden compared to mock-treated mice, except that these tumours, though, small are found invading the hepato-biliary region or the pancreas, and death is quick very likely due to bile build-up and increased inflammation.

Discussion:

Mechanistically, iNKT cells may promote tumour rejection by direct, CD1d-dependent cytolysis of the tumour cells[155,266,267], the production of pro-inflammatory cytokines to trans-activate NK cells[268–270], licensing of dendritic cells to promote anti-tumour CTLs[271,272], or by rendering the tumour microenvironment less immunosuppressive by killing tumour-associated macrophages (TAMs)[273],regulatory T-cells (T_{regs})[274] or Myeloid-Derived Suppressor Cells (MDSCs)[275,276].

The evidence collected in this study so far does not make explicit exactly how the iNKT cells are mediating anti-tumour responses, but investigating the functions of endogenous T-cells in the context of immunotherapy appears to be fertile ground for further investigations. I am inclined to disregard direct cytolysis because EBV-infected B-cells downregulate CD1d very quickly[277,278], and if direct cytolysis were a major mechanism of iNKT-mediated tumour control, we likely would have seen anti-tumour responses at early administration of immunotherapy when CD1d on transformed cells is still present.

It is known that B-cells, upon EBV infection, secrete IL-12[279–281]which we and others have shown can prompt iNKT cells to make IFNγ independently of TCR triggering. IFNγ is known to promote T-cell responses in chronic viral and bacterial infections[282,283]—and we can certainly argue that ours is as much a model of EBV-driven lymphoma as it is a model of chronic EBV infection resulting in B-cell hyperplasia. Thus, it is possible that IL-12-driven IFNγ from iNKT cells 'frees' anti-tumour endogenous T-cells from the immunosuppressive environment of the tumours. Given the contents of Chapter I, I am personally more invested in the idea of ICAM-1 expressed on transformed B-cells[284] also contributing to iNKT IFNγ which rejuvenates T-cells directly or at the level of antigen-presenting cells[285]. Though, of course, this does require that we measure serum IL-12 over time in tumour-bearing mice, and evaluate CD1d and ICAM-1 expression on tumours to assess whether some or all of these pathways could be working in concert to drive tumour-rejection. It is established that IL-12 production and ICAM-1 expression in EBV-infected B-cells is under control of Latent Membrane Protein-1 (LMP-1) expressed by EBV[281,284], so it would be valuable to evaluate the effects of iNKT immunotherapy using LMP1-deficient-EBV.

There are very clearly issues in this model which need to be explored in greater granularity so as to better understand/tailor immunotherapeutic regimens. Why is day 18 post-infection too early for iNKT cells to be effective even though tumours are present? What has to happen in the system for it to be ready to accept the adjuvanticity that iNKT cells are bringing to the system? Given that the lymphoid aggregates we see in the model are made up of a considerable amount of $CD4^+$ and $CD8^+$ T-cells, it is possible that a critical mass of tumour-infiltrating T-cells must be reached for iNKT immunotherapeutic effects to be apparent. Unpublished data from our group shows that endogenous T-cells arrive in waves upon engraftment, and the T-cell compartment is formed ~3

weeks after introducing human cells into NSG mice. It would be valuable to see how iNKT immunotherapy works in the presence of an already established T-cell compartment.

That iNKT immunotherapy is not associated with increased survival is more an issue of tumour 'real estate' than tumour burden. In our survival studies, iNKT-treated mice who eventually succumbed to tumours had smaller tumours but invading the pancreas or the hepato-biliary region. While not exactly pancreatic cancer or cholangiosarcoma/cholangiocarcinoma, it is true that invasion of these organs by transformed cells is associated with rapid death[247,248]. There were individual cases of iNKT-treated mice which died and seemingly had no visible macroscopic tumours. Yet, necropsy revealed the presence of subtle lymphoid aggregates embedded in the liver or invading the common bile duct. This indicates that iNKT cell immunotherapy is effective in our given 30-33 day timeframe of the model, but there are chances of relapse or that residual neoplasia can bounce back once the effects of iNKT-mediated adjuvanticity dwindle. This isn't surprising given that iNKT cells can be overhwhelmed by aggressive cancers . Thus, an immunotherapeutic regimen where 'booster' doses of iNKT cells are administered can also be a way forward for this study.

Materials and Methods

EBV Preps: The experiments described herein were all performed with M81, a lytic strain of EBV made to express Green Fluorescent Protein (GFP) and a hygromycin resistance gene via bacterial artificial chromosome technology. Virions were produced from 293 stably infected cell lines, and titre of infectious virus was determined by enumerating the number of fluorescent Raji cells derived from serial dilutions of virus stock[170,171]

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In vivo Modeling: The process of engrafting mice with human cord blood-derived mononuclear cells has been detailed in chapter II. Briefly, CBMC were purified via density gradient centrifugation using Ficoll-Hypaque PLUS (GE Healthcare), and exposed to EBV at the given concentrations for 2 hours at 37°C, 5% CO_2 to promote viral attachment. CBMC were then washed, resuspended in sterile PBS and ~$1x10^7$ infected CBMC were injected into 6-8 week old NSG mice intraperitoneally.

iNKT cell Expansion: Peripheral blood mononuclear cells (PBMC) were purified from blood drawn from healthy adult donors via density gradient centrifugation, as described above. The PBMC were then stained with fluorescently labeled αGC-loaded CD1d tetramer (NIH Tetramer Core), αCD3 and αCD4 (OKT3 and OKT4, respectively; Biolegend). iNKT cells were then sorted on a FACS Aria (BD Biosciences) based on staining positive for the given three markers. The sorted iNKT cells were then co-incubated with irradiated PBMC (allogeneic), 5μg per ml of phytohaemagluttanin (Sigma-Aldrich) and 200 U per ml IL-2 (Peprotech) for 2-3 weeks at which point the purity of the expanding cultures was evaluated via flow cytometry.

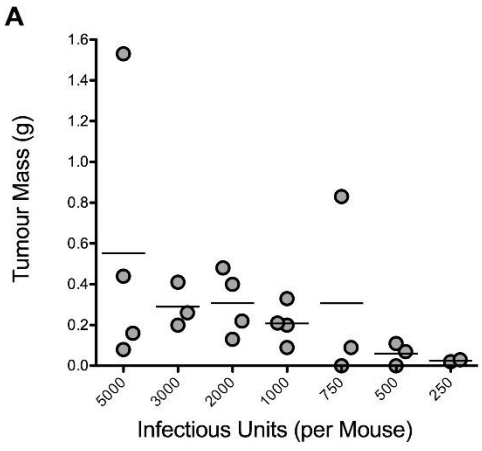
Ex vivo Peptide Challenge: To interrogate the ability of iNKT-instructed T-cells to respond ex vivo to synthetic EBV peptide, splenocytes from immunotherapy or control mice were prepared by pressing excised spleens with the plunger end of a 3 mL syringe. Splenocyte suspensions from mice in like groups were pooled, and untouched T-cells were magnetically purified using Miltenyi Biotec's Pan T-cell Isolation Kit. Magnetically sorted T-cells were held in a medium containing RPMI-1640, 10% heat-inactivated bovine calf serum (Hyclone), 3% human AB serum (Atlanta Biologicals), 1% Penicillin-Streptomycin (Sigma-Aldrich) 1% L-Glutamine (Gibco) and 200 U per ml IL-2 (Peprotech) for 72 hours to relieve tumour-mediated immunosuppression.

Following this, the T-cells were co-cultured with autologous uninfected CBMC (serving as APCs) and the given concentrations of EBV peptides (Miltenyi Biotec) for 24 hours. Supernatants were then evaluated for IFNγ via sandwich ELISA as described in chapter I.

In vivo T-cell Recall Experiments: To investigate of iNKT-instructed T-cells were mediating protection against subsequent EBV infections in vivo, we purified untouched T-cells as described above, and co-injected iNKT-instructed T-cells or T-cells from mock-treated animals and infected autologous cord cells. Tumour burden was evaluated at day 29 post engraftment.

Statistical Analyses: In experiments where treatment groups from independent analyses were aggregated, statistical analysis was performed using the van Elteren test to account for the effect of stratification, while datasets that consisted of replicate analyses of samples from the same mouse were analyzed using an unpaired, 2-tailed parametric *t* test.

Figures:



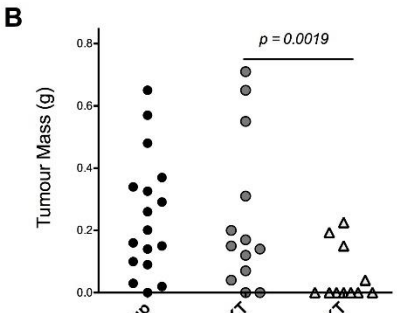


Figure 6. EBV-infected CBMC successfully engraft onto NSG mice, and result in tumour burden which is successfully controlled by late administration of iNKT immunotherapy. A) EBV-dose was titrated as shown, and tumour burden in infected mice was evaluated on D29 post-engraftment. Aggregated results from two separate analyses are shown. **B)** Introduction of iNKT immunotherapy at D25 ('late') results in dramatic tumour regression while injection of iNKT cells between days 0-3 post engraftment results in a failure to control tumour burden. The plot shows aggregated results of tumour mass from six independent experiments. Each data point represents an individual mouse. The p-value has been calculated using the van Elteren analysis: a stratified, two-tailed nonparametric test.

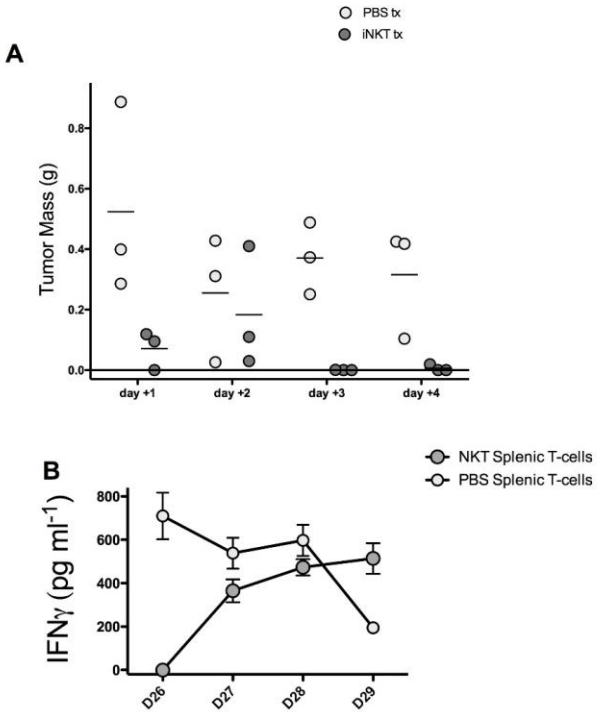


Figure 7. Tumour regression begins to occur within 24-36 hours of iNKT immunotherapy, and is associated with an increase in IFNγ from endogenous T-cells. **A)** Engrafted mice were either given immunotherapy (iNKT Tx) or mock treated (PBS Tx) on day 25 post engraftment followed by euthanasia, and evaluation of tumour burden on days +1, +2, +3 or +4 after immunotherapy. Each data point represents a single mouse. A reduction in tumour burden was seen within 24-36 hours of iNKT administration. **B)** T-cells were magnetically purified from the mouse spleens at corresponding time-points, and incubated for 72h in medium containing IL-2, followed by a 24h co-incubation with T-cell depleted, EBV-infected splenocytes as APCS. The IFNγ released into the supernatant was quantified by ELISA. There is an increase in the IFNγ response which corresponds to tumour regression.

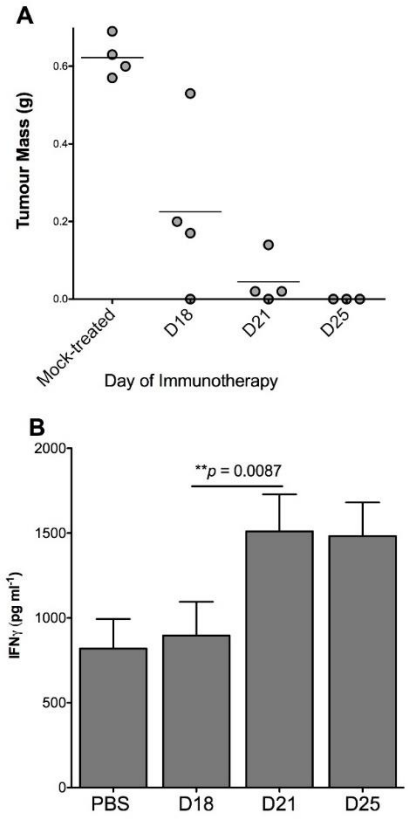


Figure 8: Immunotherapy is only effective at 21 days post-engraftment, and tumour regression is once again associated with a sustained increase in IFNγ. A) Engrafted mice were given iNKT cells at days 18, 21, or 25 post-engraftment or were mock-treated followed by sacrifice and evaluation of tumour burden on day 29 post-engraftment. iNKT immunotherapy fails to control tumour burden at day 18 post engraftment, but tumour regression is seen at days 21 and 25. **B)** Correspondingly, splenocytes harvested from mice at the designated timepoints show an increase in IFNγ at D21 which is sustained on D25. The plot shows the means of 4 replicates from pooled splenocytes from mice in each group. The p-value was calculated using an unpaired parametric *t* test

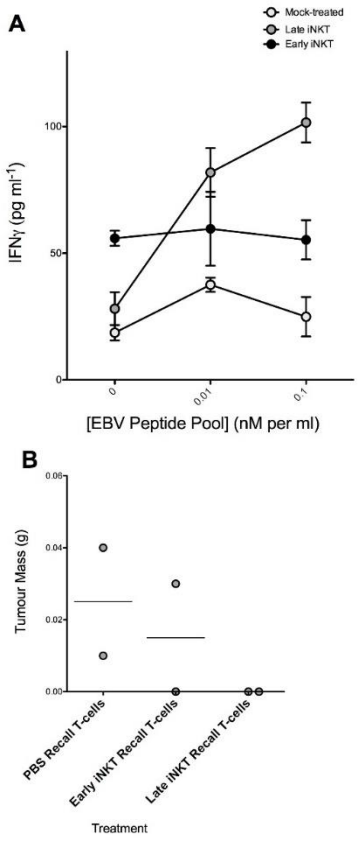


Figure 9. iNKT-instructed T-cells mount a dose-dependent IFNγ response against EBV-peptides, and may mediate protective 'recall' responses *in vivo*. A) T-cells were magnetically purified from mock-treated, early immunotherapy or late iNKT immunotherapy-treated mice (n=3), pooled, and held for 72h in culture medium containing IL-2. They were then co-incubated with autologous uninfected CBMC and the designated concentrations of a synthetic mixture of EBV peptides for 24h. IFNγ released into supernatant was evaluated by sandwich ELISA, and the plot shows the means of 4 replicates at each given concentration. Late iNKT immunotherapy which mediates tumour rejection is associated with dose-dependent IFNγ release from endogenous T-cells. **B)** When iNKT instructed T-cells are used as immunotherapeutic agents at an early timepoint (Day 3, post-engraftment) in NSG mice engrafted with autologous EBV-infected CBMC, the state

of tumour-burden at day 29 post-engraftment suggests that iNKT-instructed T-cells may mediate protection from subsequent EBV tumourigenesis.

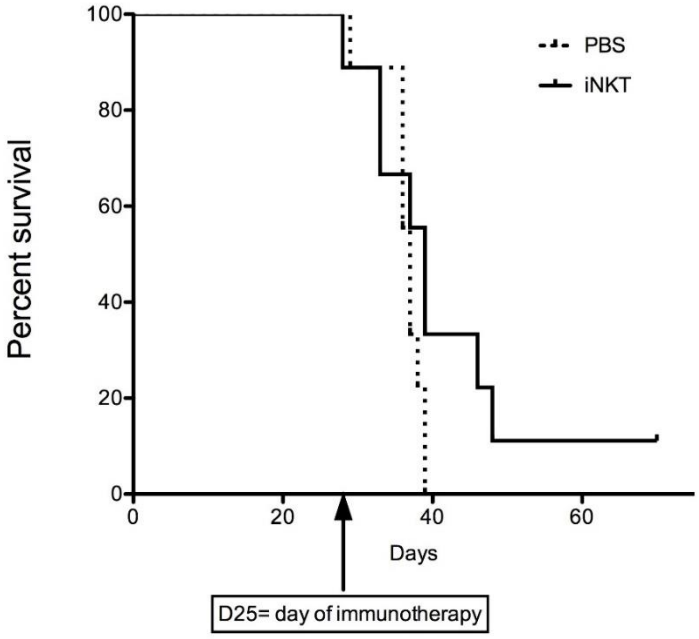


Figure 10. iNKT immunotherapy drives tumour regression, but is not associated with enhanced survival. Engrafted (EBV-infected) mice were either mock-treated or given iNKT immunotherapy at day 25 post-engraftment, and euthanized when moribund. While immunotherapy treated mice had lower tumour burden, the tumours were often invading the pancreas or the common bile duct. The plot shows aggregated results from two independent survival curves (n=4 and n=5 per group).

Appendix: Evaluation of the Immunotherapeutic Potential of Double Negative (DN) iNKT Cells.

So far, I have documented the ability of $CD4^+$ iNKT cells to mediate tumour rejection, and the data seem to suggest that $CD4^+$ iNKT cells may not necessarily be direct cytolytic effectors, but are a source of adjuvanticity for endogenous T-cells. Human iNKT subsets can be broadly divided into $CD4^+$ or $CD4^-CD8^-$ (double negative; DN). $CD4^+$ iNKT cells are capable of co-secreting Th1 and Th2 cytokines, promote more IL-12-producing pro-inflammatory dendritic cells and are highly responsive to non-TCR, 'cytokine-alone' cues of activation. In contrast, DN iNKT cells have been shown to be more Th1 biased in comparison, making large amounts of IFNγ and TNFα, are comparatively more cytolytic and may promote more suppressive or immunomodulatory dendritic cell phenotypes[123,289,290].

I shall show here a single experiment where tumour-bearing NSG mice engrafted with EBV-infected CBMC were given DN iNKT cells as immunotherapy on day 25 post-engraftment, and then evaluated for tumour burden on day 29 or 4 days after immunotherapy. The mice failed to control their tumours, and in many cases had some of the largest tumour masses I have seen working this model (Figure 11).

It has been suggested that DN iNKT cells may thwart tumours via immunosurveillance rather than at the level of marshalling immunocytes to drive tumour rejection, and this might very well be the case in this model. It would be useful, then, to administer DN iNKT cells at early timepoints, and see if they do, indeed, root out neoplasia at the level of immunosurveillance.

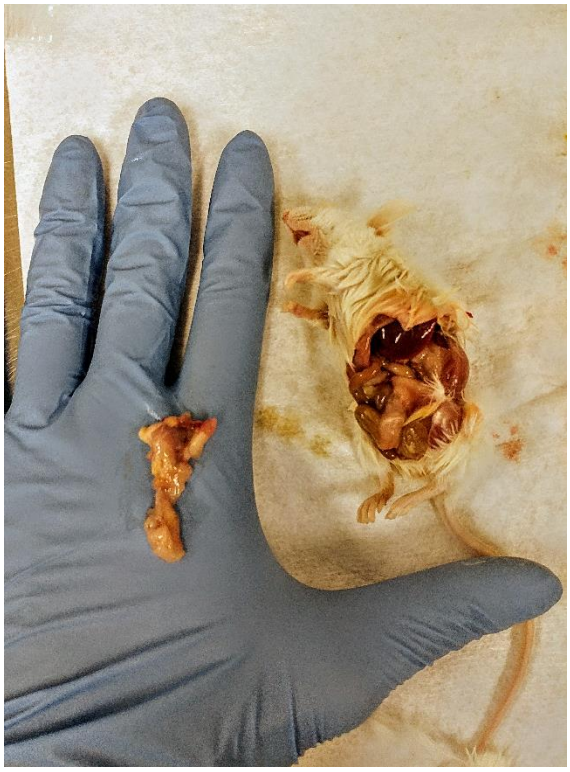


Figure 11. Tumour-bearing NSG mice fail to control tumour burden when administered DN iNKT cells as immunotherapy at late timepoints. The image is an example of tumour burden recovered from DN iNKT-treated mice.

Other Future Directions:

Roles of LFA-1:ICAM-1-driven IFNγ from iNKT Cells:

In general, I see this story as one that has found a natural conclusion. Indeed, at the time of writing this, the paper put together from these data has already undergone one round of revisions, and has been re-submitted to *The Journal of Immunology* for publication. There are intriguing and unanswered questions that remain from this particular data-set, however. At one point, I, after a series of abortive qRT-PCR attempts on ICAM-1 or non-sstimulated iNKT cells got the sense that LFA-1 ligation may be prompting the transcription of IFNγ message in iNKT cells ~90 minutes into stimulation. Future work in this model could look at both transcriptional as well as epigenetic

regulation of the *IFNG* gene locus, as well as the ability of ICAM-1:LFA-1 interactions to promote T-bet, and thus T_H1 occupancy in iNKT cells, especially given that ERK phosphorylation is posited to be upstream of ERK.

Apart from the cytokine secretion aspect, there are data that I generated which looked at the difference in adhesion and migration behaviour between high LFA-1-expressing iNKTs and lower LFA-1-expressing classical MHC-restricted T-cells. Those data are noisy given the generally messy nature of adhesion assays. Yet, it seemed like the modulation of LFA-1-mediated adhesion required IL-12 since cells seemed 'stickier' in the presence of this cytokine i.e. by showing increased adhesion to ICAM-1 coated surfaces as well as slowing down, and clustering when imaged via time-lapse microscopy. A combination of both static adhesion assays and live-cell imaging could shed more light on the interplay between cytokine and adhesion receptor interactions in modulating the functionality of iNKT cells. This work could then be extended to iNKT-APC interactions, and could be a predictor of how iNKT cells instruct different APCs.

<u>iNKT-mediated Immunotherapy:</u>

The data generated in service of this project so far strongly indicate that iNKT adjuvanticity is directed towards rejuvenating endogenous T-cells in the system, thus enabling them to promote tumour rejection. Future work would be focused on shedding light on how this is occurring. Some hypotheses worth testing include examining the status of antigen-presenting cells from iNKT-treated mice to ask if dendritic cells have upregulated activation markers such as CD70 or CD86 which would promote downstream adaptive anti-tumour responses or to ask if suppressive cells such as myeloid-derived suppressor cells or tumour associated macrophages are reduced in the presence of immunotherapy, and if iNKT cells may be killing them.

There also remains the question of how long-lasting is iNKT-mediated immunotherapy given how thoroughly invasive EBV-driven lymphoma appears to be. Necropsy performed on an iNKT-treated mouse which died 12 days after receiving immunotherapy but had no visible macroscopic tumours reveals the presence of infiltrates reaching into the adrenal gland, the reproductive apparatus, the airways, the adrenal gland and the spleen. The liver appears heavily infarcted, and the spleen, too, appears to have lost the follicular architecture associated with a healthy spleen (Figure 12).

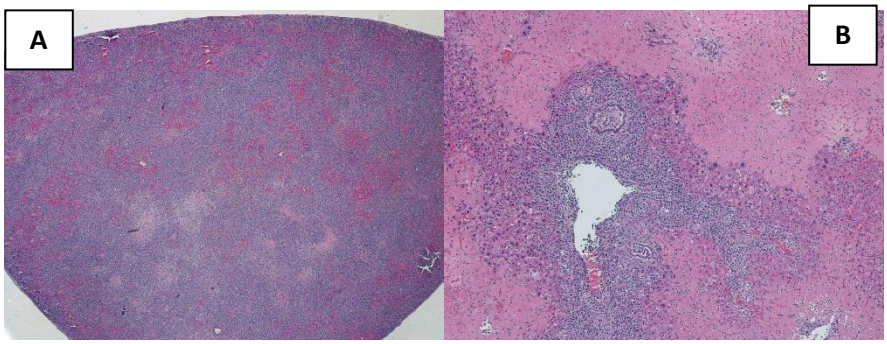


Fig 12. Invasion and infarction is visible in spleen and liver tissues in immunotherapy-treated mice despite no evidence of macroscopic tumours. Haematoxylin-Eosin stained sections from a mouse which died 12 days post-iNKT immunotherapy reveal invasion of spleen, and loss of splenic architecture (**A**) as well as an infarcted liver with the presence of Kupffer cells (purple dots in a fuschia expanse) and obstructed bile ducts (**B**).

So far, it is not obvious if this pathology seen in iNKT-treated mice is due to lymphoma relapse, or a consequence of tumour lysis syndrome. Future experiments would focus on attempting to chart the kinetics of this relapse (if that is what it is) as well as the means to mitigate it. The key may lie in titrating the dose of iNKT cells so as to achieve tumour clearance, but in a less inflammatory way.

General Discussion

The CD1 antigen presentation is an ancient system of lipid and glycolipid antigen presenting molecules found in mammals, birds and reptiles[291,292]. The natural history of the CD1 system is abstruse, controversial and beyond the scope of this dissertation. Suffice it to say, however, that the CD1 family of antigen presenting molecules is ancient and highly conserved in several mammalian orders. Of the five isoforms of CD1—CD1a, CD1b, CD1c, CD1d and CD1e—CD1d holds my primary interest, given the nature of my studies with human iNKT cells.

A bigger and perhaps existential question one has found oneself pondering is: why? Why would Nature hold onto a system of antigen-presentation and T-cell ontogeny that gives rise that T-cells whose TCRs, educated on CD1d, behave like germline encoded receptors, and recognise both self and non-self lipids? Can autoreactivity—always sold to undergraduate students in immunology as "bad!"—actually be beneficial in the right contexts? Add to this the knowledge that iNKT cells have a thriving life outside the CD1d-TCR system i.e. their responses to solitary cytokine cues and integrins (as shown here), one moves from a T-cell-mediated existential crisis to a clearer picture of not just an incredibly resourceful lymphocyte population which may very well pound the alarm very early on indeed when there is insult, but also a view of the immune system that goes beyond just fighting foreign/non-self threats.

Oftentimes, a military metaphor is invoked to talk about immunity, and if we think of immunocytes as The Troops, we must remember that The Troops are people too. They make up the fabric of society. Similarly, immunocytes are cells, too. Much like the troops, they will fight if needed, but an investment in homeostasis so as to avoid the sequelae and energetic cost of an all-out battle is

best avoided. To extend the military metaphor, diplomacy may be rife with intricacy and many moving set-pieces, but if executed astutely, diplomacy/homeostasis is preferred over war.

To me, my insights into iNKT biology reveals them as excellent arbiters of diplomacy via their ability to interact with a diversity of cells in non-traditional ways to result in outcomes which avoid pathology or take care of them swiftly. Examples of this I have encountered while working on projects which show iNKT-driven IL-6 and IL-1β from monocytes in a partially CD1d-dependent mechanism[293], or how iNKT cells 'train' DCs via sterile inflammation to rapidly handle opportunistic fungal pathogens[207], or how iNKTs may respond to ambient ICAM-1 for rapid and early IFNγ responses, or even their ability to bring relief/adjuvanticity to beleaguered tumour-dwelling lymphocytes. Figure 12 is a graphical summation of some of the projects I have had the privilege of working on which , in turn, have facilitated the view of innate T-lymphocytes as resourceful, chatty and able to use both self and non-self-motifs to orchestrate a return to homeostasis.

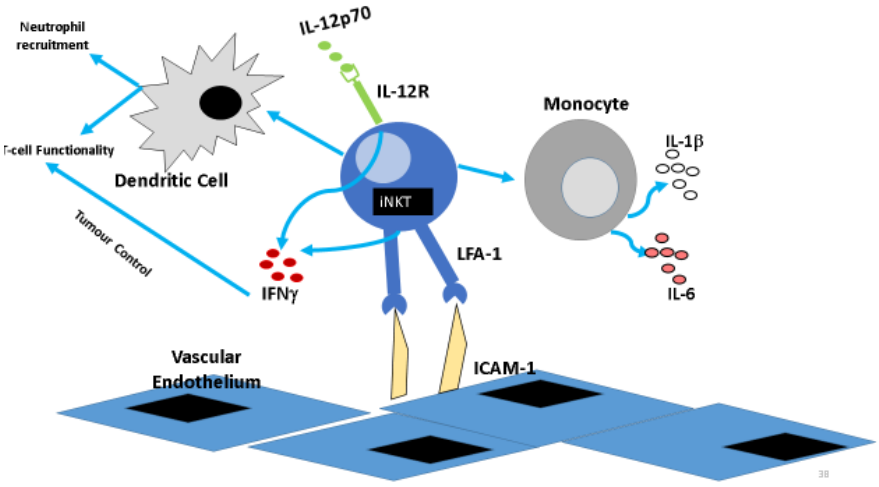


Figure 13. Graphical abstract of Akshat's PhD work. iNKT cells shape downstream responses of many immunocytes via cell:cell interactions as well an acute awareness of soluble signals and cell-associated ligands .

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